GENETIC REPORTER ASSAYS TO STUDY THE REGULATION OF INTERLEUKIN-7 RECEPTOR ALPHA GENE EXPRESSION

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GENETIC REPORTER ASSAYS TO STUDY THE REGULATION OF INTERLEUKIN-7 RECEPTOR ALPHA GENE EXPRESSION

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ABSTRACT

Interleukin-7 (IL-7) signaling through IL-7 receptor controls survival and proliferation of B- and T- lymphocytes at early stages of development. IL-7 is also a critical survival factor for naïve T-lymphocytes and memory T-cells circulating in the peripheral organs. Throughout T-cell development, IL-7Ralpha expression on distinct T-cell populations is strictly regulated. However, the mechanisms that regulate differential expression of the IL-7Ralpha chain on T- lymphocytes are poorly defined.

Here, we used bioinformatic tools to determine possible cis-acting regulatory elements at the downstream of mouse IL-7Ralpha gene and we tried to analyze the transcriptional activities of these possible cis-regulatory elements in a mouse T lymphoma cell line by means of genetic reporter systems.

We took advantage of the luciferase assay system for functional analysis of noncoding sequences at the downstream of mouse IL-7Ralpha gene. We modified luciferase reporter vector by inserting weak promoter into the upstream of luciferase cDNA and cloned the mouse noncoding sequences into this modified vector. We electroporated mouse T lymphoma cells with luciferase reporter vectors to evaluate the effects of these noncoding regions on the transcription of luciferase cDNA. Although the luciferase activities in T lymphoma cell lysates were low due to low sensitivity of the detection system used, we observed that efficient transfections with luciferase reporter vectors resulted in high luciferase activities in a different cell line. We concluded that different reporter systems or different cell lines were required to determine possible transcriptional activities of the cloned mouse noncoding sequences.

ÖZET

Interlökin-7 (IL-7) reseptör aracılığıyla harekete geçirilen IL-7 sinyalleri B ve T lenfositlerin gelişimlerinin ilk aşamalarında hayatta kalmalarını ve çoğalmalarını sağlar. IL-7, ayrıca ikincil lenfatik organlarda sirküle eden naif T lenfositlerin ve bellek T hücrelerinin hayatta kalmasını sağlayan kritik bir faktördür. T hücrelerinin gelişimi boyunca, IL-7 reseptör alfa zincirinin farklı T hücre popülasyonlarında üretilmesi sıkı bir denetim altındadır. Ancak, IL-7 reseptör alfa zinciri geninin T lenfositlerde proteine çevrilmesini denetleyen mekanizmalar çok net açıklanamamıştır.

Bu çalışmada, biyoinformatik yöntemlerle fare IL-7 reseptör alfa geninin 3' tarafındaki genomik bölgede bulunan muhtemel denetim öğelerini belirleyip, bu öğelerin transkripsiyonel aktivitelerini fare T lenfoma hücre kültürlerinde genetik raportör sistemlerle analiz etmeye çalıştık.

Fare IL-7 reseptör alfa geninin 3' tarafındaki kodlamayan sekansların fonksiyonel analizi için lusiferaz enziminin katalitik aktivitesinden yararlandık. Lusiferaz enzimini kodlayan cDNA' lerin 5' tarafına zayıf bir tetikleyici yerleştirerek lusiferaz raportör vektörlerini değiştirdik. Belirlediğimiz kodlamayan fare sekanslarını değiştirilmiş lusiferaz vektörlerinin içine klonladık ve bu sekansların lusiferaz geninin transkripsiyonu üzerindeki etkisini görmek için vektörleri fare T lenfoma hücrelerine transfekte ettik. Lusiferaz aktivitesini ölçmekte kullandığımız detektör sistemin hassasiyetinin yetersizliğine bağlı olarak T lenfoma hücrelerinde düşük lusiferaz aktiviteleri ölçmemize rağmen; yüksek verimle transfekte olan diğer bir hücre tipinde, yüksek lusiferaz aktiviteleri gözlemledik. Bu yüzden, klonladığımız fare sekanslarının transkripsiyonel aktivitelerini test edebilmek için farklı hücre tiplerine ya da farklı genetik raportör sistemlerine ihtiyaç olduğu sonucuna vardık.

To my family

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ABBREVIATIONS

-/-	Double negative
APC	Antigen presenting cell
BAC	Bacterial artificial chromosome
BCR	B-cell receptor
bp	Base pair
BSA	Bovine serum albumin
Cap(1…8)	Conserved noncoding regions from 1 to eight
Capsl	Calcyphosine like protein
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DEAE-Dextran	Diethylaminoethyl-dextran
D	Diversity gene segments
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DP	Double Positive
EB	Elution Buffer
EDTA	Ethylenediaminetetraacetic acid
GABP	GA binding protein
GFP	Green fluorescent protein
HSC	Hematopoietic stem cell
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain

IL-	Interleukin-
IL-7R	Interleukin-7 receptor
ISP	Intermediate Single-Positive
Jak	Janus kinase
J	Joining gene segments
kb	kilobase
luc+	Luciferase
MHC	Major Histocompatibility Complex
MPP	Multipotential progenitors
NCBI	National Center for Biotechnology Information
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylenglycol
PI-3	Phosphatidylinositol-3
PPBSF	pre-pro-B cell-growth stimulating factor
RAG	Recombination-activating genes
RLU	Relative light unit
rpm	Revolution per minute
SCF	Stem cell factor
SOCS	Suppressor of cytokine signaling
SP	Single Positive
STAT	Signal transducer and activator of transcription
SV 40	Simian virus 40
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TN	Triple negative
TSLP	Thymic stromal lymphopoietin
UCSC	University of California at Santa Cruz
V	Variable gene segments
SCID	Severe combined immunodeficiency
γc	common γ chain

1 INTRODUCTION

1.1 IL-7 as a nonredundant cytokine

1.1.1 IL-7

The development of an effective immune response requires complex interactions and a communication network between lymphoid cells, inflammatory cells, and hematopoietic cells. Cytokines are mediators of cell-to-cell communication in the immune system. Cytokines are involved in numerous physiologic responses among which are proliferation and differentiation of lymphocytes, activation of innate and adaptive immunity and regulation of hematopoiesis (Janeway *et al.*, 2005). Cytokines exert their effects through binding their own receptors on cell membranes and thus initiating a signaling cascade. Signal transduction is mediated by kinase mediated phosphorylation of cytoplasmic proteins. Actions of cytokines are generally transient and local. Functions of cytokines can be overlapping or redundant, loss of one cytokine may be compensated by other cytokines that achieve the same functions (Janeway *et al.*, 2005).

Interleukin-7 (IL-7), previously named as lymphopoietin-1, is a cytokine that has a critical and nonredundant role in the development and survival and homeostatic maintenance of lymphocytes. IL-7 is a glycoprotein with a molecular weight of 25 kDa and belongs to the hematopoietin family of cytokines along with IL-2, IL-4 and IL-15. Cytokines in the hematopoietin family have a similar structure consisting of four α helices (Goldsby *et al.*, 2000). Stromal and epithelial cells in the thymus and bone marrow are major sources for IL-7 in the body. Intestinal epithelium, keratinocytes,

fetal liver and follicular dendritic cells also produce IL-7 to a minor extent. The signals that control IL-7 production are not well understood, but it has been shown that Interferon-γ (IFN-γ) can upregulate and Transforming growth factor-β (TGF-β) can downregulate IL-7 production (Hofmeister *et al.*, 1999).

Developing lymphocytes require external stimuli for survival, proliferation and differentiation. IL-7 was first identified in 1988 (Namen *et al.*, 1988) as a B-cell growth stimulating factor in mice. Thereafter, it was demonstrated that IL-7 also has indispensable roles in the survival and proliferation of early T-cell progenitors. Injection of anti-IL-7 monoclonal antibodies into mice resulted in severely impaired T- and B-cell development (Grabstein *et al.*, 1993). Mice with a targeted IL-7 gene have greatly reduced peripheral lymphocyte numbers because of a block in B- and T-cell development (von Freeden-Jeffry *et al.*, 1995). IL-7 was also shown to regulate rearrangement of immunoglobulin heavy chain locus in B-cells that results in antibody production (Corcoran *et al.*, 1998) and the rearrangement of T cell receptor (TCR)γ-chain locus in γ:δ T-cells (Durum *et al.*, 1998). IL-7 is not only essential for the development and survival of immature lymphocytes, but naïve and memory T-cells in peripheral lymphoid organs also require this cytokine for long-term maintenance (Schluns *et al.*, 2000).

1.1.2 IL-7 Receptor

IL-7 signaling is initiated through binding of IL-7 to its heterodimeric receptor consisting of IL-7 Receptor α- chain (CD127) and a common γ chain (γc) that both belong to the type I cytokine receptor family (also called the IL-2 receptor family). γc is constitutively expressed on lymphocytes. Type I cytokine receptors have conserved amino acid motifs consisting of four conserved cysteine residues and a WSXWS (tryptophan–serine-any amino acid–tryptophan-serine) motif in their extracellular domains (Goldsby *et al.*, 2000).

γc was first identified as a component of IL-2 receptor and was later found to be a subunit shared by the receptors for IL-7, IL-4, IL-9, IL-15 and IL-21 (Figure 1.1). Mutations in the γc gene which is located on the X chromosome cause a disease called

X-linked Severe combined immunodeficiency (X-linked SCID) (Noguchi *et al.*, 1993). Children with X-linked SCID can only survive in a pathogen free environment unless treated with bone marrow transplantation. X-linked SCID results in greatly reduced numbers of mature T-cells and Natural killer (NK) cells, B-cell numbers are not affected. The defects in T-cell development due to γc gene mutations are also observed in patients with IL-7Rα deficiency (Puel *et al.*, 1998). Deficiencies in the other receptor subunits that dimerize with γc subunit do not result in such a severe defect in T-cell development, suggesting a nonredundant role for IL-7 and its receptor IL-7Rα for T-cell development. For example, disruption of IL-2Rβ or IL-2 gene in mice does not result in reduced number of thymocytes (Schorle *et al.*, 1991; Suzuki *et al.*, 1995) and in IL-4$^{-/-}$ knock-out mice normal T-cell numbers are observed as well (Keegan *et al.*, 1995). It should also be noted that unlike mice, IL-7 seems to be not critical for the development of B-cells in humans since X-linked SCID and IL-7Rα deficient patients do have a normal B-cell development.

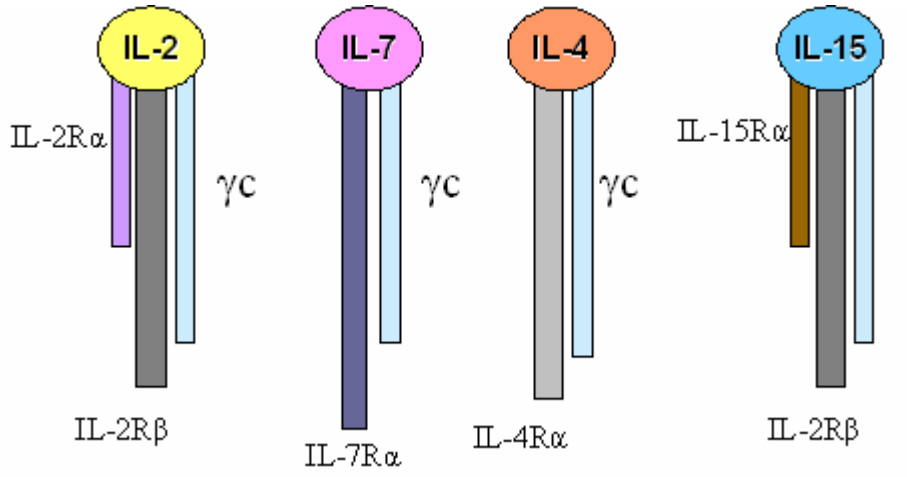


Figure 1.1: Type I cytokine receptors: Cytokine receptors in type I cytokine family share γc chain as a subunit.

IL-7Rα is a membrane glycoprotein with a molecular weight of 49.5 kDa. IL-7Rα consists of 438 amino acids and its extracellular domain contains four conserved cysteine and WSXWS motifs, as other members of type I receptor family. It has a single transmembrane domain consisting of 25 amino acids and an intracellular domain containing 195 amino acids (Jiang *et al.*, 2005). Cytoplasmic tail of IL-7Rα does not have an intrinsic tyrosine kinase activity, but it contains potential docking sites for several nonreceptor kinases and adaptors to initiate multiple signaling pathways.

Cytoplasmic tail of IL-7Rα contains functional regions; one rich in acidic residues (A region), one rich in serine residues (S region) and a tyrosine (T) region. T region contains three tyrosine residues; Ty401, Ty449 and Ty456 that are conserved between human and mice. In addition, IL-7Rα contains a membrane proximal region, 'Box1' region, that is conserved between the type I receptor family (Hofmeister *et al.*, 1999).

IL-7Rα gene is located on human chromosome 15 and on murine chromosome 8. IL-7Rα gene spans 19.79 kb in humans and 22.17 kb in mice with eight exons and seven introns. IL-7Rα is expressed on common lymphoid progenitors, thymic dendritic cells, developing B-cells, developing T-cells in the thymus and mature T-cells in the periphery. It is also expressed on keratinocytes, intestinal epithelium and bone marrow derived macrophages (Jiang *et al.*, 2005).

IL-7Rα subunit is also a part of a receptor for thymic stromal lymphopoietin (TSLP) (Park *et al.*, 2000). In IL-7Rα deficient mice, development of B-cells as well as T-cells is blocked at an earlier step of development compared to IL-7 deficient mice (Peschon *et al.*, 1994). This difference is explained by the loss of both IL-7 and TSLP signaling in IL-7Rα deficient mice. TSLP may be also important in early lymphocyte development.

1.1.3 IL-7 signaling pathways

1.1.3.1 JAK/STAT pathway

Oligomerization of type I family cytokines activates a class of protein tyrosine kinases called Janus kinases (Jaks), specifically Jak1 and Jak3. Jak3 is pre-associated with γc subunit and deficiency in Jak3 shows similar characteristics as in the case of X-linked SCID (Macchi *et al.*, 1995). Jak1 is associated with the Box1 region of IL-7Rα. Like IL-2, IL-7 can induce tyrosine phosphorylation of Jak1 and Jak3. Engagement of IL-7 to IL-7Rα recruits γc chain, bringing together Jak1 and Jak3 and causing activation by either autophosphorylation or crossphosphorylation of the Jaks. Jak1 is also associated with another protein tyrosine kinase, Pyk2 which is also phosphorylated by

IL-7 stimulation. However, the role of Pyk2 in IL-7 signaling is not known yet (Kang and Der, 2004).

The activated Jaks phosphorylate the tyrosine residue Ty449 of the IL-7Rα chain which allows recruitment of members of signal transducer and activator of transcription (STAT) proteins. STATs mediate cytokine induced responses in hematopoietic cells. Cytokine-specificity of STATs is determined by their SH2 domains that recognize different phosphorylated motifs on the cytoplasmic tails of cytokine receptors. IL-7 is shown to induce phosphorylation and activation of STAT1, STAT3 and STAT5. STAT1 and STAT3 seem not to be involved in the regulation of lymphocyte development or they have redundant roles (Hofmeister *et al.*, 1999). On the other hand, STAT5 is known to upregulate expression of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-X_L) to promote survival of lymphocytes in an IL-7 dependent manner. STAT5 refers to either of two STAT isoforms; STAT5a and STAT5b which are functionally redundant and the genes encoding for these proteins are closely linked. STAT5 binds to phosphorylated tyrosine Ty449 on IL-7Rα subunit via its SH2 domain and is phosphorylated by the Jaks (Jiang *et al.*, 2005). Phosphorylated STAT5s form homo- or hetero-dimers and translocate into the nucleus where they regulate transcription of several genes. STAT5 has been related to assembly of antigen receptors on lymphocytes by recruiting histone acetyltransferases (p300 and CBP) to the immunoglobulin gene segments. In spite of STAT5's important roles in survival and differentiation of lymphocytes, STAT5 deficiency in mice does not alter the development of lymphocytes significantly (Teglund *et al.*, 1998). Thus, IL-7 signaling through Jaks may activate other targets as well.

JAK/STAT pathway also induces expression of inhibitory proteins that provide negative-feedback of the signaling. Activated STAT5 has been shown to induce transcription of Pim-1 (a serine/threonine kinase) which can in turn inhibit STAT5 activation. Pim-1 has been suggested to inhibit STAT5 activation by increasing stability of members of suppressor of cytokine signaling (SOCS) proteins via phosphorylation. SOCS proteins compete with STAT proteins to bind to the docking sites on the cytokine receptors and they inhibit Jak activation. Two members of SOCS family; SOCS1 and CIS have been demonstrated to negatively regulate IL-7 signaling (Jiang *et al.*, 2005).

1.1.3.2 PI3 Kinase Pathway

Phosphatidylinositol-3 (PI3) kinase proteins are a family of lipid and serine/threonine kinases that are found to be important in lymphocyte development. PI3 kinase proteins can be recruited to plasma membranes by different cytokines and they phosphorylate phosphoinositides on the 3-position of the inositol ring, converting into inositol 1, 4, 5 triphosphate which is a second messenger of the signaling in the cells. Although PI3 kinases are activated by different cytokines and growth factors, IL-7Rα is the only cytokine receptor in type I receptor family which carries a direct binding site for PI3 kinase. Upon IL-7 stimulation, PI3 kinase is recruited to Ty449 residue of IL-7Rα chain via its p85 subunit and is activated by phosphorylation. Jak3 seems to mediate tyrosine phosphorylation of p85 subunit, since Jak3 is associated with p85 subunit in IL-7 activated cells (Khaled and Durum, 2002).

Although the targets of PI3 Kinase pathway are not clearly known yet, the serine/threonine protein kinase Akt (encoded by akt proto-oncogene) is known to be a key downstream substrate of PI3 Kinase. Activated Akt promotes survival by inhibiting apoptotic activity. Akt is shown to induce phosphorylation of pro-apoptotic protein Bad and inhibit its translocation to mitochondria. 14-3-3 protein binds to phosphorylated Bad protein and keeps it in the cytoplasm (Khaled and Durum, 2002). A second mechanism that Akt uses to inhibit apoptotic pathways is phosphorylation of forkhead transcription factors and inhibition of their translocation into nucleus. Once in the nucleus, forkhead transcription factors activate expression of apoptotic protein Bim and cyclin-dependent kinase inhibitor $p27^{kip1}$. Akt also induces glucose uptake through upregulation of glucose transporter proteins Glut-1 and Glut-4. It is shown that IL-7 withdrawal also reduces glucose uptake and metabolism which results in translocation of apoptotic proteins such as Bax to the mitochondria (Khaled and Durum, 2002).

PI3 Kinase pathway may be important in IL-7 regulated survival of early thymocytes to overcome inhibitory effects of phosphatase and tensin homologue (PTEN). PTEN counteracts pro-survival effects of PI3 Kinase by dephosphorylating its lipid substrates (Khaled and Durum, 2002).

1.1.4 IL-7 signaling in B-cell development

Lymphocytes diverge from hematopoietic stem cells (HSCs). During embryonic stages HSCs differentiation take place in fetal liver and spleen. After that, bone marrow becomes the major source for hematopoiesis. HSCs give rise to multipotential progenitors (MPPs) which diverge into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CMPs give rise to erythrocytes, granulocytes and megakaryocytes and CLPs give rise to B and T lymphocytes and Natural Killer (NK) cells (Janeway *et al.*, 2005).

B-cell development takes place in the bone marrow from CLPs. Pu.1 and Ikaros act at the top of genetic network that regulates B-cell development (Scott *et al.*, 1994; Georgopoulos *et al.*, 1994). Ikaros is suggested to inhibit expression of T-cell specific genes and activate expression of the receptor tyrosine kinase FLK2/FLT3 on MPPs that will give rise to early B-cell precursors (Adolfsson *et al.*, 2001). FLK2/FLT3 deficient mice show severe defects in the development of B-cell progenitors (Mackarenhtschian *et al.*, 1995). Pu.1, on the other hand is a member of ets family transcription factors that are important in hematopoiesis (Dekoter and Singh, 2000). While high levels of Pu.1 expression favor macrophage lineage, low levels of Pu.1 direct multipotent progenitors into the lymphoid lineage. Pu.1 is implicated to exert its functions in B-cell development through activation of expression of IL-7Rα in CLPs (Dekoter *et al.*, 2002; Medina *et al.*, 2004). A binding site for Pu.1 approximately 800 bp downstream of the transcriptional start site of the IL-7Rα gene has been identified and Pu.1 has been shown to activate IL-7Rα expression in pro-B cells through binding to this site (Dekoter *et al.*, 2002). In one study, it is seen that generation of B-cell precursors in mice is completely blocked when deficiency of FLK2/FLT3 receptor is combined with IL-7Rα deficiency (Sitnicka *et al.*, 2003). Thus IL-7R and FLK2/FLT3 signaling may be complementary in B-cell development.

IL-7 was first identified and cloned based on its ability to promote short-term proliferation of B-cell progenitors in the absence of stromal factors in mice (Namen *et al.*, 1988). IL-7 promotes expansion of B-cell precursors as indicated by the fact that administration of exogenous IL-7 causes an increase in mouse B-cell progenitors and may result in B-cell lymphomas (Morrissey *et al.*, 1991).

Studies with mice deficient for IL-7 or its receptor emphasize the proliferative effect of IL-7 on the B-cell progenitors undergoing B-cell receptor heavy-chain rearrangement (Peschon *et al.*, 1994). Proliferative effect of IL-7 in B-cell precursors is achieved by upregulation of anti-apoptotic genes; such as bcl-2; but it is not restricted to bcl-2 upregulation (Maraskovsky *et al.*, 1998).

The development of B-cells is divided into several stages according to the acquisition of sequential rearrangements of the B-cell receptor (BCR) immunoglobulin heavy (IgH) and light chains (IgL) and their expression on the cell surface (Hardy *et al.*, 1991). Developing B-cells as well as T-cells assemble their antigen receptors from different gene segments by a recombination event. Heavy-chain and light-chain loci contain arrays of variable region gene segments that are assembled in a different combination by somatic DNA recombination in each B-cell. These variable regions in each chain cause variations in the antigen binding regions of the BCR providing a wide range of antigen specificity. The rearrangements that result in in-frame rearrangements are called productive rearrangements (Janeway *et al.*, 2005).

Rearrangement starts at the IgH locus and involves recombination of one of Diversity (D_H) gene segments to one of Joining (J_H) gene segments first; followed by the recombination of a Variable (V_H) gene segment at the 5' end to D_H-J_H (Figure 1.2). Rearrangement of the IgH locus takes place at the pro-B cell stage. D_H-J_H rearrangement occurs at the pre-pro-B cell stage; which is followed by V_H to DJ_H rearrangement at the late pro-B cell stage. Productive VDJ_H rearrangement results in expression of heavy chain in combination with a surrogate light chain to form pre-B cell receptor (pre-BCR) which is an indicator of large pre-B cell stage. Pre-BCR signaling causes an arrest of the V_H to DJ_H rearrangement of the heavy-chain allele on the other chromosome which is called allelic exclusion. Pre-BCR induces several cell divisions resulting in small resting pre-B cells which then undergo V_L-J_L rearrangement of the IgL locus. Upon successful rearrangement of the light chain; cells develop into immature B-cells expressing the B-cell receptor (BCR) at their surface (Janeway *et al.*, 2005).

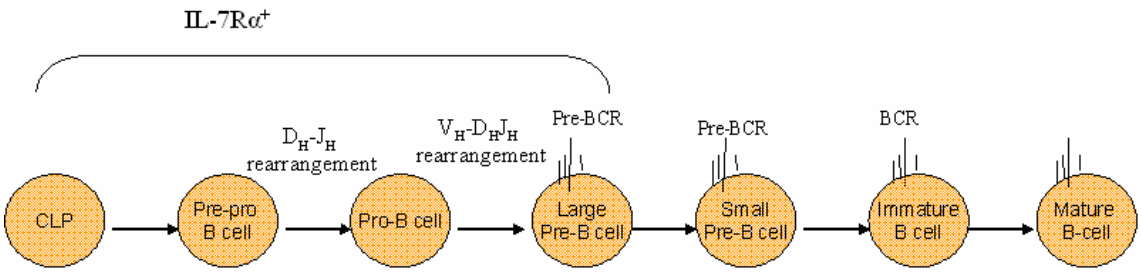


Figure 1.2: Developmental stages of B-cell development

Mouse B-cells are responsive to IL-7 at the pro-B cell stage (Lee *et al.*, 1989) (Figure 1.2). It is observed that while pre-pro-B cells that did not achieve D-J_H rearrangement can respond to IL-7 in a stromal-contact dependent manner (Hardy *et al.*, 1991); IL-7 responsiveness becomes contact-independent after D-J_H rearrangement in pro-B cells (Sudo *et al.*, 1989). Requirement for stromal contact at pre-pro B cells is explained by the identification of pre-pro-B cell-growth stimulating factor (PPBSF) which supports B-cell growth at the pre-pro-B cell stage. PPBSF is a heterodimeric cytokine that is formed by IL-7 and β chain of hepatocyte growth factor (Lai and Goldschneider, 2001).

There is an overall reduction in B-cell population with paucity at the transition from pro-B cell to pre-B cell stage in IL-7 deficient mice (von Freeden-Jeffry *et al.*, 1995). In IL-7Rα$^{-/-}$ mice, B-cells are arrested at pre-pro B-cell stage (Peschon *et al.*, 1994). This difference is likely due to the lack of TSLP signaling in IL-7Rα deficient mice since TSLP is shown to signal through IL-7Rα also. IL-7 responsiveness is gradually decreased after pre-BCR complex is formed since IL-7 does not induce proliferation of small pre-B cells or immature B cells (Hardy *et al.*, 1991). IL-7Rα expression is also downmodulated at the pre-B cell stage (Marshall *et al.*, 1998). Dependence of pro-B cells to IL-7 is due to the fact that IL-7 is involved in the regulation of rearrangement of IgH locus and promotes survival in pro-B cells.

Rearrangement is regulated by the accessibility of the locus to the proteins encoded by Recombination-activating genes (RAG) (Hesslein and Schatz 2001). RAG proteins initiate rearrangement by their endonuclease activity operating at the DNA motifs that they specifically recognize. This results in double-strand breaks in the

chromosome. And by the activity of other proteins involved in rearrangement, the gene segments of the variable region are joined together. The motifs necessary for RAG recognition and binding are similar in all rearranging loci including T-cell receptor loci. In addition, different regions of heavy-chain locus are rearranged in different stages of development (Durum *et al.*, 1998). Thus, the timing of the expression of RAG genes and the accessibility of these specific motifs to RAG proteins' binding are vital for the regulation of rearrangement in all lymphocytes. Changes in the structure of chromatin are believed to regulate the accessibility of a locus to RAG proteins. Active regions of chromatin show some characteristics that differ from inactive regions. Histones associated with active regions are acetylated and CpG dinucleotides of active regions are hypomethylated (Chowdhury and Sen, 2001).

Allelic exclusion at the IgH locus is shown to be regulated by the level of chromosome accessibility to RAG genes. It is found that in pre-B cells, IgH locus has a reduced nuclease sensitivity compared to pro-B cells (Maes *et al.*, 2001) meaning that after expression of pre-BCR, IgH locus becomes inaccessible to RAG proteins and V_H to DJ_H rearrangement is inhibited so that only one allele of heavy-chain is expressed in BCR complexes. IgH locus accessibility is regulated by the chromatin structure of three different regions of V_H gene segments (Chowdhury and Sen, 2001). Largest of these regions is V_HJ558 gene family which spans two third of the V_H gene segments (Corcoran *et al.*, 1998; Chowdhury and Sen, 2001). IL-7's role in the regulation of rearrangement of V_HJ558 genes is shown by the studies that mice deficient in IL-7R lack rearrangement of V_HJ558 genes (Corcoran *et al.*, 1998). Subsequently; Chowdhury and Sen (2001) have reported that IL-7 activates histone hyperacetylation and chromosome accessibility of V_HJ558 genes in pro-B cells. Very recently, it has been demonstrated that Oct-1 transcriptional factor can recruit STAT5 to V_HJ558 promoters as a coactivator and $STAT5^{-/-}$ knock-out mice have impaired rearrangement in V_HJ558 genes with reduced histone acetylation and germline transcription (Bertolino *et al.*, 2005). It has been shown that loss of IL-7 signaling in pre-B cell stage converts these regions back to inaccessible stage (hypoacetylated) and sustained IL-7 signaling results in the rearrangement of allelically excluded V_H genes (Chowdhury and Sen, 2003). These results altogether suggest that in mice IL-7 induces expansion of pro-B cells and rearrangement of IgH locus and at the pre-B cell stage; IL-7 responsiveness is downregulated for allelic exclusion to occur.

Despite the fact that IL-7 is very important in B-cell development in mice; such dependence to IL-7 is not observed in humans. In Severe Combined Immunodeficiency (SCID) patients that have IL-7R mutations, any reduction in B-cell numbers has not been seen (Puel *et al.*, 1998). In addition, human immature B-cells could develop in the bone marrow without a requirement to IL-7 (Prieyl and LeBien, 1996). Although human B-cell development is not dependent on IL-7; human B-cell precursors at the pro-B cell stage expand in response to IL-7 (Dittel and LeBien, 1995).

1.1.5 IL-7 signaling in T-cells

1.1.5.1 Roles of IL-7 in T-cell development

T-cell generation begins at the embryonic stages and declines by aging. T-cell development takes place in the thymus which provides a unique microenvironment in a network of thymic epithelia. Some of the common lymphoid progenitors migrate from fetal liver, bone marrow or peripheral blood to the thymus and give rise to T-cells and intrathymic dendritic cells, but these precursors are still capable of generating NK cells as well as B-cells (Donskoy and Goldschneider, 1992). Several transcription factors have been defined to regulate commitment to T-cell lineage. The most significant of these seems to be Notch-1 (Radtke *et al.*, 1999) whose over-expression in the bone marrow caused HSCs to choose T-cell lineage instead of B-cell fate (De Smedt *et al.*, 2002).

The stages in lymphoid differentiation can be followed by the expression of cell surface molecules. CLPs possess IL-7Rα, c-kit (receptor for stem cell factor; SCF), CD44 adhesion molecule and Sca-1 on their surface. Since CLP precursors do not express any of the T-cell receptor (TCR) associated surface molecules CD3, CD4 and CD8 at this stage, they are called as triple negative-1 (TN1) thymocytes (Figure 1.3). In α:β T-cells, TCR is composed of heterodimer antigen-binding subunits; α and β associated with CD3γδε and ζ invariant signaling complex and with the co-receptor CD4 or CD8. Apart from α:β T-cells; there is a minor T-cell subset bearing TCR

composed of γ and δ chains instead of α:β chains. γ:δ T-cells are a distinct lineage of T-cells differing from α:β T-cells (Janeway *et al.*, 2005).

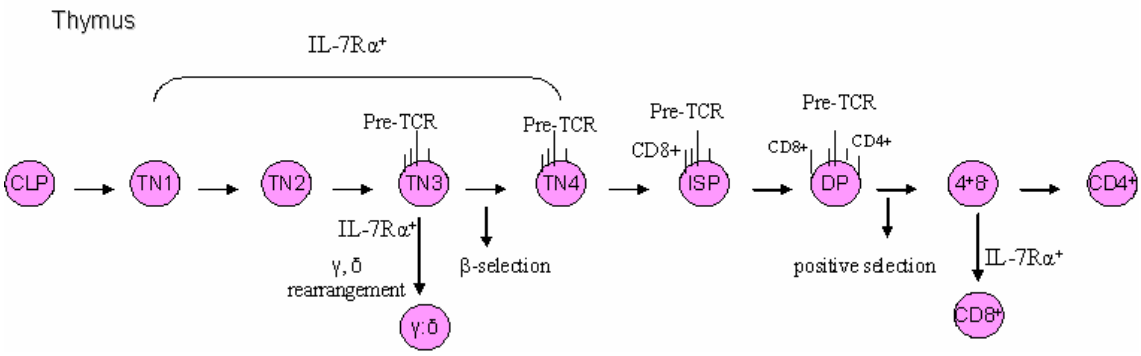


Figure 1.3: Stages of T-cell development in the thymus

Potency of TN1 ($CD44^+CD25^-$c-kit^+) thymocytes is narrowed by the expression of CD25 (β-chain of IL-2 receptor) on the cell-surface which precedes cells into TN2 ($CD44^+CD25^+$c-kit^+) stage (Godfrey *et al.*, 1993) (Figure 1.3). TN1 and TN2 thymocytes' survival and expansion is largely due to the presence of stem cell factor; SCF (Rodewald and Fehling, 1998) in conjunction with IL-7. At TN3 stage, CD44 and c-kit disappear from the cell surface ($CD44^-CD25^+$) and the precursors are fully committed to T-cell lineage. Expression of RAG genes and subsequent V-DJ rearrangement of β-chain is initiated at TN3 stage (Godfrey *et al.*, 1993). Notably, γ-chain and δ-chain rearrangements are also initiated at this stage. At the end of TN3 stage, thymocytes that successfully rearranged their β-chain transit to TN4 stage where they express pre-TCR complex (β-chain pairs with surrogate pre-TCR α-chain and is associated with CD3 signaling complex). During the transition from TN3 to TN4, β selection takes place (Hoffman *et al.*, 1996). β selection means that TN3 thymocytes should make productive rearrangements in β-chain loci so that signaling through pre-TCR begins and rescues cells from apoptosis (Fehling *et al.*, 1995; Saint-Ruf *et al.*, 1994). Pre-TCR signals in β selection commit thymocytes into α:β lineage instead of γ:δ lineage (Aifantis *et al.*, 1998). Pre-TCR signals first downregulate CD25 expression and enforce allelic exclusion. Then pre-TCR signals induce cell proliferation and maturation and lead cells to Double-Positive stage (DP) through an intermediate Immature Single-Positive stage (ISP) by first upregulating expression of co-receptor CD8, followed by

upregulation of CD4 (Wiest *et al.*, 1999). When the cell proliferation ceases, DP cells are now small and resting cells that undergo the rearrangement of α chain locus.

At small resting DP stage, cells pass from a checkpoint that their TCRs are tested for self reactivity and their capability to recognize Major Histocompatibility Complexes (MHCs). Unlike BCR, TCR can recognize antigens when presented on MHCs on the cell surface. MHCs help T-cells to discriminate between self and non-self peptides. There are two classes of MHC; MHC Class I and MHC Class II. MHC Class I molecules present peptides processed in the cytoplasm of the cell. These peptides derive from viruses. MHC Class I molecules can be found on the surface of all cell types, except red blood cells. MHC Class I molecules and the bound peptide are recognized by $CD8^+$ cytotoxic T-cells. On the other hand, MHC Class II molecules are found primarily on antigen presenting cells which are dendritic cells, macrophages and B-cells. They are recognized by $CD4^+$ helper T-cells. MHC Class II molecules present peptides that are internalized by phagocytosis or endocytosis and associated with the vesicles of the endocytic processing pathway (Janeway *et al.*, 2005).

At normal conditions, in the thymus, MHC Class I and II molecules display self peptides so that Double Positive T-cells are negatively or positively selected based on their TCR's ability and affinity to recognize self peptides. The ones which express receptors that recognize self-peptide/MHC molecules on the thymic epithelial cells with low-affinity are positively selected, that is they are permitted to survive and differentiate into single positive $CD4^+$ or $CD8^+$ T-cells. During positive selection, if TCR cannot recognize self-peptide/MHC, cells have the opportunity to undergo different rearrangements at the α-chain locus since RAG proteins are still expressed and allelic exclusion at α-chain locus is not as strict as β-chain locus. If these rearrangements are not sufficient for low-affinity interaction with self-peptide/MHC, then these cells can not receive survival signals through their TCRs and die because of neglect. If TCR on the cell surface binds self-peptide/MHC with a high affinity, then these cells are negatively selected meaning that they receive signals from TCR/self-peptide-MHC complexes to undergo apoptosis (Abbas *et al.*, 2003).

Positively selected DP cells undergo maturation and differentiation into either $CD8^+$ or $CD4^+$ lineage. The ones that recognize MHC Class II bound self peptides

differentiate into $CD4^+$ SP cells; whereas the ones that recognize MHC Class I bound peptides differentiate into $CD8^+$ SP cells. These two lineages are distinct from each other with respect to their functions in the immune system. $CD4^+$ T-cells are helper T-cells. Upon activation by the interaction between their TCR and foreign peptide/MHC Class II they can produce various kinds of cytokines that will activate macrophages or B-cells. On the other hand, $CD8^+$ T-cells produce cytotoxic effector molecules to kill the cells infected mostly by viruses; when they are activated (Abbas *et al.*, 2003).

Although IL-7 was identified as a cytokine that is important for B-cell development, soon it was realized that IL-7 can also induce proliferation of mouse T-cells (Conlon *et al.*, 1989; Murray *et al.*, 1989). In fetal thymic organ cultures, IL-7 could maintain the viability of $CD3^-CD4^-CD8^-$ T cells (Watson *et al.*, 1989; Suda and Zlotnik, 1991). Long-term treatment with anti-IL-7 monoclonal antibodies resulted in 99% decrease in thymic cellularity and an arrest of most of the thymocytes at the $CD44^+CD25^+$ (TN2) stage (Bhatia *et al.*, 1995). In $IL\text{-}7^{-/-}$ knock-out mice, thymic cellularity was decreased 20-fold and thymocytes were arrested at the same stage (Moore *et al.*, 1996). With respect to $IL\text{-}7^{-/-}$ knock-outs, in $IL\text{-}7R\alpha^{-/-}$ knock-out mice thymic cellularity was reduced to 10% of normal thymus population and most of the cells were arrested at an earlier stage that corresponds to the transition from TN1 to TN2 stage (Peschon *et al.*, 1994). This difference again seems to be correlated with impaired TSLP signaling which may be important in T-cell development as well as B-cell development. One of the mechanisms by which IL-7 maintains and induces expansion of T-cell precursors is the upregulation of expression of the anti-apoptotic genes and inhibition of the pro-apoptotic genes. In IL-7 deficient mice, overexpression of the anti-apoptotic gene *Bcl-2* (Akashi *et al.*, 1997; Maraskovsky *et al.*, 1997) or deletion of the pro-apoptotic genes *Bax* or *Bad* (Khaled, 2002) partially restored T-cell development.

These studies reveal that IL-7 maintains the survival of early thymocytes during TN stages and induces their proliferation to provide an adequate number of T-cells before a massive loss of T-cells at β selection, positive and negative selection. At TN3 stage; after pre-TCR formation -as a result of productive rearrangement at β-chain locus- expression of IL-7Rα decreases which is demonstrated by a decrease in STAT5 phosphorylation until the positive selection of the cells at the DP stage (Van De Wiele

et al., 2004). However, it should also be noted that at late TN3 stage, signaling through the pre-TCR complex is claimed to maintain a low level of IL-7Rα expression in the cell to ensure IL-7 mediated survival of the thymocyte during β selection (Trigueros *et al.*, 2003). During the transition from TN4 to DP stage through the intermediate ISP stage, IL-7Rα expression is nearly absent in the thymocytes (Van De Wiele *et al.*, 2004). The necessity for downregulation is explained by the finding that IL-7R signaling inhibits the expression of the transcription factors required for differentiation of ISP into the DP stage; namely TCF-1, LEF-1 and RORγ/ RORγt (Yu *et al.*, 2004). As expected, introduction of IL-7Rα transgene into mice resulted in a higher proportion of ISP compared to DP supporting the idea that IL-7Rα expression in ISP inhibits differentiation into DP (Yu *et al.*, 2004). It is also demonstrated that continuous expression of IL-7Rα gene in mice decreases the number of the thymocytes as a function of age (Munitic *et al.*, 2004). This decrease is related to the depletion of the limiting amounts of IL-7 whose production also decreases with age (Andrew and Aspinall, 2001). Competition for limiting IL-7 between TN thymocytes and DP thymocytes (that normally do not express IL-7Rα) causes IL-7 starvation in TN thymocytes and subsequent decrease in thymocyte population. Thus, termination of IL-7Rα expression in ISP stage is both necessary for expression of transcription factors and availability of IL-7 to TN population that are dependent on IL-7 for survival.

After cells transit to small-resting DP stage, they are negatively or positively selected as mentioned above. IL-7Rα expression is reactivated in DP cells that are designated to become single positive $CD8^+$ T-cells. DP cells are under a series of events named co-receptor reversal, to differentiate into single positive cells. Co-receptor reversal model indicates that DP cells first differentiate into $CD4^+CD8^-$ intermediates by transiently terminating CD8 expression under the influence of TCR signals. Persistence of TCR signals causes differentiation into $CD4^+$ SP cells; whereas cessation of TCR signals causes reexpression of CD8 gene accompanied by termination of CD4 expression and IL-7Rα reexpression that leads cells into CD8 SP lineage (Brugnera *et al.*, 2000). IL-7Rα reexpression seems to be important for differentiation of intermediate $CD4^+CD8^-$ into $CD8^+$ SP. It is demonstrated that IL-7 signaling maintains viability of intermediate $CD4^+CD8^-$ cells which cannot receive TCR signals anymore through upregulation of bcl-2 expression. IL-7 also contributes to termination of CD4 expression and promotes maturation of $CD8^+$ SP cells (Yu *et al.*, 2003).

Although Pu.1 is shown to regulate IL-7Rα expression in pro-B cells, termination of Pu.1 expression is necessary at the early stages of T-cell development (Dekoter and Singh, 2000). Another Ets family transcription factor GA binding protein (GABP) is demonstrated to regulate the expression of IL-7Rα in T-cells through binding to the same site that Pu.1 binds (Xue *et al.*, 2004). However, timing and importance of its regulatory activity on IL-7Rα expression throughout all stages of T-cell development remains to be established.

1.1.5.2 Roles of IL-7 in peripheral T-cell homeostasis

Peripheral T-cell population bears naïve T-cells, effector $CD4^+$ helper and $CD8^+$ cytotoxic T-cells and also memory T-cells. In normal young animals, most of the $CD4^+$ and $CD8^+$ SP cells are naive T-cells that have not yet encountered foreign peptide/self MHC molecules on antigen presenting cells (APCs). As a result, they are not yet activated and differentiated into effector cells (Janeway *et al.*, 2005). Naïve T-cells leave the thymus to the peripheral lymphoid organs such as lymph nodes and spleen and circulate throughout the body, but their circulation is regulated so that T-cells can encounter their specific antigen /MHC on APCs. Naïve T-cells in mice are distinguished by their low expression of CD44 on their surface and these cells are largely quiescent. The half-life of naïve T-cells in the periphery is estimated to be three to six months in mice and a few years in humans. If naïve T-cells do not encounter their specific antigen, they eventually die by apoptosis. Long-term survival of naïve T-cells requires environmental signals (Roitt *et al*., 2001).

Survival signals required for maintenance of naïve T-cells are mediated by TCR-self peptide/MHC low affinity interactions and cytokines. Competition for limiting amount of these signals keeps the peripheral T-cell pool at a constant size. The disruption of the signaling through TCR eliminated long-term survival of naïve T-cells in the periphery. In addition to low affinity TCR signals, naïve T-cells also require IL-7 mediated signals (Rathmell *et al.*, 2001). As T-cells leave the thymus, higher expression of IL-7Rα on the surface of the cells has been shown (Boursalian *et al.*, 2004). Culturing purified naive T-cells in vitro without any cytokines results in massive loss of these cells. Addition of IL-7 or IL-2 into T-cell cultures greatly reduces the rate of death

(Kishimoto and Sprent, 1999). Likewise, overexpression of IL-7 in mice causes a considerable increase in T-cell pool size (Kieper *et al.*, 2002).

The overall size of T-cells in the periphery is normally kept at a steady state by the generation of new cells from the thymus and the death of the cells that do not encounter their specific antigen. When large numbers of thymuses were transplanted into mice, number of T-cells in the periphery did not change significantly (Matsuyama *et al.*, 1966). The turnover of these cells without changing the overall size of peripheral T-cells is called homeostatic cycling (Fry and Mackall, 2005).

Thus, IL-7-IL-7R and TCR-self peptide/MHC interactions both promote survival of naïve T-cells and maintain their number at a constant size. This is believed to be regulated by limiting amounts of IL-7 and APCs circulating in the body. Competition for the limited amounts of survival factors keeps the T-cell pool size at a constant number. The regulation of IL-7Rα is as important as the availability of IL-7. IL-7 and some other cytokines in the hematopoietin family are shown to downregulate IL-7Rα transcription in order to prevent the consumption of limiting amount of IL-7 by the T-cells that have already received survival signals through IL-7R. This type of suppression of IL-7Rα gene is observed in the naïve T-cells and this suppression is found to be mediated by the transcriptional repressor Gfi1 in $CD8^+$ SP cells, but the mechanism of the suppression in $CD4^+$ SP cells could not be revealed. Gfi1 has been shown to bind to two sites at the intronic regions of IL-7Rα (Park *et al.*, 2004).

Under lymphopenic conditions, at which most of the T-cells are depleted, homeostatic expansion of T-cells occurs (Goldrath and Bevan, 1999). Homeostatic expansion causes dramatic increase in T-cell numbers and restoration of the peripheral T-cell pool in lymphopenic hosts. Homeostatic expansion differs from homeostatic cycling in that T-cells spontaneously proliferate. Homeostatic expansion is also triggered by IL-7. Homeostatic expansion is severely absent in lymphopenic hosts in the absence of IL-7 (Tan *et al.*, 2001).

As naïve T-cells are activated by the introduction of the specific antigen by APC, the antigen-specific T-cell clones expand up to 50,000 fold from their basal levels and differentiate into effector cells. Upon activation, antigen-specific T-cell clones

upregulate IL-2 expression. Autocrine IL-2 signaling causes expansion of these clones for an effective response. However, IL-2 also induces apoptosis of cells at the end of an immune response. Interestingly, it is shown that IL-2 signaling in activated T-cells causes downregulation of IL-7Rα via PI3 kinase-Akt pathway and other undefined pathways (Xue *et al.*, 2002). This regulation may be necessary for induction of apoptotic pathways in activated T-cells at the end of an immune response.

Thereof, expression of IL-7Rα is downregulated in activated T-cells and it is reexpressed in the cells that will become a memory cell. Memory T-cells possess high amounts of CD44 on their surface. Immunological memory is provided by long-lived antigen-specific lymphocytes that were once activated by the exposure to the specific antigen and survive in the peripheral tissues in a semiactivated phenotype (Fry and Mackall, 2005). After first exposure to an antigen, numbers of T-cells specific for the given antigen expand remarkably, and then most of them die, but some of these cells persist in the body for the rest of the life as memory T-cells. In young animals, memory T-cells form 10-20 % of the T-cell population. By aging, memory T-cells start to outnumber naïve T-cells. Unlike naïve T-cells, survival of memory cells seems to be independent of TCR signaling since memory T-cells can persist in $MHC^{-/-}$ hosts (Swain *et al.*, 1999). IL-7Rα is reexpressed on both $CD4^+$ and $CD8^+$ memory T-cells (Tan *et al.*, 2001). IL-7 is essential for both survival and homeostatic divisions of memory cells (Schluns *et al.*, 2000) and overexpression of IL-7 can alone be sufficient for maintenance of memory $CD8^+$ cells (Kieper *et al.*, 2002). On the contrary, in the absence of IL-7, there is a reduction in the number of memory $CD8^+$ T-cells generated after an antigenic response (Ku *et al.*, 2000). In addition, it is also demonstrated that effector $CD8^+$ cells that have high levels of IL-7Rα expression at the peak of $CD8^+$ T cell response, preferentially turn into memory $CD8^+$ cells (Kaech *et al.*, 2003). Although, the role of IL-7 in survival of $CD4^+$ memory cells has not been clearly established, there is a lot of evidence that IL-7 also contributes maintenance of $CD4^+$ memory cells (Kondrack *et al.*, 2003; Li *et al.*, 2003; Seddon *et al.*, 2003).

1.1.5.3 Roles of IL-7 in γ:δ T-cell development

γ:δ T-cells recognize antigens independent of presentation by MHC. Although their exact functions are not well identified; it is known that they bind certain kind of ligands such as heat-shock proteins, nonpeptide antigens such as pyrophosphate derivatives, mycobacterial lipid antigens (Janeway *et al.*, 2005).

The first T cells that appear at embryonic stage are γ:δ T cells which populate in certain tissues such as epidermis, reproductive epithelium and intestinal epithelium. γ:δ cells that reside in the epidermis are named as dendritic epidermal T cells and the ones that are found in the gut are known as intraepithelial lymphocytes. After birth, thymocytes that are committed to α:β lineage predominate although γ:δ T-cells are still produced (Janeway *et al.*, 2005).

α:β and γ:δ T-cell lineages diverge from common thymocyte progenitors. There are four types of gene loci that contain genes encoding for TCR; α, β, γ and δ. Rearrangement in γ, δ and β loci occur simultaneously in TN3 cells. The fate of a thymocyte is determined based on the fact that the productive rearrangement at the γ-chain and δ-chain loci or β-chain locus takes place first (Figure 1.3). As a result of productive rearrangement at the β locus, a pre-TCR complex is expressed; and subsequent signaling results in the inhibition of the rearrangement of the γ and δ gene segments. In most of the T-cells, β-chain is rearranged before γ and δ-chains complete their rearrangements. As a result, 95% of thymocytes appear to be α:β T-cells (Janeway *et al.*, 2005).

IL-7 has an indispensable role in γ:δ T-cell development which is documented by several studies that show mice deficient in IL-7 or its receptor or IL-7 downstream signaling component Jak3 lack γ:δ T-cells both in the thymus and peripheral tissues such as skin, intestinal epithelium and spleen completely (Moore *et al.*, 1996; He and Malek, 1996; Park *et al.*, 1995). In contrast to γ:δ T-cells, α:β T cells were still present in these deficient mice albeit in a reduced number. These observations indicate that IL-7 has different effects on the development and survival of the γ:δ T-cell and α:β T-cell lineages.

IL-7 is shown to be involved in the control of the rearrangement of the TCR γ-chain locus (Durum *et al.*, 1998; Maki *et al.*, 1996; Schlissel *et al.*, 2000). IL-7 is believed to control the rearrangement of γ-chain locus by promoting the acetylation of the histones resulting in unpacking of nucleosomes (Agata *et al.*, 2001; Huang *et al.*, 2001); demethylation of the locus (Durum *et al.*, 1998) and inducing RAG expression (Muegge *et al.*, 1993) all of which cause accessibility of γ-chain locus to recombination machinery.

The molecular mechanisms that govern IL-7 mediated regulation of γ-chain locus are not well known although there are some candidates such as STAT5. There are a number of consensus STAT5 binding motifs in γ-chain locus that STAT5 has been shown to interact to induce histone acetylation and germ-line transcription from V region of γ-chain locus (Ye *et al.*, 1999; Ye *et al.*, 2001). Germ-line transcription is the transcription of receptor chain locus that has not been rearranged. Germ-line transcripts are used as a marker of a rearranging locus; because the locus becomes accessible to recombinational and transcriptional machinery along with germ-line transcription. In these studies (Ye *et al.*, 1999; Ye *et al.*, 2001) it has been shown that STAT5 overexpression restores the rearrangement of TCR γ-chain locus in IL-7R deficient T-cell precursors. It should be noted that these results are obtained from fetal thymocytes that are deficient in IL-7R. There are also reports against the role of STAT5 in IL-7 mediated γ-chain rearrangement (Porter *et al.*, 2001; Kang *et al.*, 2004) which emphasize the necessity of investigating IL-7 downstream components other than STAT5 that may be involved in TCR γ-chain locus accessibility.

1.2 Identification of *cis*-acting regulatory elements

1.2.1 *Cis*-acting regulatory elements

Cis-acting regulatory elements are control regions that regulate transcription of genes. *Cis*-acting regulatory elements that activate transcription of genes include proximal promoters, core promoters and enhancers. Core promoters contain DNA

sequence motifs that reside within -40 to 40 bp relative to the transcriptional initiation site and specify the site of transcriptional initiation by binding to basal transcription machinery. Proximal promoters reside within a couple of hundred base pairs (bp) with respect to the transcription initiation site and contain multiple recognition sites for sequence-specific DNA binding transcription factors (Blackwood and Kadonaga, 1998).

Enhancers are *cis*-acting DNA sequences that can activate the transcription in a specific cell type or in a specific stage of development. Enhancers can activate transcription independent of their orientation and their relative distance to the transcriptional start site. The enhancers that activate transcription can reside either several kilobases away from promoters or in the intronic regions of the genes. The enhancers may be located at the upstream or downstream of the gene that they regulate (Blackwood and Kadonaga, 1998). Enhancers can activate gene transcription in several ways. They contain modules that are recognition sites for different transcription factors. Transcriptionally inactive genes are organized in tightly packed chromatin structures and inaccessible to binding of transcriptional factors. Enhancers can recruit transcriptional factors carrying enzymatic activities such as acetylation of histones that can unpack nucleosomes and make the promoter region accessible to basal transcriptional machinery. Thus, enhancers may counteract the repression created by chromatin structure and initiate transcription of their target genes. Enhancers may recruit transcription factors to the promoter possibly by forming DNA loops so that the basal transcription machinery can interact with the transcription factors (Blackwood and Kadonaga, 1998).

1.2.2 Bioinformatic approaches

Bioinformatic tools are extremely valuable for the identification of such *cis*-acting regulatory elements because conserved noncoding sequences detected by the comparative analysis of the genomes of different species have shown that a high number of *cis*-acting regulatory elements are located at these conserved noncoding sequences. The conservation of gene order and position of *cis*-acting regulatory elements in different species may explain the conserved functions of genes in different species. In order to identify conserved noncoding sequences that may regulate the

transcription of the gene of interest, the orthologs of genes in other species should be defined because it is most likely that the regulation of functionally conserved genes are conserved as well. Sequence comparison analysis of ortholog genes and flanking regions are done by alignments (Nardone *et al.*, 2004).

AVID is a global alignment program available on the online VISTA Server (http://www-gsd.lbl.gov/vista). The alignment done by AVID is processed by VISTA to generate graphical representations of the conserved regions between the two aligned sequences. The program eliminates the exons of the genes and the repeat sequences found in the genomic region before it gives the conservation between two aligned sequences. Therefore, the conserved regions given by the program reside in the noncoding regions including introns of the genes. The program plots the percent identity between the aligned sequences based on one of the aligned sequences. The base sequences and their positions in the genomic region are represented at the horizontal axis of the graphic. VISTA shows the percent identity between two sequences by sliding a fixed 'window' over an alignment (Nardone *et al.*, 2004).

1.2.3 Genetic reporter systems

Conserved noncoding regions determined by bioinformatics approaches should be experimentally evaluated for possible regulatory functions. One of the functional assays to identify *cis*-acting regulatory elements is genetic reporter assay. Genetic reporter assays take advantage of the genes whose transcription, expression or enzymatic activity can be quantitatively detected, so these genes are used in reporter assays as an indicator of transcriptional activity resulting from an enhancer or promoter activity. The genomic regions that will be analyzed are cloned into reporter vectors and the resulting vectors are transfected into appropriate cell lines. Actively transcribed reporter genes are either detected by measurement of the protein or mRNA or, in the case of enzymatic reporter genes, by assaying the catalytic activity of the reporter enzyme. Reporter genes commonly used to analyze *cis*-acting regulatory elements are chloramphenicol acetyltransferase (CAT), β-galactosidase, alkaline phosphatase, luciferase and green fluorescent protein (GFP) (Sambrook *et al.*, 2001).

Luciferase reporter assays are more sensitive than other commonly used reporter assays because the half-life of luciferase is very short and its basal level activity is very low. Luciferase activity can rapidly change according to its transcription rate. Thus, luciferase assays allow the detection of the transcriptional activities of weak promoters and enhancers. Luciferase assays also allow the analysis of transcriptional activities in the cells that are inefficiently transfected with reporter vectors (Wood, 2004).

Luciferases are the enzymes which catalyze chemical reactions that generate a flash of light in bioluminescent organisms. One of the best studied luciferases is the luciferase of North American firefly *Photinus pyralis*. Firefly luciferase (*Photinus* luciferin 4-mono-oxygenase) catalyzes the oxidative decarboxylation of D(-) luciferin (6-hydroxybenzothiazole) in the presence of ATP-Mg^{2+} to generate oxyluciferin and light (Figure 1.4) (Sambrook *et al.*, 2001).

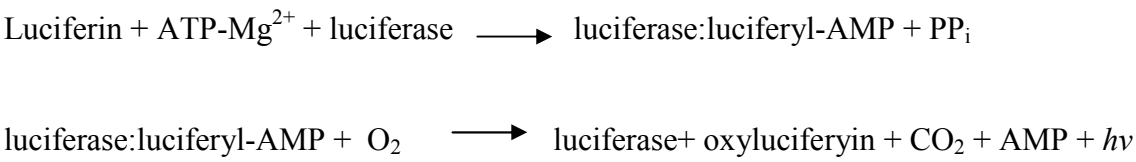


Figure 1.4: Two-step chemical reaction catalyzed by the firefly luciferase

The luciferase reporter assays are based on the detection of light generated by the luciferase catalyzed reaction. A flash of light is generated 0.3 sec after the enzyme and substrate is combined. The light decays rapidly within a few seconds due to slow enzymatic turnover and product inhibition by pyrophosphate. The emitted light is detected by luminometers or liquid scintillation counters (Sambrook *et al.*, 2001).

2 PURPOSE OF THE STUDY

IL-7 signaling is essential and indispensable at the early stages of development of T-cells and B-cells in mice. IL-7 signaling is also critical for T-cell development in humans, but it can be dispensable for the growth of human B-cells. IL-7 not only promotes survival and proliferation of lymphocytes, but also regulates some differentiation events at different stages of development. IL-7 signaling is required for the V to D-J rearrangement of heavy-chain locus in B-cells and rearrangement of γ-chain locus in T-cells. Thus, IL-7 contributes to the assembly of antigen receptors in lymphocytes. The necessity for IL-7 signaling is not restricted to the thymus and bone marrow where the development of T-cells and B-cells take place. Although IL-7 signaling is not required for B-cells after the assembly of the antigen receptor, IL-7's presence is still required for the T-cells that leave the thymus to the periphery. IL-7 maintains the naïve T-cells circulating in the periphery and regulates survival and proliferation of memory T-cells (Fry and Mackall, 2005).

Although essential and diverse roles in different stages of lymphocyte development have been attributed to IL-7, the mechanisms that regulate the production of IL-7 from stromal cells and the expression of the IL-7Rα chain on the lymphocytes are poorly defined. Regarding the IL-7Rα chain gene, two ets family transcription factors Pu.1 and GABP are shown to bind to an ets motif found 182 bp 5' of the translation start codon and activate IL-7Rα expression (Dekoter and Singh, 2000; Xue *et al.*, 2004). Pu.1 is an essential transcription factor regulating B-cell development, but the expression of Pu.1 is diminished at the early stages of T-cell development. GABP is suggested as a regulator of transcription of IL-7Rα gene in T-cells (Xue *et al.*, 2004). However, regulation via GABP can partially explain the tight regulation of IL-7Rα expression in different developmental stages of T-cells. IL-7Rα is first expressed on

common lymphoid progenitors and is found at the surface of TN cells as well as pro-B cells. IL-7Rα expression is down modulated as the pro-B cells proceed to the pre-B cell stage. In T-cells, IL-7Rα expression gradually decreases at the TN4 stage and diminishes at the intermediate single positive stage. IL-7Rα is reexpressed as the cells transit from double positive stage to $CD8^+$ single positive stage through $CD4^+CD8^-$ stage. IL-7Rα is also present on naive T-cells that leave the thymus to the periphery. IL-7Rα expression ceases in T-cells that are activated in response to an antigen. At the end of the immune response, the expression of IL-7Rα is upregulated in T-cells that are designated to become a memory T-cell.

In order to have a better understanding about the cell-specific and stage-specific expression of IL-7Rα, we aim to identify new *cis*-regulatory elements that are likely acting on the IL-7Rα gene. We think that enhancers may be responsible for the differential expression of IL-7Rα at distinct developmental stages. For this purpose, we will investigate the downstream of mouse IL-7Rα gene spanning 80 kb region via bioinformatics tools and identify noncoding regions downstream of the IL-7Rα gene that are conserved between mouse and human. Conserved noncoding regions will be tested for a transcriptional activity by luciferase reporter assays. These conserved regions will be PCR amplified and cloned into luciferase reporter vectors. Vectors constructed in this way will be transfected into mouse $CD4^+$ SP cells and analyzed for transcriptional activation of luciferase enzyme under the effect of conserved noncoding sequences.

3 MATERIALS & METHODS

3.1 Materials

3.1.1 Chemicals & media components

Chemicals and media components used throughout the project are listed in Appendix A.

3.1.2 Equipments

Equipments used are listed in Appendix B.

3.1.3 Molecular biology kits& enzymes

Molecular biology kits and enzymes used are listed in Appendix C.

3.1.4 Vectors

Vectors used are listed in Appendix D.

3.1.5 Oligonucleotides and DNA molecular weight markers

Oligonucleotides and DNA molecular weight markers are listed in Appendix E.

3.1.6 Buffers & solutions

Buffers and solutions prepared are listed in Appendix F.

3.1.7 Cell types & growth media

3.1.7.1 Bacterial strains

E.coli DH5α strain	Bio LabI, Sabancı University
E.coli DH10 strain transformed with RP23-365P6 BAC Clone	Children's Hospital Oakland Research Institute, USA

3.1.7.2 Growth media for bacterial cells

3.1.7.2.1 Solid medium

40 g of Luria Agar is dissolved in 1 L of distilled water and autoclaved. If *E.coli* DH5α is transformed with a vector, appropriate antibiotics (ampicillin or kanamycin) that the vector carries as a selection marker is included in Luria Agar at a final concentration of 100 μg/ml. For growth of *E.coli* DH10, chloramphenicol (selection marker that BAC clone carries) is included in Luria Agar at a final concentration of 12.5 μg/ml according to supplier's instructions. Luria Agar is poured on Petri dishes and cooled down.

3.1.7.2.2 Liquid medium

20 g of Luria Broth is dissolved in 1 L of distilled water and autoclaved. If necessary, appropriate antibiotics are included in Luria Broth as in Luria Agar at a final concentration of 100 µg/ml.

3.1.7.3 Mammalian cell lines

EL4 murine T lymphoma cell line and 293 T cell line (human kidney fibroblasts) are used.

3.1.7.4 Growth media for mammalian cells

EL4 cells are grown in RPMI 1640 supplemented with 2 mM L-glutamine, 5 % FBS, 100 unit/ml Penicillin and 100 unit/ml Streptomycin.
293 T cells are grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 10% FBS, 100 unit/ml Penicillin and 100 unit/ml Streptomycin.

3.1.8 Software & web-based applications

Primer3	http://frodo.wi.mit.edu/primer3
UCSC Genome Browser	http://www.genome.ucsc.edu
AVID-VISTA	http://www-gsd.lbl.gov/vista
SoftMax Pro4.3	Molecular Devices Inc., USA
VectorNTI 9.1.0	Invitrogen, USA

3.2 Methods

3.2.1 Cell cultures

3.2.1.1 Culture of *E.coli* DH10 & *E.coli* DH5α

3.2.1.1.1 Solid culture

Since *E.coli* DH10 contained RP23-365P6 BAC clone with the chloramphenicol resistance gene, cells were grown on Luria Agar + chloramphenicol plates at 37°C for 18 hrs.

E.coli DH5α cells were grown on Luria Agar plates at the same conditions. If the cells were transformed, they were grown with the appropriate antibiotics (ampicillin or kanamycin).

3.2.1.1.2 Liquid culture

E.coli strains were grown in Luria Broth for 16 hrs at 37°C with moderate shaking (270 rpm). Where necessary, antibiotics were included.

3.2.1.2 Mammalian cell cultures

EL4 cells were grown at 37°C and in a 5% CO_2 atmosphere in RPMI 1640 (supplemented as described in Materials 3.1.7.4). Cell cultures were split at a ratio of 1:10 once in 3 days.

293 T cells were grown at 37°C and in a 5% CO_2 atmosphere in DMEM (supplemented as described in 3.1.7.4). Cell cultures were trypsinized and split at a ratio of 1:10 once in 3 days.

3.2.2 Measuring EL4 and 293 T cell viability by trypan blue- exclusion method

To determine the viable cell numbers in culture, 10 µl of culture was taken and mixed with 10 µl of trypan blue dye (0.4% w/v) and incubated for a few minutes. 10 µl of the mixture was placed on a hematocytometer and covered with cover slip. Alive cells that did not permit trypan blue and appeared translucent were counted.

3.2.3 Agarose gel electrophoresis

0.2 volume of 6X loading dye was added to 1 volume of DNA samples. DNA fragments were run in 1% agarose gels at 100 V for 1hr.

3.2.4 Gel extraction of DNA fragments

DNA fragments were extracted from agarose gels into 50 µl Elution Buffer (EB) according to Qiaquick Gel Extraction Protocol.

3.2.5 Competent cell preparation

Inoculum from a single colony of *E.coli* DH5α Luria Agar streak was taken into 50 ml Luria Broth and grown. 4 ml of this culture was inoculated into 500 ml Luria Broth and grown until the optical density of the culture at 600 nm reached 0.375. *E.coli* DH5α competent cells were prepared according to Ausubel et *al*., 2002.

3.2.6 Transformation of *E.coli* DH5α competent cells

Transformations of competent *E.coli* DH5α cells with 10 to 50 ng of pGL3 Basic, Δ56fosCAT, pSP72μ170, pGL3 Enhancer, pMaxGFP were done according to Sambrook et *al.,* 2001.

3.2.7 Selection of transformants

Transformed *E.coli* DH5α cells were spread onto Luria Agar plates containing the appropriate selection marker. pMaxGFP transformed *E.coli* DH5α cells were spread on Luria Agar + kanamycin plates. Other transformants mentioned were selected with Luria Agar + ampicillin plates.

3.2.8 Isolation of BAC clones and plasmids

BAC clones were isolated from *E.coli* DH10 bacteria according to Sambrook *et al*., 2001. Plasmids were isolated from transformants (*E.coli* DH5α) according to Sambrook *et al.,* 2001 or Qiaprep Miniprep plasmid purification kit Handbook or Qiagen Midi plasmid purification kit Handbook. Concentrations of plasmids isolated were determined by measuring their optical densities at 260 nm.

3.2.9 Identification and amplification of conserved noncoding regions at the downstream of mouse IL-7Rα gene

Downstream of mouse IL-7Rα gene spanning 80 kb region was aligned with downstream regions of human IL-7Rα genes. Pairwise alignments were done using the AVID program. The alignment results were processed by VISTA program to evaluate the conservation level of the aligned sequences. The eight conserved noncoding regions determined (namely; Cap 1, Cap 2, Cap 3, Cap 4, Cap 5, Cap 6, Cap 7, Cap 8) were amplified from BAC clone RP23-365P6 with the primers designed for each conserved noncoding region by Primer3 online software (Primer sequences are in Appendix E and

sequences of noncoding regions that are amplified are in Appendix H). Primers were designed to have *Sal* I restriction sites at the 5' termini. The names of the primers were given according to the region they were designed for. For example; Cap 1 forward and reverse primers were designed to amplify Cap 1 conserved noncoding region. PCR components were prepared as follows:

PCR components	Volume	Final Concentrations
BAC clone RP23-365P6	3 µl	
dNTP Mix ,10 mM	0.5 µl	0.2 mM
$MgCl_2$, 25mM	2 µl	2 mM
10X Polymerase Reaction Buffer	2.5 µl	1X
Taq DNA Polymerase (5u/µl)	0.125 µl	0.025 u/ µl
Forward Primer, 10 µM stock	2 µl	0.8 µM
Reverse Primer, 10µM stock	2 µl	0.8 µM
Sterile Nuclease-free water	12.875 µl	
Total	25 µl	

PCR amplification of the mouse conserved regions were carried out at the conditions shown below:

PCR conditions:

Initial Denaturation:	2 min. at 95°C	
Denaturation:	0.5 min at 95°C	30 cycles
Annealing:	1 min at 56°C	
Extension:	2 min at 72°C	
Final Extension:	5 min at 72°C	
Hold	at 4°C	

Eight PCR fragments were run in 1% agarose gels and extracted from agarose gels into 50 µl Elution Buffer (EB). *Sal* I restriction sites created by the primers at the ends of the PCR fragments were digested with *Sal* I enzyme to create sticky ends:

PCR fragment in EB	50 µl
Sal I (10u/µl)	2 µl
Buffer D	6 µl
Distilled H_2O	2 µl
Total	60 µl

Digestions were carried out for 2 hrs at 37°C and *Sal* I enzyme was heat inactivated at 65°C for 20 min.

3.2.10 Vector constructions

3.2.10.1 Restriction enzyme digestions

Isolated plasmids were digested for the vector constructions as shown in the table 3.1.

vector	Length (bp)	Digested with	Insert to be ligated	Insert DNA cut from	Insert taken out by	Vector constructed
pGL3 Basic	4818	*Hind* III	fos promoter (165 bp)	Δ56fosCAT (5200 bp)	*Sal* I + *Xba* I	pGL3fos (4996 bp)
pGL3fos	4996	*Sal* I + *BamH* I	µ170 Enhancer (221 bp)	Psp72µ170 (2632 bp)	*Bgl* II + *Sal* I	pGL3fos-µ170 (5217 bp)
pGL3fos	4996	*BamH* I + *Nco* I	SV40 Enhancer + *luc*+ (2164bp)	pGL3 Enhancer (5064 bp)	*BamH* I + *Nco* I	pGL3fos-SV40 (5242 bp)
pGL3fos	4996	*Sal* I	PCR fragment			

Table 3.1: Restriction Enzyme Digestions of Vectors for Ligations:

In the last row of the table pGL3fos vector was digested with *Sal* I enzyme to perform insertion of *Sal* I digested PCR fragments (as described in section 3.2.9) into pGL3fos vector. Restriction enzyme digestion mixtures shown in the table 3.1 were prepared by adding the appropriate buffers given in Appendix C at a 1:10 ratio. Restriction enzyme units in the reaction mixture were adjusted to 1:10 or less of the total volume. The amount of vectors and insert DNA fragments required for adjusting 1:3 (vector:insert) molar ratio in ligation reactions were taken into consideration while determining DNA amounts included in the restriction digestions. Restriction digestions were carried out for 1.5- 2 hrs at 37°C.

3.2.10.2 Dephosphorylation of 5' overhangs of vectors

At the end of the digestions, phosphate groups at 5' termini of the vectors; pGL3 Basic and pGL3fos were removed by calf intestinal alkaline phosphatase (CIAP) to prevent self-circularization and religation of the vectors.

Dephosphorylation of 5' Overhangs:

Restriction digestion mixture	50µl
CIAP (diluted to 1 unit/µl)	0.5µl
10X CIAP Buffer	5.5µl
Total	56µl

Dephosphorylation reactions were done at 37°C for 30 min. Linearized and dephosphorylated vectors shown in the table 3.1 were extracted from agarose gels to remove restriction enzymes and salts. Insert fragments were also extracted from agarose gels into 50 µl EB.

3.2.10.3 Generation of blunt-ended DNA

5' protruding ends of pGL3 Basic vector (which was generated by *Hind* III digestion) and fos promoter (generated by *Sal* I and *Xba* I digestions) were filled in by the use of DNA Polymerase I Large (Klenow) fragment. Blunt-ends were generated as:

pGL3 Basic (1.25 µg) or fos promoter (125 ng) in EB	50 µl
Klenow fragment, 5 u/ µl	1 µl
Klenow 10X Buffer	10 µl
Acetylated BSA, 10mg /ml	0.5 µl
dNTP mix, 10 mM of each	1 µl
Distilled H_2O	37.5 µl
Total	100 µl

Blunt-ending reactions were performed at 24°C for 30 min. Blunt-ended pGL3 Basic and fos promoter were purified from enzymes and dNTPs by Qiaquick PCR purification kit according to the instructions of the manufacturer.

3.2.10.4 Ligations

The pairs of inserts and vectors to be ligated are shown in the table 3.1. In addition, *Sal* I digested PCR fragments were inserted to pGL3fos vector linearized with *Sal* I enzyme. Molar ratio of vector:insert was adjusted to 1:3 in ligations as described in Sambrook *et al.*, 2001. Amount of vectors were about 100 ng in the ligations and the amount of inserts were calculated accordingly. The ligation of blunt-ended DNA fragments and the ligation of DNA fragments with sticky ends were performed at different conditions.

3.2.10.4.1 Blunt-end ligations

Ligations were performed at 16°C for 18 hrs. Self-ligation of pGL3 Basic was also performed as a control. Ligation mixtures were prepared as:

pGL3 Basic (blunt-ended at HindIII site)	5 µl (125 ng)
fos promoter (blunt-ended at SalI and XbaI sites)	8 µl (20 ng)
T4 DNA Ligase (3 u/ µl)	2 µl
10X Ligation Buffer	2 µl
PEG 4000 (%50 w/v)	1 µl
Sterile distilled water	2 µl
Total	20 µl

3.2.10.4.2 Ligation of sticky ends

Restriction digestions other than *Hind* III digest of pGL3Basic and *Sal* I-*Xba* I digest of Δ56fosCAT vectors that are listed in the table 3.1 generated compatible ends for ligations.

In addition to these ligations; *Sal* I sticky ends of pGL3fos were ligated with PCR fragments that are *Sal* I digested. Vectors constructed by the insertion of PCR fragments

into *Sal* I site of pGL3fos were named according to the name of the PCR fragment inserted.

Ligation mixtures:

Vector DNA	~5 µl (100 ng, n moles)
Insert DNA	X µl (3n moles)
T4 DNA Ligase (3u/ µl)	1 µl
10X Ligation Buffer	2 µl
Sterile distilled water	up to 20 µl
Total:	20 µl

Ligations of sticky ends were carried out at 22°C for 3 hrs. Self-ligations of vectors were also performed as a control. 10 µl of ligation mixtures were transformed to *E.coli* DH5α and spread on Luria Agar + ampicillin plates and grown.

3.2.10.5 Analysis of vector constructs

Colonies formed on Luria Agar + ampicillin plates were checked for transformations with successful ligations. Colonies were analyzed by two means; colony PCR and diagnostic digestions with restriction enzymes.

3.2.10.5.1 Colony PCR

Vectors constructed by the insertion of PCR fragments into *Sal* I site of pGL3fos were analyzed by colony PCR. After the ligations were transformed into *E.coli* DH5α and grown on Luria Agar + ampicillin plates, separate colonies transformed with pGL3fos-Cap (1…8) vector constructs were picked up by sterile toothpicks into 8.5 µl sterile H_20 in PCR tubes and swirled briefly in the PCR tubes. Replica plates were also streaked with these toothpicks. Inoculations from separate single colonies in the PCR tubes were held at 95°C for 8 min. PCR mixture (same as in 3.2.9) was added to a final volume of 25 µl. Primers included in PCR mixtures were the same primers described in 3.2.9. Primers designed to amplify each PCR fragment that was inserted into pGL3fos

vector were used in colony PCR. PCR conditions were set up as the same in 3.2.9. Colony PCR products were analyzed by 1% agarose gel electrophoresis.

3.2.10.5.2 Diagnostic digestions

Single colonies were picked up into liquid media with ampicillin and grown. Plasmids were isolated and digested with restriction enzymes to confirm successful vector constructions. While choosing restriction enzymes, restriction sites uniquely created via the insertion of the DNA fragment of interest into the vector backbone were taken into consideration (maps of constructed vectors are given in Appendix G). Restriction digestions to cut out the inserted fragments were also done.

Single Restriction Enzyme Digestions	Double Restriction Enzyme Digestions
2 µl vector	2 µl vector
0.5 µl Restriction Enzyme (5 unit)	0.5 µl first Restriction Enzyme (5 unit)
-	0.5 µl second Restriction Enzyme (5 unit)
1 µl 10X appropriate Enzyme Buffer	1.5 µl 10X appropriate Enzyme Buffer
6.5 µl sterile distilled H_2O	10.5 µl sterile distilled H_2O
Total: 10 µl	Total: 15 µl

Table 3.2: Components for diagnostic digestions

Digestions were carried out at 37°C for 1.5 hrs. 2 or 3 µl 6X Loading dye was added to digestion mixtures and checked by agarose gel electrophoresis. Diagnostic digests that were performed for the vectors construsted are given in table 3.3.

Vector constructs	Diagnostic Digests	
	1. digest	2. digest
pGL3fos	*Hind* III+*Nco* I	*Pvu* II + *Sph* I
pGL3fos-µ170	*Sal* I + *Xba* I	-
pGL3fos-SV40	*Hind* III+*Xba* I	*Pvu* II + *Sph* I
pGL3fos-Cap1	*Sal* I	*Pvu* II
pGL3fos-Cap2	*Sal* I	*Xmn* I
pGL3fos-Cap3	*Sal* I	*Sph* I
pGL3fos-Cap4	*Sal* I	*Pvu* II
pGL3fos-Cap5	*Sal* I	*Xba* I
pGL3fos-Cap6	*Sal* I	*Pvu* II
pGL3fos-Cap7	*Sal* I	-
pGL3fos-Cap8	*Sal* I	-

Table 3.3: Diagnostic digests for vector constructs

3.2.11 Transient transfections of mammalian cells with plasmid DNAs

3.2.11.1 Calcium-phosphate mediated transfection of 293 T cells

293 T cell lines were transiently transfected with pMaxGFP and pGL3fos-SV40 vectors. Transfection of plasmid DNAs into 293 T cells was achieved by formation of a coprecipitate of calcium phosphate and DNA.

293 T cells are grown to 65-75% confluency and cells are harvested by trypsinization. They are replated on 60-mm tissue culture dishes. Total cells to be replated are adjusted to four different numbers ($2x10^6$, $1x10^6$, $0.5 x10^6$ and $0.25 x10^6$). Cultures on 60-mm dishes are incubated for 24 hrs at 37°C and 5% CO_2 and the medium is changed 1 hr before transfection.

Calcium phosphate-DNA coprecipitate was prepared as: 25 µl of 2.5 M $CaCl_2$, 6.5 µl of plasmid DNA (~6.5 µg) and 218.5 µl of 0.1X TE (pH 7.6) were combined with 250 µl of 2X HEPES-buffered saline and the solution was incubated at room temperature for 1 min. The solution (total: 500 µl) was transferred to culture dishes. Dishes were rocked to mix the solution with the medium. Culture dishes were transferred back to the incubator for 48 hrs.

3.2.11.2 Transient transfection of EL4 cells with plasmid DNAs

Transfection of EL4 cells was mediated by two methods; by DEAE-Dextran and by electroporation.

3.2.11.2.1 DEAE-Dextran mediated transfection

A total of 10^7 EL4 cells grown to a density of $5x10^5$ or 10^6cells/ml were harvested, centrifuged at 1000 rpm for 5 min and resuspended in 5 ml of 1X STBS solution (supplemented with 10 µl of $MgCl_2$-$CaCl_2$ solution). Cells were pelleted again at 1000 rpm and resuspended in a mixture of 1.2 ml of STBS solution, 60 µl of DEAE-Dextran (10 mg/ml) and three different amounts of DNA, 5 µg, 10 µg or 15 µg of plasmid DNA.

EL4 cells were incubated in this solution at room temperature for four different lengths of time; 5 min, 10 min, 15 min or 20 min. Cells were then washed once again with 5 ml of STBS solution and cells were either treated with chloroquine or DMSO. Cells were either transferred into 9ml RPMI medium containing 0.01% chloroquine (5.16 mg/ml) and incubated at 37°C and 5% CO_2 for 45min or they were incubated with 15% DMSO for 3 min. Cells were then pelleted and transferred into fresh RPMI 1640 medium and incubated for 48 hrs at 37°C and 5% CO_2.

3.2.11.2.2 Electroporation

EL4 cells were also transfected by electroporation. Cells were harvested at mid-late growth phase and pelleted at 1000 rpm for 5 min. Cells were resusupended in 5 ml of serum-free RPMI 1640 and repelleted. EL4 cells were then resuspended in 500 µl of serum-free RPMI 1640 mixed with 20 or 25 µg of plasmid DNA and incubated for 10 min at room temperature. Cells were transferred into electroporation cuvettes and electroporated at different field strengths and different length of pulses. Cells were then transferred back to culture plates and incubated for 48 hrs.

3.2.12 Luciferase reporter assays

Cells transfected with luciferase reporter vectors were lysed 48 hr after transfection. Cells were lysed in 400 µl of the reporter lysis buffer (RLB) provided by Promega Luciferase Assay System and incubated for 15 min. Cell debris was pelleted at 12000x *g* for 1.5 min at 4°C in a microcentrifuge. Supernatants were removed for luciferase assays. An aliquot of supernatants were taken for Bradford assays. 50 µl of supernatant was mixed with 100 µl of luciferase reagent (supplied in luciferase assay system) in black 96-well plates and immediately inserted in the luminometer to detect the light generation.

3.2.13 Protein content determination by Bradford assays

Standard curves for Bradford assays were plotted by measuring absorbance of serial dilutions of bovine serum albumin (BSA) mixed with Bradford reagent at 595 nm (Figure 3.1). For determination of protein concentration in cell lysates, 5 µl of cell lysates were mixed with 250 µl of Bradford reagent and incubated for 15 min. Optical densities at 595 nm was measured in spectrophotometers.

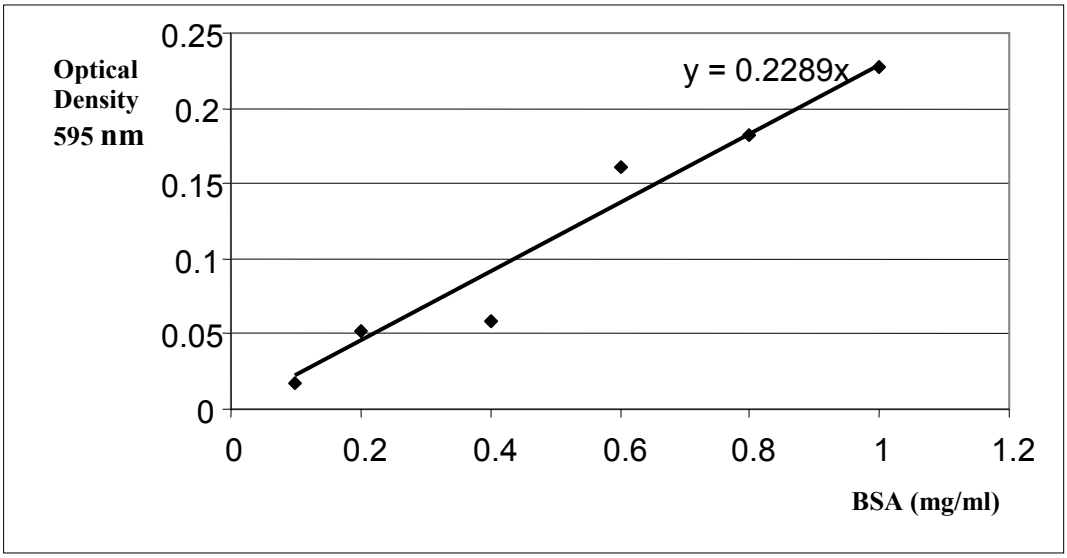


Figure 3.1: Standard curve for protein concentration determination

3.2.14 Frozen stocks of cells

3.2.14.1 Glycerol stocks of bacterial cells

150 µl of 87% glycerol was added to 1 ml of bacterial culture in Luria Broth and the bacterial cells were frozen in liquid nitrogen. The glycerol stocks were stored at -80°C.

3.2.14.2 Frozen stocks of mammalian cells

A total of 10^7 EL4 cells were spun down and resuspended in1 ml of ice-cold freezing medium. The cells were immediately taken to -80°C and frozen for 2 days. Frozen cells were then stored in liquid nitrogen.

4 RESULTS

As summarized in the Introduction, the cell-specific and stage-specific expression of IL-7Rα definitely requires identification of new regulatory elements that act on the IL-7Rα gene. This led us to search for an enhancer region that would regulate IL-7Rα gene.

In order to find an enhancer region that may regulate IL-7Rα transcription, the downstream of mice IL-7Rα gene spanning 80 kb region was investigated. Using bioinformatics tools, noncoding regions downstream of the IL-7Rα gene that are conserved between mouse and human were identified. These regions were PCR amplified from bacterial artificial chromosome (BAC) clones containing the mouse IL-7Rα gene and its downstream regions. PCR amplified noncoding conserved regions were cloned into luciferase reporter vectors and transfected into mouse EL4 cells expressing IL-7Rα gene. Luciferase assays were performed to detect transcriptional activation of luciferase enzyme by the conserved noncoding sequences.

4.1 Constructions of pGL3fos, pGL3fos-SV40 and pGL3fos-µ170 Vectors

4.1.1 pGL3fos

pGL3fos vector was constructed by the insertion of fos promoter into the pGL3 Basic vector. pGL3 Basic is a luciferase reporter vector possessing *luc*+ cDNA encoding for firefly luciferase enzyme (vector map is given in Appendix D). pGL3 Basic lacks any promoter or enhancer in order to study the effects of the *trans*-acting or

cis-acting regulatory elements on the gene expression. The fos promoter is a weak promoter and was cloned to the upstream of *luc*+ cDNA to minimize the level of gene expression driven in the absence of an enhancer (sequence of fos promoter is given in Appendix H). pGL3fos vector is a luciferase reporter vector with a weak promoter and was constructed to search for possible enhancers that would activate luciferase gene expression (map of pGL3fos vector is given in Appendix G).

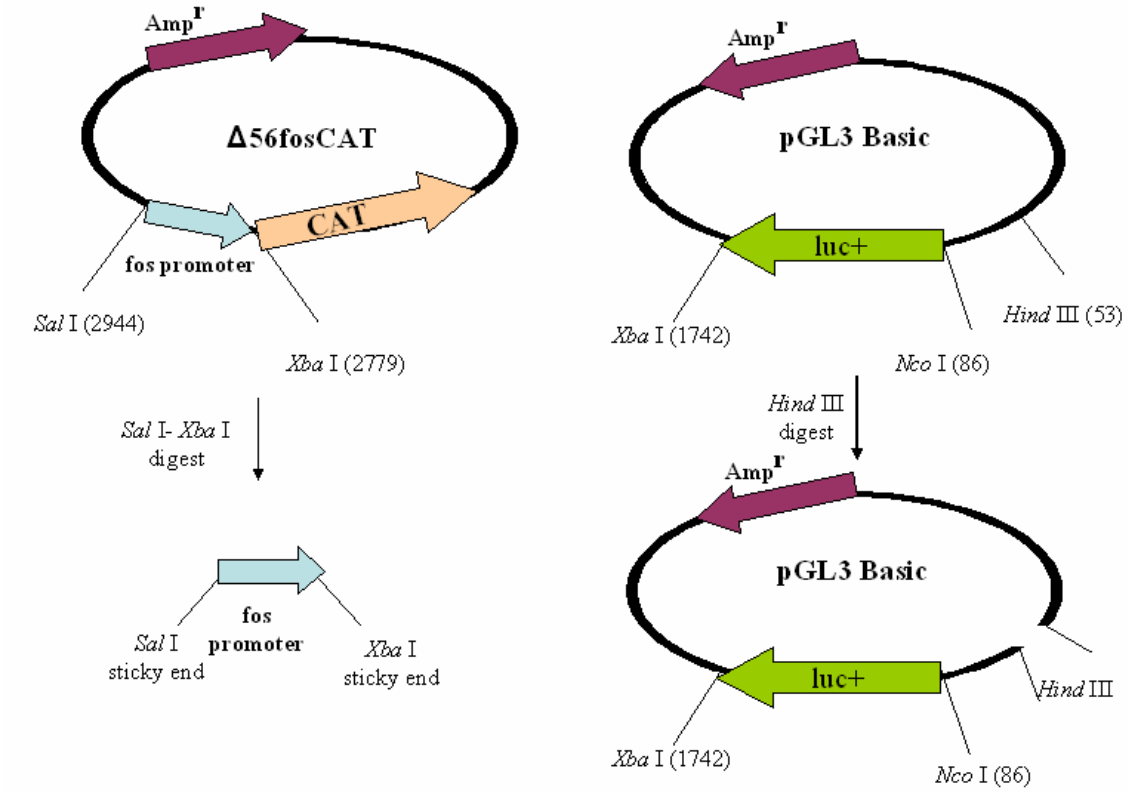


Figure 4.1: The schematic representation of the pGL3 Basic and Δ56fosCAT digestions showing how the fos promoter was cut from Δ56fosCAT vector by Sal I and Xba I digests. pGL3 Basic vector was linearized with Hind III digest.

fos promoter was directionally cloned into *Hind* III site of pGL3 Basic via blunt-end ligation. Since *Sal* I- *Xba* I digestions required to cut out the fos promoter did not generate compatible ends with *Hind* III site, both *Sal* I and *Xba* I sticky ends of fos promoter and *Hind* III sticky ends of pGL3 Basic were blunt-ended for cloning. Directional cloning of fos promoter into *Hind* III site was checked according to the regeneration of a *Hind* III site by the ligation of blunt-ended *Hind* III site with the blunt-ended *Sal* I site. In addition, ligation of blunt-ended *Hind* III site with blunt-ended *Xba* I site regenerated *Xba* I site.

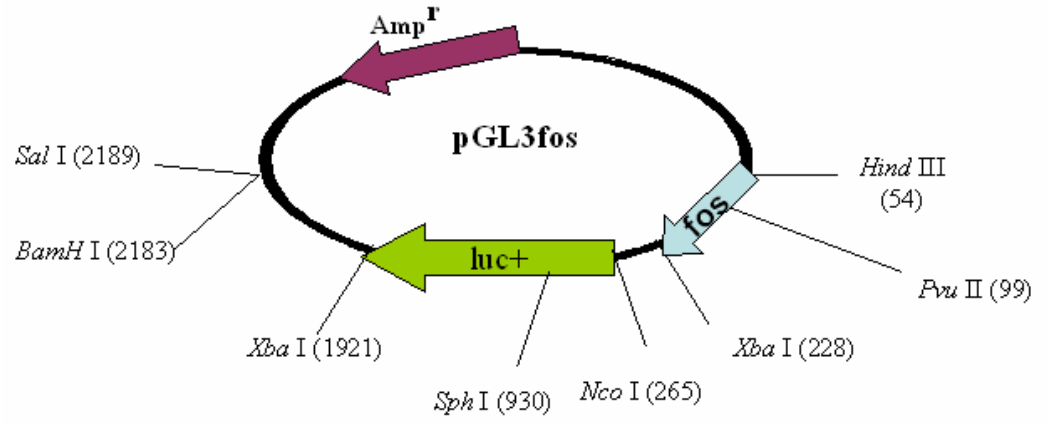
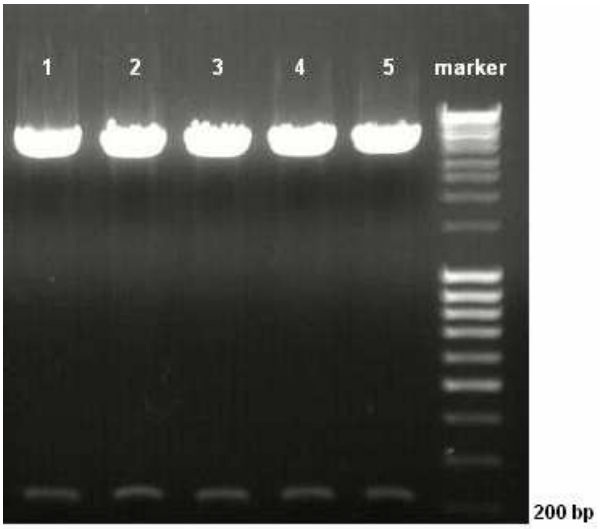


Figure 4.2: Schematic representation of pGL3fos vector:
Directional insertion of fos promoter by the ligation of blunt-ends regenerated *Hind* III and *Xba* I sites at the ends of the promoter. In addition, a unique *Pvu* II site was introduced into pGL3 Basic by the insertion of fos promoter

To confirm the presence of the appropriate insert and to select inserts in the appropriate orientation, pGL3fos vectors isolated from transformants were digested with *Hind* III and *Nco* I. This digest gave the 211 bp fragment containing the fos promoter (Figure 4.3a). Cloning in the reverse direction would have generated a *Hind* III site at the position 228 and *Xba* I site at the position 54. Thus, in the case of an insert in the reverse orientation, a *Hind* III- *Nco* I digest would have given a 37 bp instead of 211bp fragment.

We confirmed the identity of the insert also by comparing pGL3 Basic and pGL3fos vectors. We digested both vectors with *Hind* III-*Xba* I to show the absence of the fos promoter of 165 bp length in the pGL3 Basic vector (Figure 4.3b; lanes 3 and 6). Figure 4.3b also shows *Pvu* II-*Sph* I digestion of the pGL3 Basic and pGL3fos vectors (lanes 2 and 5). *Pvu* II- *Sph* I digestion of pGL3 Basic only linearized the vector since the vector was only cut at the position 930 by *Sph* I whereas digested pGL3fos gave two fragments because of a unique *Pvu* II site found in the fos promoter.

a)



b)

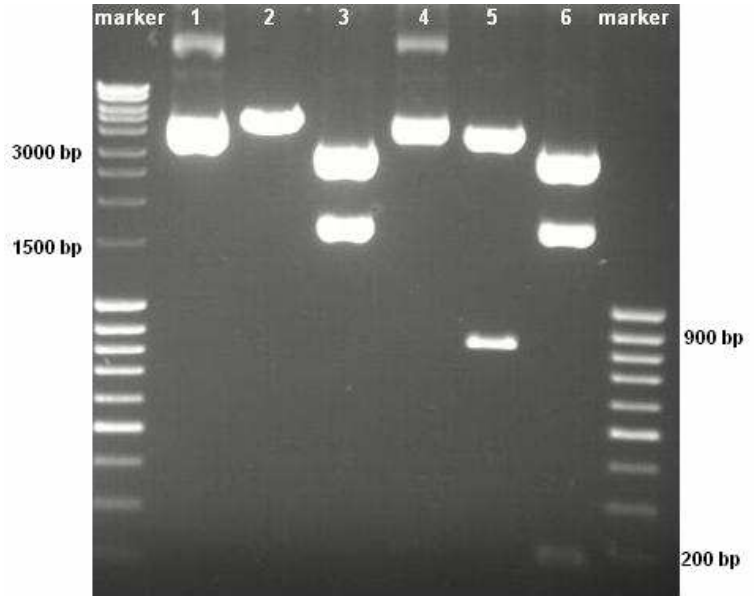


Figure 4.3: Diagnostic digests for pGL3fos vector:
a) Demonstration of the presence of inserts in pGL3fos: *Hind* III –*Nco* I digests of five plasmids isolated from five different colonies (lanes 1-5). The marker is Mass Ruler mix range. The fragments at the bottom of the gel are 211 bp, indicating the presence of an insert in all plasmids.
b) Confirmation of the orientation of inserts; the marker on the left is Mass Ruler mix range and the marker on the right is Mass Ruler low range. Lane 1: undigested pGL3 Basic. Lane 2: *Pvu* II-*Sph* I digestion of pGL3 Basic which gave linearized vector.
Lane 3: *Hind* III-*Xba* I digest of pGL3 Basic; bands corresponded to 3129 and 1689 bp. Lane 4: undigested pGL3fos, 4996 bp. Lane 5: *Pvu* II-*Sph* I digestion of pGL3fos vector which gave two fragments of length, 4165 and 831 bp. Lane 6: *Hind* III-*Xba* I digest of pGL3fos; bands corresponded to 3129 bp, 1693 bp and 174bp.

4.1.2 pGL3fos-SV40

pGL3fos-SV40 vector was constructed as a positive control of luciferase reporter assays, because the SV40 enhancer has a high transcriptional activation capability in most cell lines. SV40 enhancer and *luc*+ cDNA were cut out of pGL3 Enhancer vector (map of pGL3 Enhancer is given in Appendix D) and ligated to pGL3fos at *BamH* I and *Nco* I sites (Figure 4.4). The reason of cutting out the *luc*+ cDNA together with the SV40 enhancer was that there was not any restriction site at the 3' end of the SV40 enhancer to cut it out. pGL3fos-SV40 was constructed to ensure that fos promoter could drive the transcription of the *luc*+ cDNA under the influence of a strong enhancer such as SV40 (map of pGL3fos-SV40 is given in Appendix H).

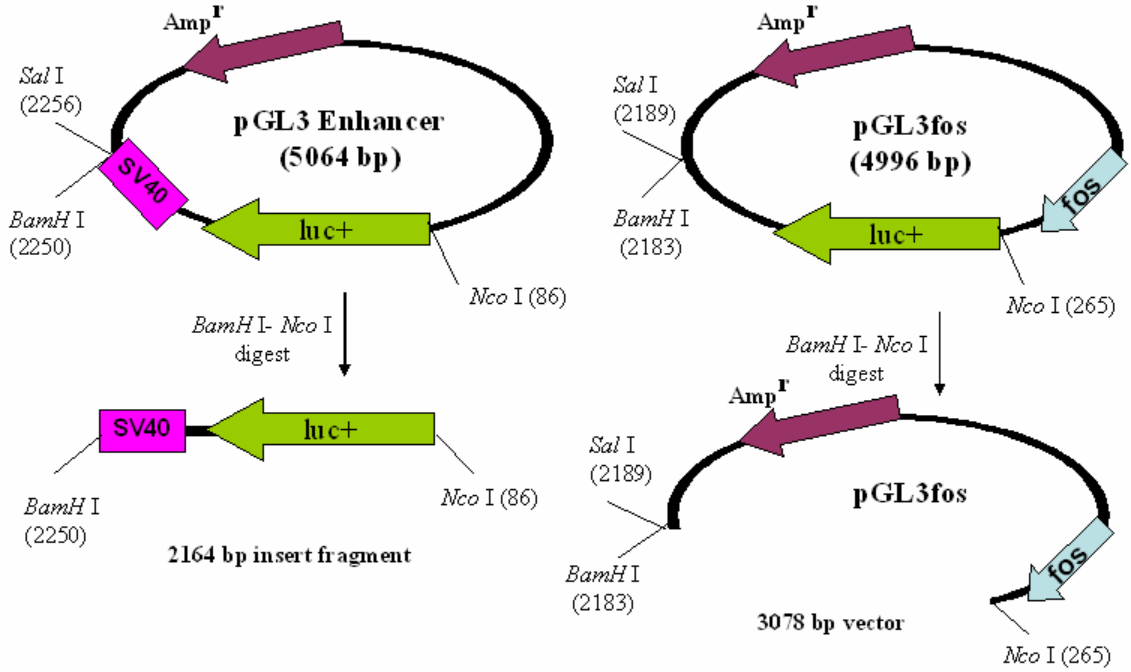


Figure 4.4: Schematic digestions of pGL3 Enhancer and pGL3fos vectors and formation of fragments to be ligated

As can be seen from figure 4.4, *BamH* I-*Nco* I digestions of pGL3 Enhancer and pGL3 Basic vectors were required to obtain the fragments to be ligated. In figure 4.5, *BamH* I-*Nco* I digestions of pGL3 Enhancer and pGL3 Basic vectors are shown.

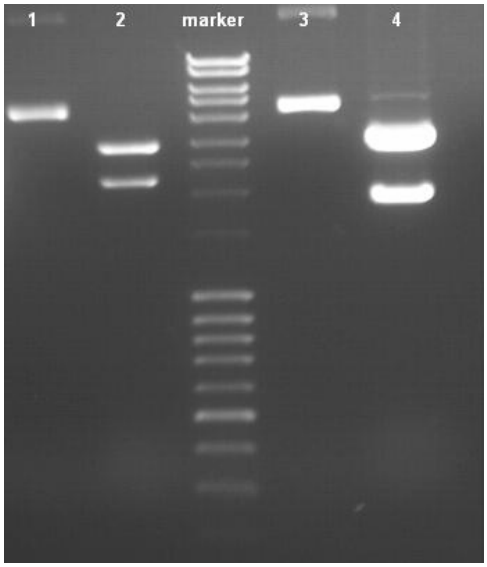


Figure 4.5: *Bam*H I-*Nco* I digests of pGL3 Enhancer and pGL3 Basic vectors:
Marker: Mass Ruler mix range Lane 1: undigested pGL3 Enhancer vector.
Lane 2: *BamH* I-*Nco* I digest of pGL3 Enhancer giving fragments of length 2900 bp and 2164 bp. 2164 bp fragment containing SV40 and *luc*+ cDNA sequences was extracted from gel. Lane 3: undigested pGL3fos vector. Lane 4: *BamH* I-*Nco* I digest of pGL3fos, the bands correspond to 3078 bp and 1918 bp. 3078 bp fragment was extracted from the gel.

pGL3fos-SV40 vector constructed by the ligation of the fragment of length 2164 bp (figure 4.5; lane 2) and the pGL3fos vector of length 3078 bp (figure 4.5; lane 4).

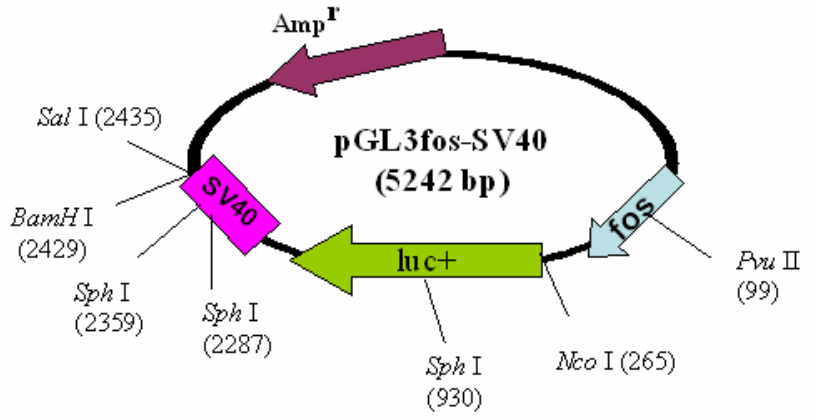


Figure 4.6: pGL3fos-SV40 vector

pGL3fos-SV40 vector was *Pvu* II-*Sph* I digested. *Pvu* II site was uniquely found in the fos promoter and *Sph* I site was found in the SV40 enhancer region (Figure 4.6).

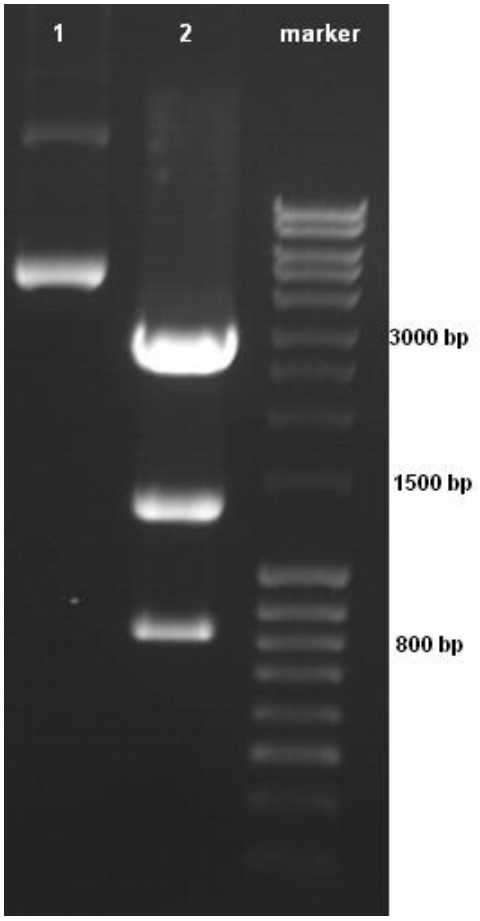


Figure 4.7: *Pvu* II-*Sph* I digestion of pGL3fos-SV40:
Marker: Mass Ruler mix range. Lane 1: undigested pGL3fos-SV40. Lane 2: *Pvu* II-*Sph* I digestion of pGL3fos-SV40. *Pvu* II-*Sph* I digest gave four fragments of length; 2982, 1357, 811 and 72 bp. Three of the fragments can be seen except for the fragment of 72 bp length.

4.1.3 pGL3fos-µ170

pGL3fos-µ170 was constructed as a positive control of luciferase assays as well as pGL3fos-SV40. In fact, µ170 is a minimal domain of µ enhancer which regulates rearrangement of immunoglobulin heavy-chain in developing B-cells. µ170 contains motifs that are binding sites for the transcription factors that will mediate the rearrangement processes in B-cells. pGL3fos-µ170 was constructed in order to see whether µ170 could show enhancer activity in T-cells (EL4) as well. If µ170 enhancer was found to activate gene transcription in T-cells, pGL3fos-µ170 vector would be used as a positive control of enhancer activity in luciferase reporter assays.

We digested pSP72- µ170 vector with *Bgl* II and *Sal* I enzymes to obtain a 211 bp fragment containing µ170 enhancer (map of pSP72 vector is given in Appendx D). pGL3fos was digested with *Sal* I and *BamH* I enzymes. *Sal* I sticky ends of the insert and vector were ligated to each other. *Bgl* II and *BamH* I sites were ligated to each other since digestions with these two enzymes generated compatible sticky ends (Figure 4.8).

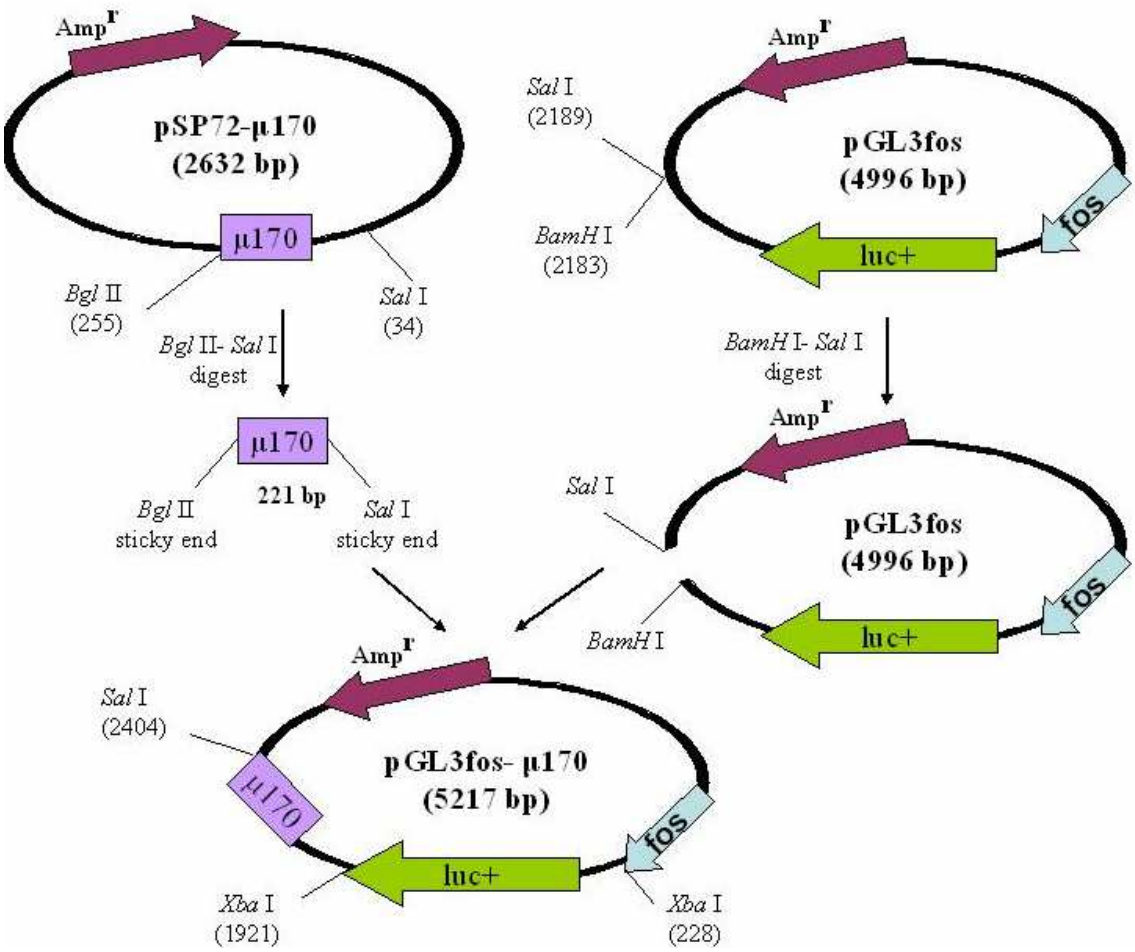


Figure 4.8: Construction of pGL3fos-µ170 vector

We isolated pGL3fos-µ170 vectors from transformants and digested with *Sal* I and *Xba* I enzymes to confirm the insertion of µ170. As can be seen in figure 4.9, these digestions verified the insertion of µ170 into pGL3fos vector.

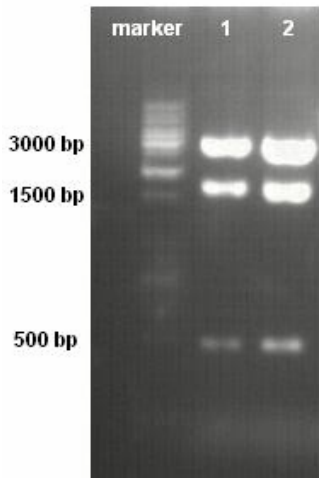


Figure 4.9: *Sal* I-*Xba* I digest of pGL3fos-µ170 vector verifying the insertion µ170 enhancer into pGL3fos: marker: Gene Ruler 1 kb. Lanes 1 and 2 show two plasmids that were isolated from two different colonies. *Sal* I-*Xba* I digests gave three fragments of length 3041 bp, 1693 bp and 483 bp which were the expected fragments according to the vector map given in Appendix G.

4.2 Identification and Amplification of Conserved Noncoding Regions at the Downstream of Mouse IL-7Rα gene

Mouse IL-7Rα gene and the downstream genomic region up to 80 kb was investigated to determine possible conserved noncoding regions that could activate the transcription of IL-7Rα gene. Genomic regions conserved between mouse and human were determined and amplified from mouse BAC clone in order to clone into luciferase reporter vectors and search for transcriptional activity.

4.2.1 Identification of conserved noncoding regions at the downstream of mouse IL-7Rα gene

The mouse IL-7Rα gene resides on the chromosome 15 between positions 9,402,535 – 9,426,409. Human and mouse IL-7Rα genes are homologous to each other as given in the NCBI HomoloGene database with the call number 1646. Human IL-7Rα gene is located on chromosome 5. Investigation of the mouse IL-7Rα gene locus

showed that the genomic region at the downstream of IL-7Rα gene contained a gene (1700028N11Rik gene) encoding for calcyphosine like protein (Capsl). According to NCBI HomoloGene Database (call number 16959), Capsl is homologous to human RefSeq gene CAPSL (encoding calcyphosine like protein). Human CAPSL gene is located at the downstream of IL-7Rα as well. Thus, IL-7Rα gene and the flanking gene are conserved between human and mouse in the same order. This brings the possibility that *cis*-acting regulatory elements residing in IL-7Rα locus of human and mouse may be conserved as well. The sequences of mouse IL-7Rα gene and its downstream region including Capsl gene were taken from University of California at Santa Cruz (UCSC) Genome Browser (www.genome.ucsc.edu) (Figure 4.10). The sequences of human IL-7Rα gene and its downstream region including CAPSL gene were taken from UCSC Genome Browser as well (Figure 4.11).

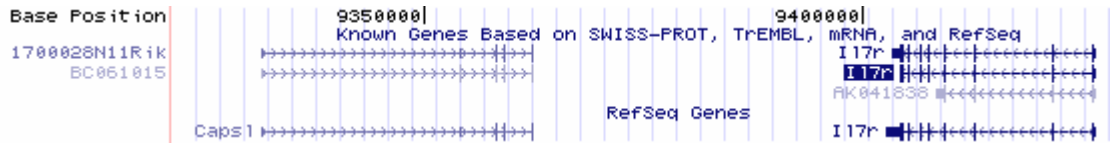


Figure 4.10: Mouse genomic regions including IL-7Rα gene and downstream Capsl gene: The genomic sequences were taken from UCSC Genome Browser. The genomic region corresponds to positions 9,321,248 – 9,426,409 on mouse chromosome 15. A total of 105 kb region of mouse chromosome 15 were retrieved from the database.

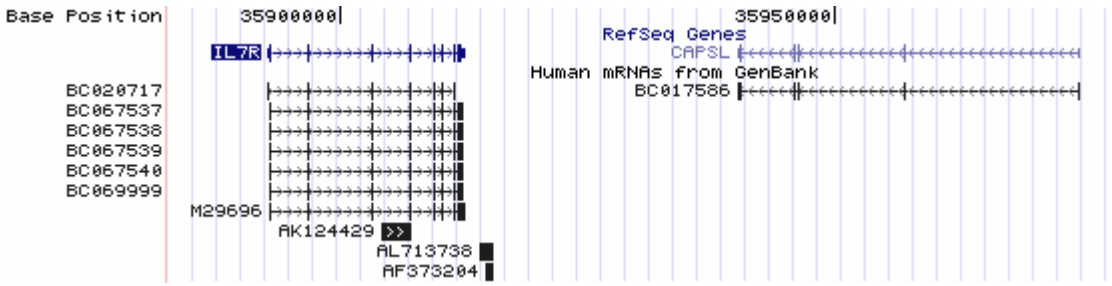


Figure 4.11: Human genomic regions including IL-7Rα gene and downstream CAPSL gene: The genomic sequences were taken from UCSC Genome Browser. The genomic region corresponds to positions 35,892,748 – 35,984,638 on human chromosome 5. A total of 92 kb region of human chromosome 5 were retrieved from the database.

As can be seen in figure 4.10, the orientation of mouse IL-7Rα gene is found in the negative direction with respect to the 15th chromosome direction in the database. Thus, the reverse complement of the sequences (9,321,248 – 9,426,409; on

chromosome 15) were taken in order to get the sequences in the direction of the coding strand of IL-7Rα gene. After the sequences of the genomic regions shown in figures 4.10 and 4.11 were retrieved from UCSC Genome Browser; global pairwise alignments of mouse and human sequences were done with the AVID program. Mouse genomic sequences were chosen as the base in the graphical output of VISTA program in figure 4.12. Therefore, numbers given on the x-axis in figure 4.12 indicate the base positions (in kb) of genomic region of mouse containing IL-7Rα gene and Capsl gene. The numbers start from 0 to 105 kb. 0 refers to the genomic position 9,426,409 and 105 kb refers to the genomic position 9,321,248 on the mouse chromosome 15, since the reverse complement of the mouse sequences were aligned with human sequences.

Window size was set as 50 bp so that the conserved noncoding regions given as the output would be at least 50 bp in length. The percent identity was set as 85%; so the noncoding conserved regions given by VISTA were at least 85% identical. Peaks higher than 85% and longer than 50 bp were presented in pink color (figure 4.12) and were considered to be conserved between the two aligned sequences. The output of VISTA included ten noncoding conserved regions at the downstream of mouse IL-7Rα gene. Conserved noncoding regions and their lengths are given in table 4.1.The locations of conserved noncoding regions are also shown in table 4.1. The base positions of mouse are given in the interval of 0 - 105 kb which refers to the region 9,321,248 - 9,426,409 on mouse chromosome 15. The base positions of human are given in the interval of 0 - 92 kb which refers to the region 35,892,748 – 35,984,638 on human chromosome 5 (Table 4.1).

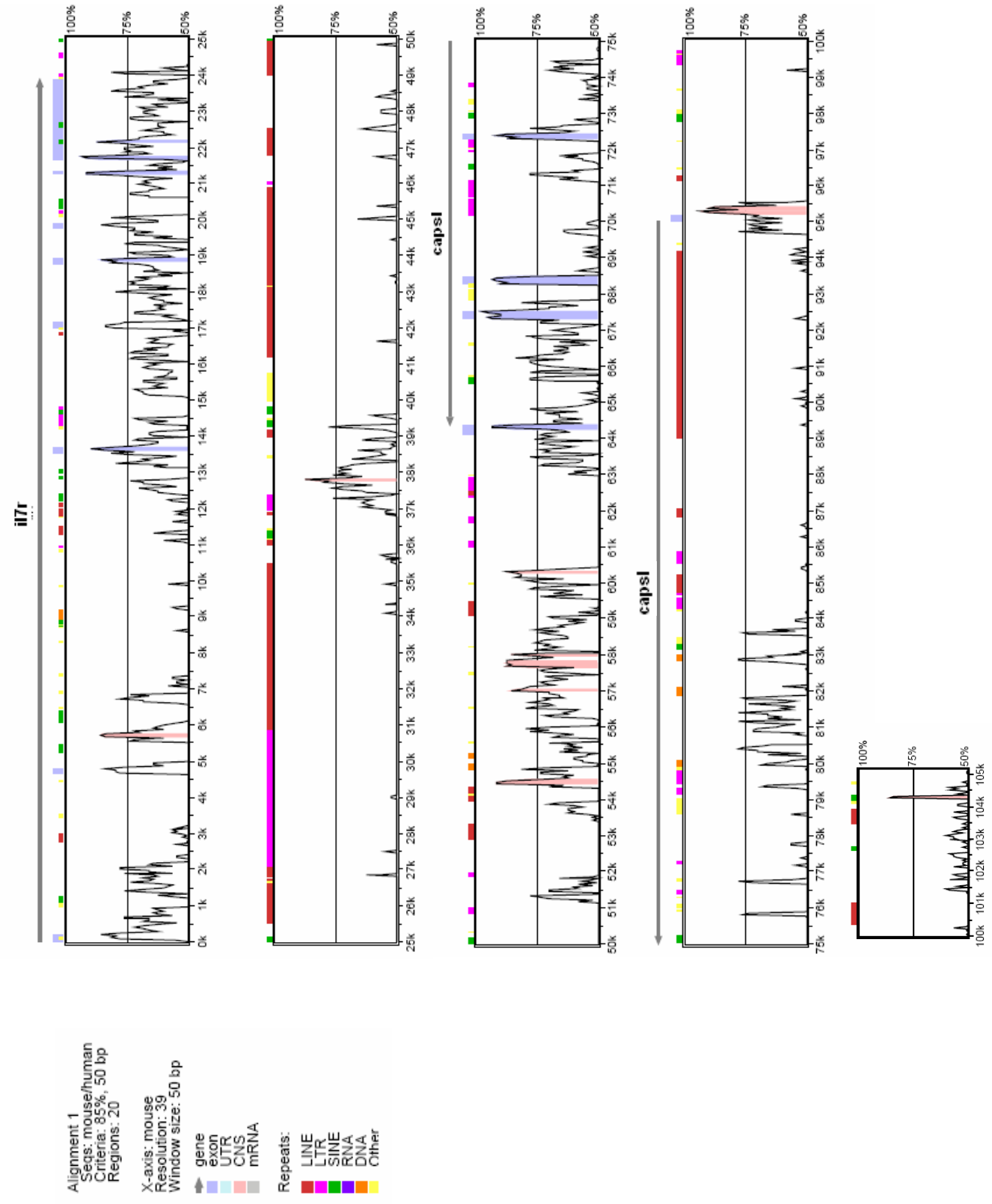


Figure 4.12: VISTA output showing the noncoding conserved sequences between downstream regions of mouse and human IL-7Rα genes: Peaks in pink color show the noncoding conserved regions. Exons and repeat sequences are shown in different colors at the top of the graph. The locations of IL-7Rα and Capsl genes are shown by grey arrows at the top of the graph.

Mouse noncoding regions	Human noncoding regions	Length of the region (bp)	Percent identity (%)
37769 - 37819	27797 - 27847	51	88.2
54427 - 54550	39284 - 39408	125	86.4
56993 - 57042	42051 - 42100	50	86.0
57664 - 57770	43032 - 43138	107	83.2
57781 - 57849	43149 - 43218	71	85.9
57955 - 58004	43315 - 43364	50	86.0
60252 - 60301	45590 - 45639	50	88.0
95207 - 95300	81843 - 81936	95	89.5
95339 - 95405	81972 - 82038	67	86.6
104278 - 104327	88540 - 88590	51	86.3

Table 4.1: VISTA output showing conserved noncoding regions between mouse and human and their percent identity

The mouse genomic regions shown in the table 4.1 were retrieved from UCSC Genome Browser after an enlargement of the region approximately 200 bp in both 5' and 3' directions. The regions were enlarged to preserve a reasonable distance between the possible enhancers and the luciferase gene after the conserved noncoding sequences were cloned into the reporter vectors. In addition to the regions shown in the table 4.1, one more region located at the first intron of Capsl gene was considered as conserved between mouse and human. Although the conservation between human and mouse was not as high as 85% in this intronic region, it contained conserved sequences with a length of 450 bp.

After the conserved noncoding sequences were retrieved from UCSC Genome Browser, some of the regions that were proximal to each other were combined into a single region to be amplified from mouse BAC clones. The regions amplified from BAC clones are given in table 4.2 with the locations of the regions on both VISTA output and the mouse chromosome 15 (sequences of noncoding regions amplified are given in Appendix H).

Name given to the conserved region	Locations relative to VISTA output	Locations relative to mouse chromosome 15	Length of the region
Cap 1	37452 – 37971	9388438 – 9388957	520
Cap 2	54193 – 54792	9371617 – 9372216	600
Cap 3	56825 - 57275	9369134 – 9369584	451
Cap 4	57521 – 58141	9368268 – 9368888	621
Cap 5	59725 – 60444	9365965 – 9366684	720
Cap 6	66038 – 66946	9359463 – 9360371	909
Cap 7	95125 – 95600	9330809 – 9331284	476
Cap 8	104010 - 104425	9321984 – 9322399	416

Table 4.2: Conserved noncoding regions amplified from BAC clone

4.2.2 Amplification of conserved noncoding regions

The regions in the table 4.2 were amplified from BAC clone RP23-365P6 which contains mouse IL-7Rα gene and its downstream. The PCR amplified fragments can be seen in figure 4.13.PCR amplifications were performed with the primers that were designed for each region and the sequences of these primers are given in Appendix E. The lengths of the fragments in figure 4.13 were consistent with the lengths given in table 4.2.

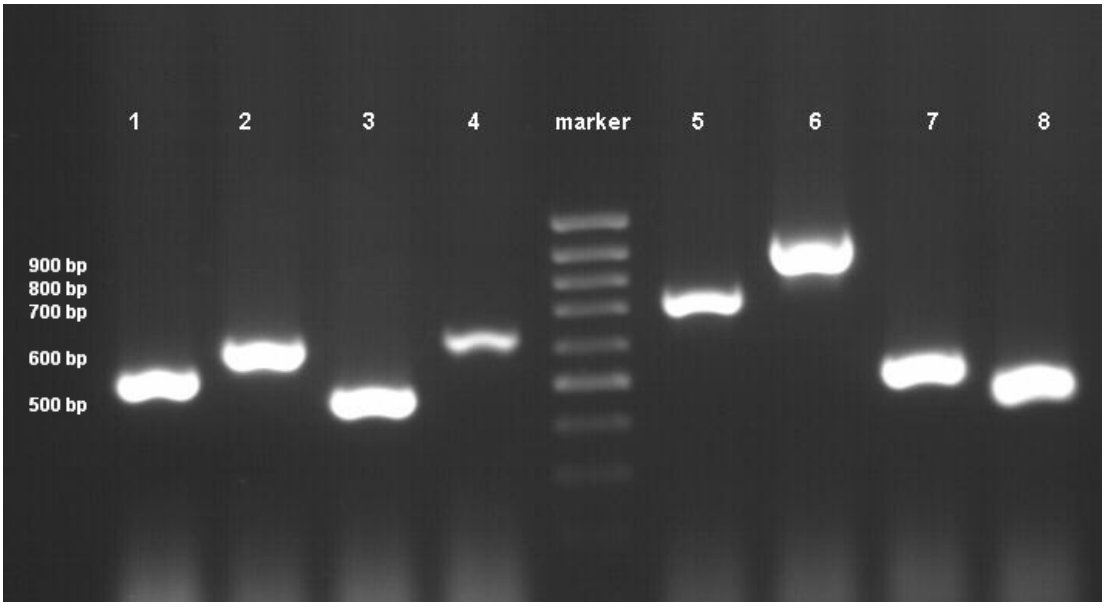


Figure 4.13: PCR amplified conserved noncoding sequences: marker is Mass Ruler low range. Lane1: Cap 1. Lane 2: Cap 2. Lane 3: Cap 3. Lane 4: Cap 4. Lane 5: Cap 5. Lane 6: Cap 6. Lane 7: Cap 7. Lane 8: Cap 8.

4.3 Cloning of Conserved Noncoding Sequences into pGL3fos Vector

Conserved noncoding sequences were inserted into pGL3fos luciferase reporter vectors to analyze the regulatory functions of these regions on *luc*+ cDNA expression. Conserved noncoding sequences were inserted into *Sal* I site of pGL3fos vector. For this purpose, the primers used to amplify each region were designed to contain *Sal* I sites at 5' termini. As a result, there were *Sal* I sites at the ends of PCR fragments. PCR fragments were digested with *Sal* I enzyme to clone into pGL3fos luciferase reporter vector as shown in figure 4.14.

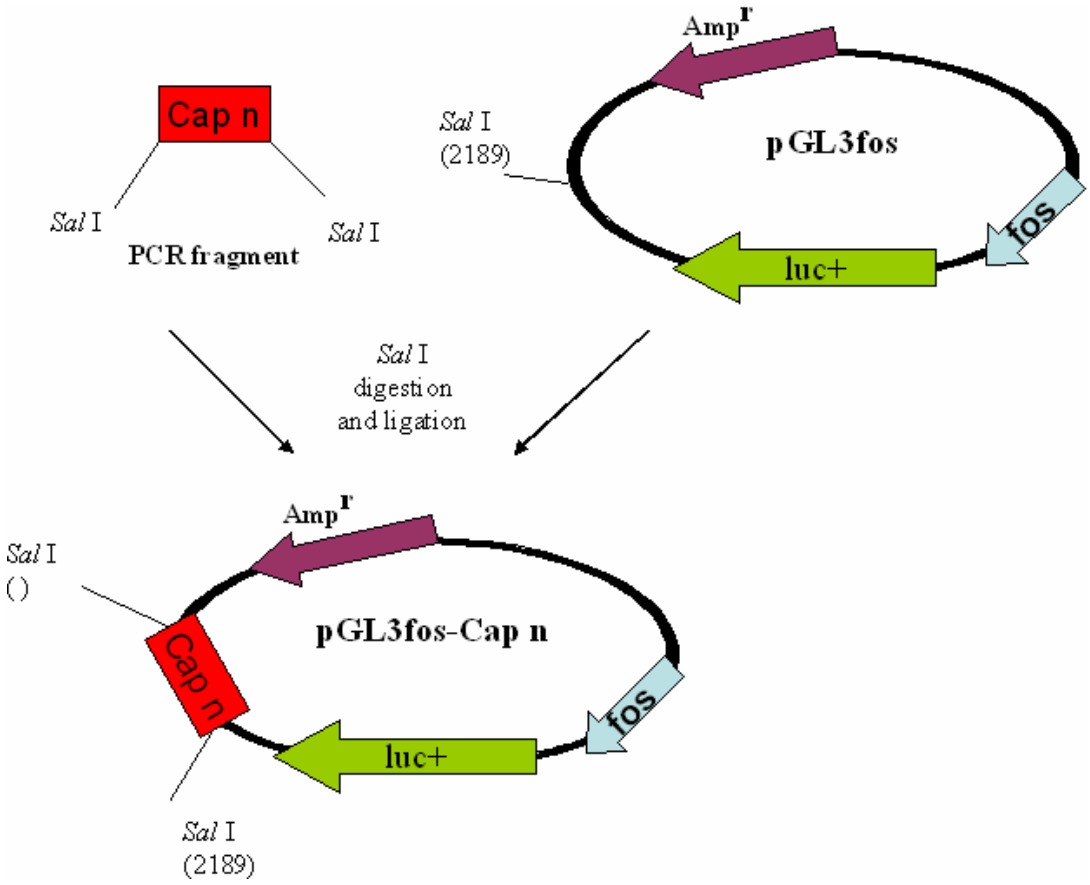
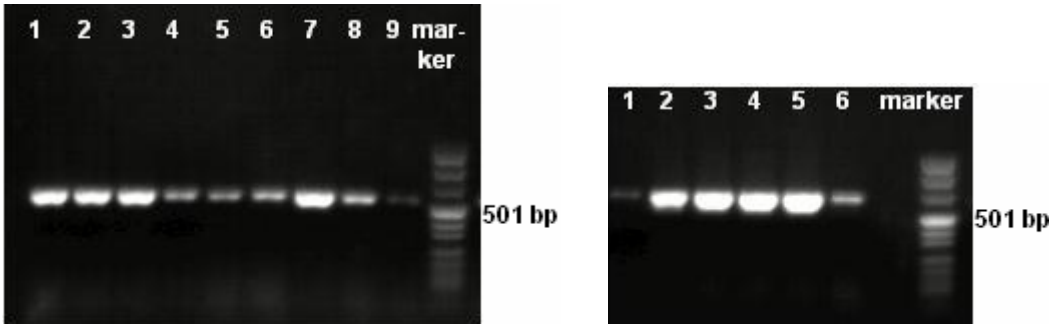


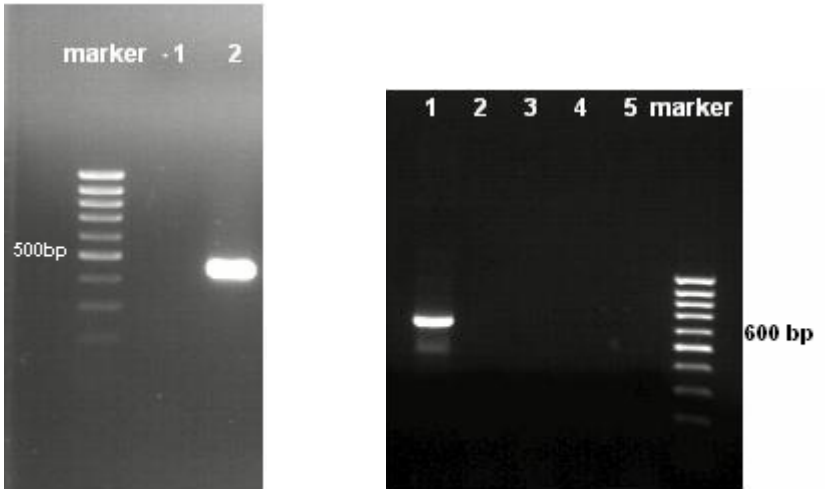
Figure 4.14: Cloning of conserved noncoding sequences into *Sal* I site of the pGL3fos luciferase reporter vector. 'Cap n' indicates one of the eight conserved noncoding sequences. Each vector constructed by the insertion of one of the Cap n (1…8) regions were named as pGL3fos-Cap 'n'.

Eight conserved noncoding regions were inserted into pGL3fos vectors and they were named as pGL3fos-Cap1, pGL3fos-Cap2, pGL3fos-Cap3, pGL3fos-Cap4, pGL3fos-Cap5, pGL3fos-Cap6, pGL3fos-Cap7, pGL3fos-Cap8 according to the inserted fragment (sequences of inserts are in Appendix H). The presence of the insert fragments in pGL3fos vector was confirmed by colony PCR (described in Methods, 3.2.10.5.1). Figures 4.15 - 4.19 show insertion of each fragment into pGL3fos.



a) b)

Figure 4.15: Colony PCR from colonies transformed with pGL3fos-Cap1 and pGL3fos-Cap2. The markers are Puc mix marker 8 in both a and b. a) Confirmation of pGL3fos-Cap1 construction; the amplified fragments in all lanes are at the length of Cap 1 (520 bp). The fragments in all lanes were amplified from separate colonies. b) Confirmation of pGL3fos-Cap2 construction; in lanes 2-6, the inserted Cap 2 was amplified from pGL3fos-Cap2 plasmids of six different colonies. The length of Cap 2 is 600 bp.



a) b)

Figure 4.16: Colony PCR from colonies transformed with pGL3fos-Cap3 and pGL3fos-Cap4. The markers are Mass Ruler low range in both gels. a) Colony PCR amplification of Cap 3: Cap 3 with a length of 451 bp was amplified from the colony in lane 2, showing successful construction of pGL3fos-Cap3. b) Amplification of Cap 4 from colonies: Cap 4 was amplified from the colony in lane 1. The length of the fragment is 621 bp.

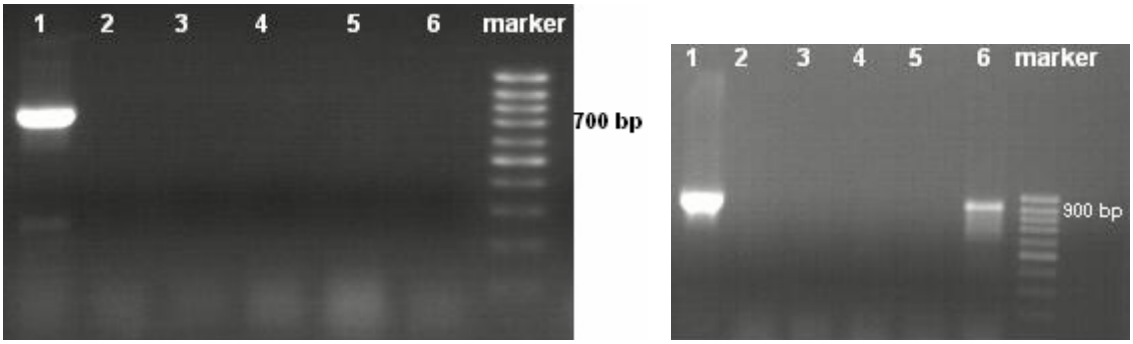


Figure 4.17: Colony PCR amplification of Cap 5 and Cap 6: The markers used in both gels are Mass Ruler low range. a) Colony PCR for Cap 5: In lane 1, a fragment between 700 and 800 bp is seen; resulted from PCR from pGL3fos-Cap5 vectors. This fragment confirmed the insertion of Cap 5 with a length of 720 bp into pGL3fos vector.
b) Colony PCR for Cap 6: In lane 1 and 6, Cap 6 with a length of 909 bp was amplified from pGL3fos-Cap6.

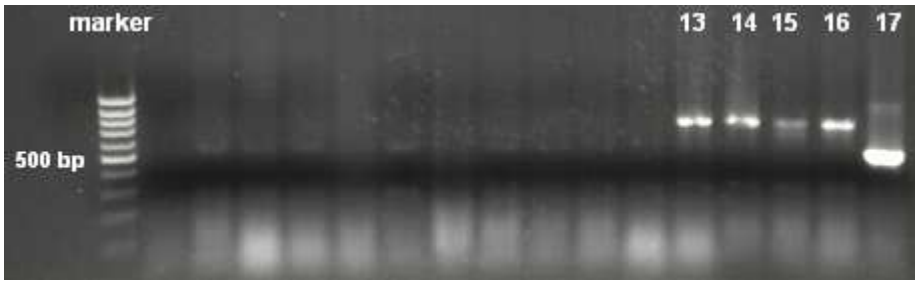


Figure 4.18: Colony PCR to confirm insertion of Cap 7: The marker used is Mass Ruler low range. Lanes 13-17 shows PCR amplified fragments from five different colonies. However, only the PCR fragment in lane 17 was at the length of Cap 7; 476 bp. Thus, pGL3fos-Cap7 was successfully constructed and transformed into the colony in lane 17.

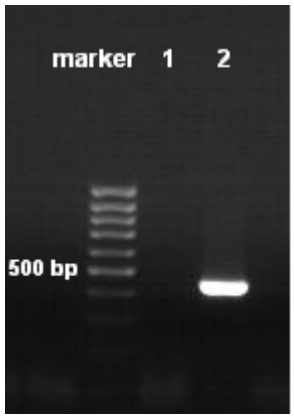


Figure 4.19: Colony PCR of Cap 8 from pGL3fos-Cap8 plasmids: The marker used is Mass Ruler low range. In lane 2, Cap 8 was amplified from the colony showing that pGL3fos-Cap8 was successfully constructed. The length of Cap 8 is 416 bp, as seen in lane 2.

Plasmids were isolated from the colonies that were confirmed to carry pGL3fos-Cap vectors in figures 4.15-4.19. These plasmids were digested with *Sal* I enzyme to confirm the presence of the inserts in the plasmids. A second digestion was done with the restriction enzymes whose recognition sites were found in the Cap sequences. The digestions are shown in figures 4.20-4.22.

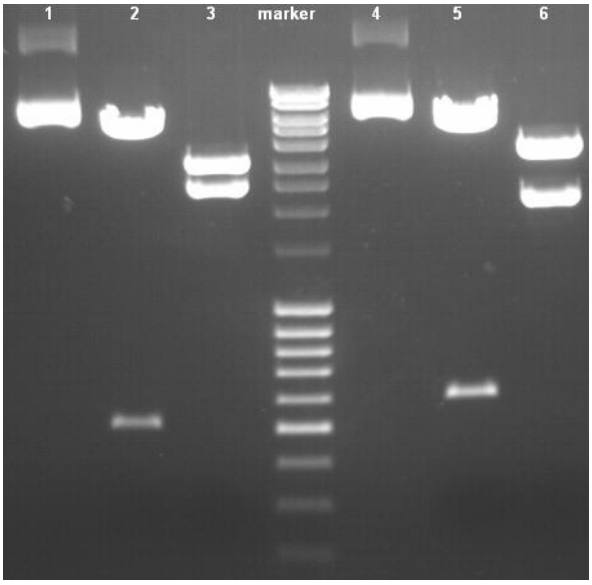


Figure 4.20: Digestions that confirmed pGL3fos-Cap1 and pGL3fos-Cap2 vectors:
The marker is Mass Ruler mix range. Lane 1: undigested pGL3fos-cap1. Lane 2: *Sal* I digest of pGL3fos-Cap1 showing that Cap 1 fragment with a length of 520 bp was cut out from pGL3fos-Cap1. Lane 3: *Pvu* II digest of pGL3fos-Cap1, the bands correspond to 3107 and 2415 bp which were the expected fragments since the vector was cut at at two sites one in the Cap1 region and one in the fos promoter. Lane 4: undigested pGL3fos-Cap2. Lane 5: *Sal* I digest of pGL3fos-Cap2 that gave a 600 bp fragment which is the size of Cap 2. Lane 6: *Xmn* I digest of pGL3fos-Cap2 confirmed Cap 2 insertion. The fragments in the gel are 3597 and 2005 bp in length which is consistent with the vector map.

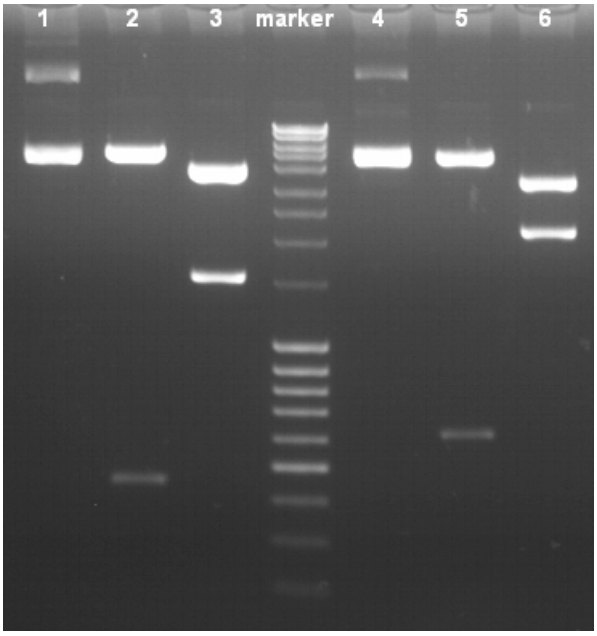
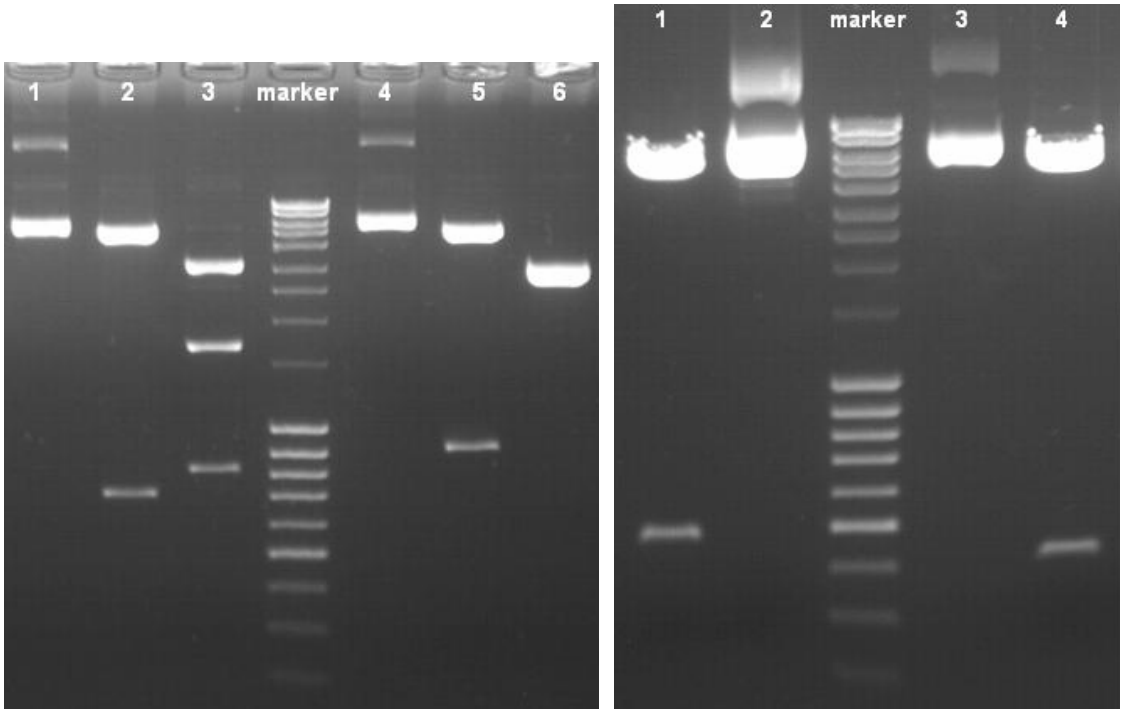


Figure 4.21: Digestions that confirmed pGL3fos-Cap3 and pGL3fos-Cap4 vectors:
The marker is Mass Ruler mix range. Lane 1: undigested pGL3fos-cap3. Lane 2: *Sal* I digest of pGL3fos-Cap3 showed that Cap 3 fragment in the length of 451 bp was ligated to pGL3fos. Lane 3: *Sph* I digest of pGL3fos-Cap3 showed that a second *Sph* I site was introduced to the pGL3fos vector by the insertion of Cap 3. The lengths of bands are 4031-1422. Lane 4: undigested pGL3fos-Cap4. Lane 5: *Sal* I digest of pGL3fos-Cap4 giving the fragment of length, 621 bp. Lane 6: *Pvu* II digestion of pGL3fos-Cap4 showed that the vector was cut at two sites one in the Cap4 and one in the fos promoter. The bands are in the length of 3467 and 2188 bp.



a) b)

Figure 4.22: Digestions that confirmed pGL3fos-Cap5, pGL3fos-Cap6, pGL3fos-Cap7 and pGL3fos-Cap8 vectors: The marker is Mass Ruler mix range in both gels.
a) Confirmation of pGL3fos-Cap5 and pGL3fos-Cap6 vectors
Lane 1: undigested pGL3fos-cap5. Lane 2: *Sal* I digest of pGL3fos-Cap5 showing that Cap 5 was inserted into pGL3fos, the length of the band is 720 bp. Lane 3: pGL3fos-Cap5 was digested with *Xba* I giving three fragments of length; 3194, 1693 and 835 bp. The bands in lane 3 confirmed the pGL3fos-Cap5 vector construction. Lane 4: undigested pGL3fos-Cap6. Lane 5: *Sal* I digest of pGL3fos-Cap6 gave the Cap 6 fragment in length of 909 bp. Lane 6: *Pvu* II digestion of pGL3fos-Cap6 showed that the Cap 6 was inserted in the reverse direction because the fragments in the gel were in the length of 2900 bp which would be resulted from reverse orientation of Cap 6.
b) Confirmation of pGL3fos-Cap7 and pGL3fos-Cap8 vectors.
Lane 1: *Sal* I digest of pGL3fos-Cap7 showing Cap 7 fragment with a length of 476 bp was cut out from pGL3fos-Cap7. Lane 2: undigested pGL3fos-cap7. Lane 3: undigested pGL3fos-Cap8. Lane 4: *Sal* I digest of pGL3fos-Cap8 which gave the fragment of length 416 bp.

4.4 Transfections of EL4 Cells and Luciferase Assays

EL4 cells are mouse T lymphoma cells that are $CD4^+$ single positive. EL4 cells possess IL-7Rα on their cell surface that makes it possible to study the regulation of IL-7Rα gene in these cell lines. Eight different pGL3fos-Cap luciferase reporter vectors

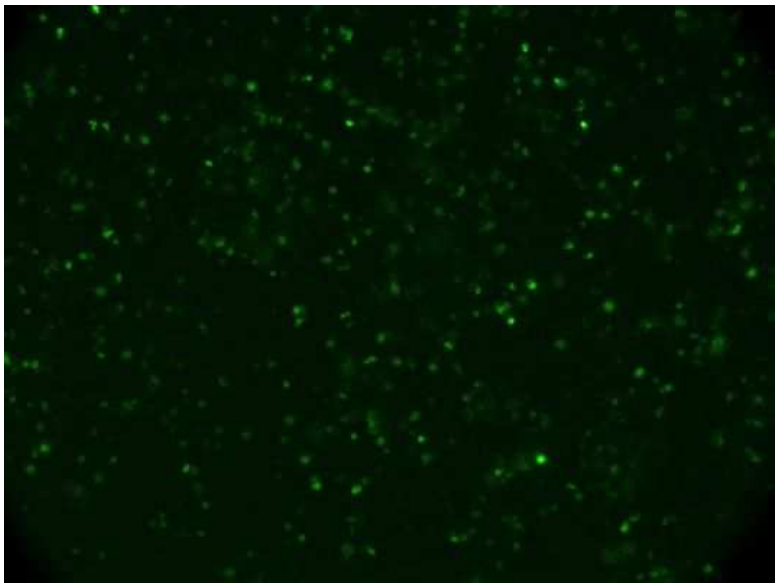
each containing one of the conserved noncoding sequences of IL-7Rα downstream region were constructed in order to introduce into EL4 cells. Transfected EL4 cells would be assayed for activation of luciferase expression under the influence of a possible enhancer residing in one of the eight conserved noncoding sequences. Before transfection of EL4 cells with pGL3fos-Cap constructs, we needed to optimize both transfection efficiency of EL4 cells, the protein content in each cell lysate and the measurement of luminescence generated by luciferase activity. Although the cell numbers transfected and the conditions of transfections were considered to be constant in each transfection experiment, total number of cells transfected might be different in each experiment due to variations in application of electroporation and different physical conditions of the cells before and after transfection.

4.4.1 Optimization of transfection efficiencies of EL4 cells

To optimize the efficiency of transfection, we transfected EL4 cells with pMaxGFP vector that contains cDNA encoding for green fluorescent protein (GFP). The percent of GFP expressing cells in total viable cell culture (transfection efficiency) was easily determined under a fluorescent microscope since GFP emits green light when excited with blue light. Transfection of EL4 cells were first tried by DEAE-Dextran mediated method. The parameters we tried to optimize were the concentration of DEAE-Dextran, the length of incubation time of EL4 cells with DEAE-Dextran, the amount of plasmid DNA transfected and the growth phase of EL4 cells (described in Methods). Since we could not optimize the conditions for an efficient transfection mediated by DEAE-Dextran, we tried to transfect EL4 cells by electroporation. At first set of transfection experiments, a broad range of electroporation conditions were tried. The optimum conditions were found to be electroporation of 10^7 cells with 20 μg of pMaxGFP at 260 V, 1050 μF and 100 Ω. The efficiency of transfections (percentage of cells expressing GFP over total cells alive) were calculated 24 hr after transfection by trypan blue exclusion method and was found to be lower than 10% (Figure 4.23a). Although we changed the amount of DNA transfected, total cell numbers electroporated and the conditions of electroporation, the efficiency of transfection could not be improved over 20%. In figure 4.23b, GFP expressing EL4 cells electroporated at 290 V,

1400 µF and 100 Ω. A total of 10^7 cells were transfected with 20 µg of plasmid DNA and the efficiency of transfection was approximately 15-20 % (Figure 4.23b).

a)



b)

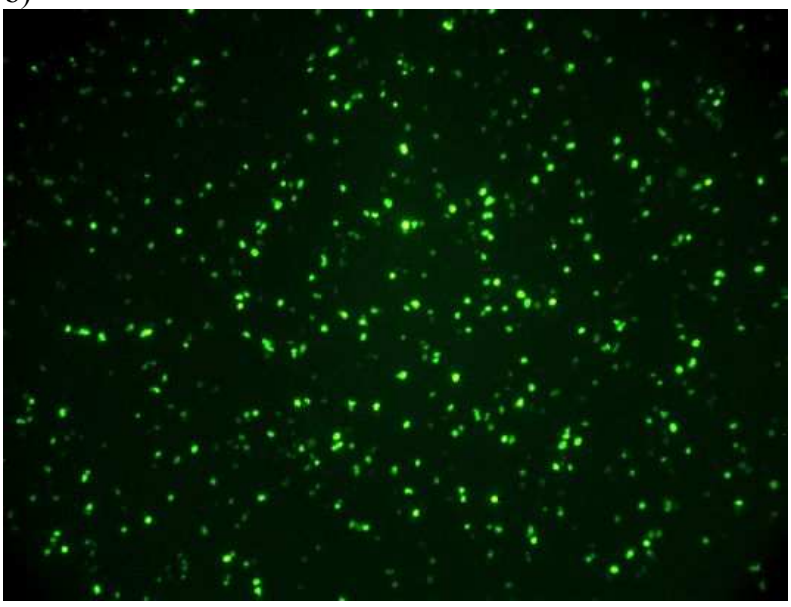


Figure 4.23: Visualization of GFP expressing EL4 cells electroporated with pMaxGFP
a) EL4 cells were electroporated with 20 µg of pMaxGFP at 260 V, 1050 µF and 100 Ω and visualized under fluorescent microscope 24 hrs later. Transfection efficiency was below 10%.
b) EL4 cells were electroporated with 20 µg of pMaxGFP at 290 V, 1400 µF and 100 Ω and visualized under fluorescent microscope 24 hrs after transfection. Transfection efficiency was 15-20 %.

4.4.2 Standard curve for luciferase activity

Preparing standard curves of luciferase activity is necessary because the linear range of light detection of the luminometer used should be determined. The relative light units given by the luminometer that are out of the linear range are not reliable because fold activation or inhibition of luciferase activity can not be determined proportionally out of the linear range. For this purpose, serial dilutions of recombinant luciferase were prepared and standard curve was plotted by measuring luminescence generated by activities of recombinant luciferase. The measurements of the luminometer are given in relative light unit (RLU). RLU values according to different amounts of recombinant luciferase are given in table 4.3. The standard curve according to the data in table 4.3 is shown in figure 4.24.

Luciferase (pg)	RLU
750000	153016.6
250000	88910.95
83333.33	49986.7
25000	20047.24
16666.67	12878.77
5555.56	3926.955
1851.85	1183.389
617.28	353.451
205.76	169.312
68.59	56.637
22.86	114.672
0	54.013

Table 4.3: RLU measured for different amounts of recombinant luciferase

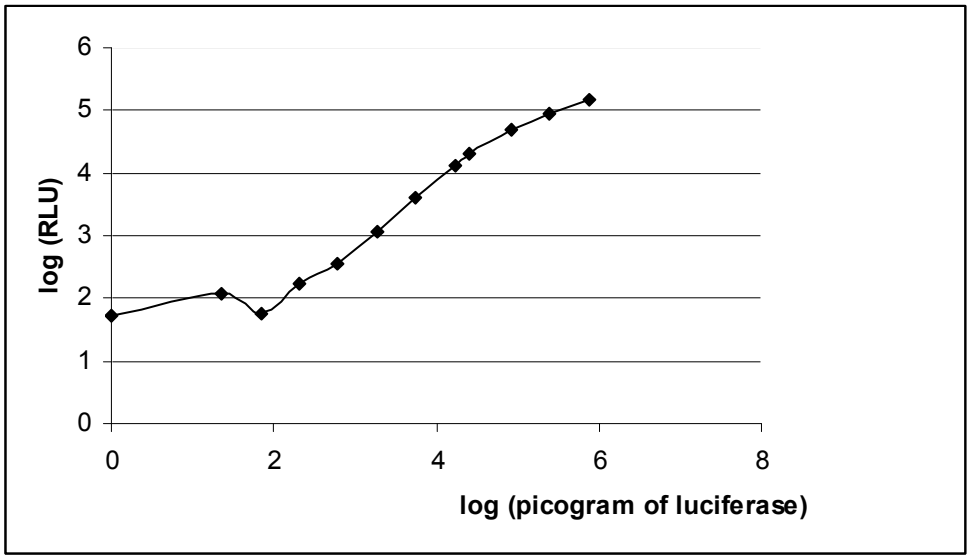


Figure 4.24: Standard curve of log (RLU) vs log (picogram of recombinant luciferase)

The curve in figure 4.24 shows that linear range of light detection is even higher than 10^6 RLU whereas the lower limit of detection is 10^2 RLU. Thus, luminometer can not detect the light generated by the activity of luciferase which is less than 100 picograms.

4.4.3 Luciferase assays of EL4 cells transfected with pGL3fos-SV40

EL4 cells were electroporated with pGL3fos-SV40 at the conditions given in figure 4.23b. We lysed EL4 cells 48 hrs after transfection with RLB and luciferase assays were performed to measure the transcriptional activity of SV40 enhancer. These assays were done as a positive control of both fos promoter and luminescence detection. The light generated by the cell lysates would mean that fos promoter we inserted into pGL3 Basic was functional to initiate transcription of *luc*+ cDNA under a strong enhancer activity. At the same time, luminescence detection would ensure that luciferase reporter system worked properly when a strong enhancer acted on the *luc*+ cDNA.

Aliquots of cell lysates were assayed with Bradford reagent and the protein concentration of EL4 cell lysates were 500 μg/ml. The aim of Bradford assays was to

keep the total amount of protein constant in each cell lysate that would be assayed for luciferase activity.

50 µl of cell lysates were mixed with luciferase assay reagent (containing the substrate of luciferase) and luminescence was measured in the luminometer. Negative controls containing only luciferase reagent in RLB were also measured. In addition, lysates of cells that were not transfected were also measured in the luminometer. Both negative controls and the pGL3fos-SV40 transfected cell lysates gave RLU of approximately 50.

4.5 Transfection and Luciferase assays of 293 T cells

Since we could not achieve luciferase assays with EL4 cell line, we wanted to see whether there was a problem with the luciferase assay or the EL4 cell line. For this reason, we transfected another cell line, human kidney fibroblast cells (293 T cells) with pGL3fos-SV40 vectors and assayed for luciferase activity. For transfection of 293 T cells, we used calcium-phosphate mediated transfection. To test the efficiency of transfection mediated by calcium-phosphate method, we first transfected 293 T cells with 6.5 µg of pMaxGFP vector and visualized cells under fluorescent microscope 48 hrs after transfection (Figure 4. 25).

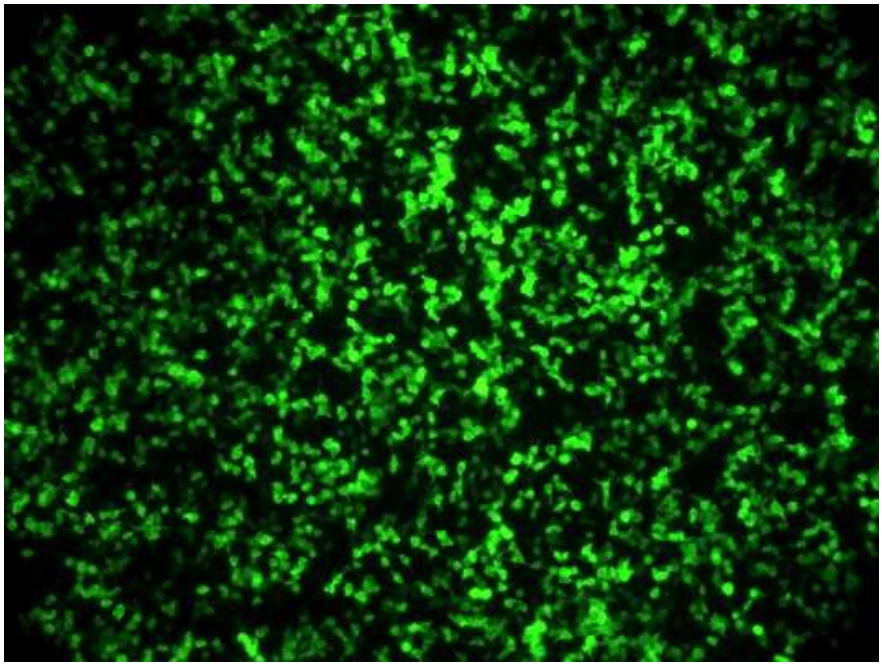


Figure 4.25: Visualization of GFP expression in 293 T cells 48 hrs after transfection

We observed that 293 T cells were transfected with an efficiency of 30-35 % and we examined the luciferase assay system with this cell line. We transfected 293 T cells with 6.5 μg of pGL3fos-SV40 vectors by calcium-phosphate method. We seeded 293 T cells at four different densities before the day of transfection to achieve highest efficiency in transfection and accordingly in luciferase assays. The total cell numbers seeded on plates were; $2.5x10^5$, $5x10^5$, $1x10^6$ and $2x10^6$. Luciferase assays were done 48 hrs post-transfection and protein concentrations were also determined by Bradford assays. The graph in figure 4.26 shows the luminescence generated as a result of SV40 enhancer activity. RLU negatively correlated with changes in cell numbers. In addition, Bradford assays showed the increasing protein concentration proportional to the cell numbers (Table 4.4). Thus, highest luciferase activity was observed in the cell lysate that had the lowest protein content. The cell culture seeded at a density of $2.5\ x10^5$ cells/plate were the most effectively transfected population among the four cell cultures. These results show that pGL3fos-SV40 can enhance the transcription of *luc*+ cDNA by interacting weak fos promoter that we inserted.

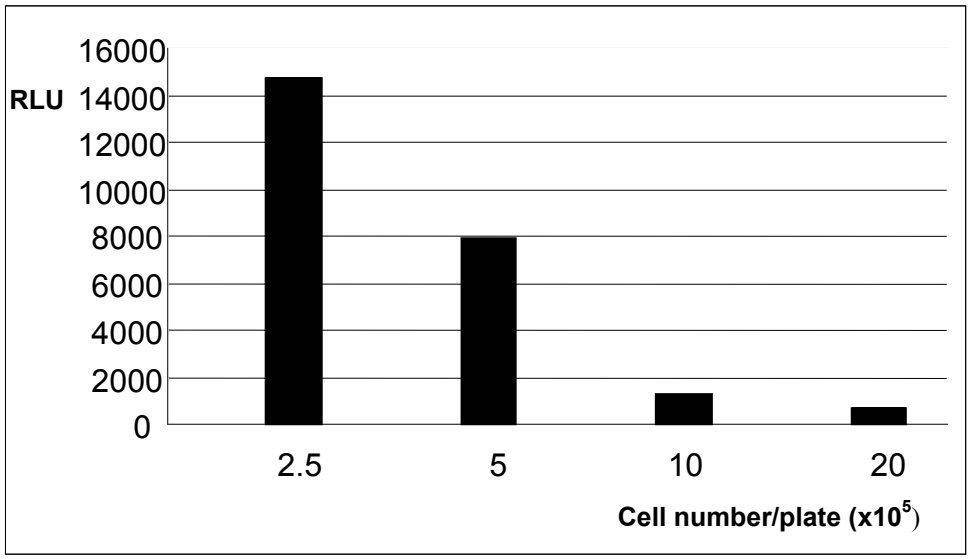


Figure 4.26: 293 T cell numbers seeded / plate show different levels of luciferase gene expression as assessed by RLU measurements: 293 T cells that were seeded at four different densities/plate before transfection with pGL3fos-SV40 showed a negative correlation between cell numbers and luciferase activities. Luciferase assays were done 48 hrs post-transfection.

293 Tcell numbers ($x10^5$)	2.5	5	10	20
$OD_{595\ nm}$	0.100	0.189	0.240	0.295
Protein conc (μg/ml)	0.44	0.83	1.05	1.29

Table 4.4: Protein concentrations of 293 T cell lysates assayed for luciferase activity

5 DISCUSSION

As outlined in the Introduction, IL-7 signaling through IL-7 Receptor has profound effects on B- and T- lymphocyte development and maintenance of T-lymphocytes in the periphery and IL-7 signaling is strictly regulated by the local availability of IL-7 in the lymphoid organs and expression of IL-7Rα chain in lymphocytes. IL-7Rα is differentially expressed depending on the lymphocyte type and developmental stage of the lymphocyte. However, the signals that regulate differential expression of IL-7Rα are poorly defined. To have a better understanding about the mechanisms regulating cell-specific and stage-specific expression of IL-7Rα, we tried to identify possible *cis*-acting elements activating transcription of IL-7Rα gene. For this purpose, we examined downstream of IL-7Rα gene spanning 80 kb region to determine noncoding sequences conserved between human and mouse. The reason why we tested such a huge region for conserved regions was that enhancers can regulate cell-specific and developmental stage-specific expression of genes even if they are located as far as 85 kb away from the promoter of the gene. Human and mouse IL-7Rα genes are homologous to each other and the gene flanking IL-7Rα gene is also homologous in mouse and human with conserved order of the genes in the locus. Human and mouse sequences were globally aligned by the AVID program. Mouse Capsl and human CAPSL genes flanking IL-7Rα genes were also included in the aligned sequences due to a possibility of coordinated regulation of IL-7Rα and Capsl genes by an enhancer located at the introns of the Capsl gene. Conservation degree of noncoding sequences downstream of the IL-7Rα gene were identified by the VISTA program according to the alignments done by AVID. The parameters were set to find conserved sequences at least 50 bp in length and 85% similar. In this way, ten conserved noncoding regions were found. The conserved regions that were proximal to each other in the genome were joined into a single region, thus a total of seven conserved noncoding regions were

chosen to examine for transcriptional activity. However, we also searched conserved sequences at least 100 bp in length and 75% similar to detect the sequences which are less conserved but occupying a remarkable size in the genomic region. Comparison of 75% conserved 100 bp long regions that VISTA found (data not shown) with 85% conserved 50 bp long regions showed that one more conserved region was located at the first intron of Capsl gene. These intronic sequences were 78% conserved between mouse and human but they occupied a region with a length of 450 bp. Since the enhancers can be located at the intronic regions, we considered the intronic region of Capsl as a conserved noncoding region. As a result, we determined a total of eight conserved noncoding regions at the downstream of IL-7Rα gene to be assayed for transcriptional activity.

Conserved noncoding sequences were amplified from mouse BAC clone RP23-365P6 by the primers carrying *Sal* I sites at 5' termini which was required for cloning into pGL3fos vector. While choosing primers, we eliminated the ones that were complementary to genomic regions in the BAC clone other than conserved noncoding sequences. Conserved noncoding sequences were made sure not to contain any *Sal* I site. Conserved noncoding sequences were named as Cap 1…8 as shown in table 4.2. Each of the Cap regions were cloned into pGL3fos vectors at the *Sal* I site located at the downstream of *luc*+ cDNA encoding for luciferase enzyme. The reason why we constructed pGL3fos vector was to include a weak promoter (fos) to the upstream of *luc*+ cDNA of pGL3 Basic reporter vector. Thus, in the absence of an enhancer activity, the promoter would not be sufficient for inducing the transcription of *luc*+ cDNA. Since we inserted a weak promoter to the upstream of *luc*+ cDNA, we would be able to detect the light generated even under the regulation of a low enhancer activity.

The vectors constructed by the insertion of conserved noncoding regions were named as pGL3fos-Cap(1…8) and the colonies transformed with pGL3fos-Cap vectors were identified by colony PCR method since we had the primers to specifically amplify each conserved noncoding region from pGL3fos-Cap vectors. Plasmids were isolated from the colonies. We analyzed Cap regions and characterized restriction sites found in each Cap region. Plasmids were digested with appropriate restriction enzymes to confirm the insertion of Cap regions. We did not intend to check the orientation of the

inserts because enhancers are *cis*-acting sequences that can regulate transcription independent of orientation with respect to the gene they regulate.

EL4 cells were chosen as the cell line to be transfected with pGL3fos-Cap constructs because EL4 cells are mouse $CD4^+$ single positive (SP) T-cells. As mentioned in the introduction part, both $CD4^+$ and $CD8^+$ SP T-cells that are not activated by specific antigen, express IL-7Rα at their cell surface. Enhancers can activate transcription of genes by recruiting transcription factors or coactivators to the promoter of the gene. Cell-specific and developmental stage-specific activity of enhancers can be explained by the fact that expression and modifications of transcription factors which bind to specific sites in the enhancer sequences are also regulated by cell-specific and developmental stage- specific signals. Hence, to study the enhancer activities of conserved noncoding sequences that may be regulating IL-7Rα gene expression, transcription factors possibly binding to these conserved regions should be found in the cell line studied. For this purpose, we chose EL4 cells that possess IL-7Rα on their surface.

We tried to optimize transfection efficiencies of EL4 cells before transfection with pGL3fos-Cap constructs. We first performed DEAE-Dextran mediated transfection of EL4 cells with pMaxGFP, but we could not observe any GFP expression even though we changed several parameters of the protocol (described in Methods). So we decided to transfect EL4 cells by electroporation. Although various changes in electric field strength, length of the electrical pulse and growth phase of the cells were examined, the efficiency of transfection was around 15% as determined by percentage of GFP expressing cells in total cell number.

Before transfection of EL4 cells with luciferase reporter constructs, we prepared the standard curve for protein concentration determination. We would determine the protein concentration in each cell lysate before measuring luminescence in order to eliminate the variances between each cell lysate. Although the transfection conditions would be considered to be the same in each transfection experiment, each transfection of luciferase constructs into a different cell culture would have resulted in different numbers of cells transfected and, as a consequence, different luminescence

measurements. Therefore, we aimed to take cell lysates that had equal protein content before measuring luminescence in each cell lysate.

Although the efficiency of transfection of EL4 cells by electroporation was low, we electroporated EL4 cells with pGL3fos-SV40 vector constructs. pGL3fos-SV40 plasmids contain SV40 enhancer that has a strong transcriptional activity for most genes. pGL3fos-SV40 were constructed as positive controls of luciferase assays. Luciferase assays of EL4 cells transfected with pGL3fos-SV40 generated the same RLU as blanks. Although we repeated the transfection with pGL3fos-SV40 several times, we measured luminescence under 100 RLU. The absence of transcriptional activity of SV40 on *luc*+ cDNA might have been due to low transfection efficiency of EL4 cells or the absence of a functional promoter. We needed to check the promoter activity of fos which was inserted to the upstream of *luc*+ cDNA. For this purpose we used another cell line 293 T cells which can efficiently uptake foreign DNA. In this cell line, we observed generation of a high luminescence as a result of SV40 activity on fos promoter to induce expression of *luc*+ cDNA. When we decreased the cell numbers to be transfected, we observed that transfection efficiency and luciferase activity increased. This result may be explained by the fact that cells proliferate rapidly when they have not reached confluency, so the numbers of the transfected cells also increase exponentially.

Since we confirmed that pGL3fos-SV40 plasmid construct was an efficient positive control of luciferase assays, the explanation of the absence of luminescence in EL4 cells transfected with pGL3fos-SV40 could be low transfection efficiency. However, luciferase reporter system is highly sensitive and can report the luminescence generated by the activity of even a few femtograms of luciferase, so transfection of EL4 cells with low efficiency should not have resulted in absence of a luminescence. We thought that luminometer we used might be the reason of the insensitivity in the measurements. When we reexamined the luminescence data of recombinant luciferase (Table 4.3), we realized that the luminometer could not detect the luminescence generated by the activity of 100 picograms or lower amounts of luciferase. Hence, the luminescence generated in small numbers of cells could not be detected by the luminometer we used. In addition, the activities of weak enhancers on luciferase expression can not be detected with this luminometer.

6 CONCLUSION

IL-7 / IL-7R signaling controls survival and proliferation of B- and T-lymphocytes at early stages of development and positive selection of $CD8^+$ single positive T-cells. IL-7 also regulates homeostatic maintenance of naïve T-lymphocytes in the peripheral organs and contributes to the generation of memory T-cells at the end of an immune response. IL-7Rα is found at the surface of lymphocytes at early stages of development. IL-7Rα expression is absent in B-cells after pre-B cell stage. In developing T-cells, IL-7Rα expression is absent at between TN4 and double positive stage. Expression of IL-7Rα is upregulated in the cells that differentiate into $CD8^+$ and $CD4^+$ single positive cells and is present on the cells as they leave to the periphery. IL-7Rα expression diminishes in activated T-cells and again upregulated in memory T-cells.

Although the expression of IL-7Rα in T-cells is tightly regulated, little is known about the signals that regulate IL-7Rα expression. Pu.1 and GABP are shown to bind to an ets motif at the promoter region of IL-7Rα and activate transcription. However, regulatory effect of Pu.1 on lymphocytes is restricted only in early stages of lymphocyte development and the role of GABP as a transcriptional activator of IL-7Rα in T-lymphocytes has not been shown to correlate with IL-7Rα expression in different stages of T-lymphocytes development.

The aim of this project was to identify and characterize possible enhancer sequences at the downstream of IL-7Rα gene that may explain the distinct regulation of IL-7Rα in different subsets of lymphocyte population. Enhancers can activate transcription even if they reside several kilobases upstream or downstream away from promoters so we examined up to 80 kb region in the downstream of mouse IL-7Rα

gene. Enhancers contain specific binding sites for different transcription factors and these sites are mostly conserved between different species. Human and mouse IL-7Rα genes are functionally conserved and their regulation by a possible enhancer region may be conserved as well. For this reason, we aligned human and mouse IL-7Rα gene downstream sequences and we identified conserved noncoding sequences in an 80 kb genomic region at the downstream of mouse IL-7Rα gene.

We aimed to analyze the regulation of mouse IL-7Rα gene by conserved noncoding sequences in $CD4^+$ Single Positive (SP) cells. We intended to use luciferase enzyme as a reporter of possible transcriptional activity of these regions. For this purpose, we modified pGL3 Basic luciferase reporter vectors to carry a weak promoter (pGL3fos). Weak promoter would induce basal level of luciferase transcription in the absence of an enhancer region. Conserved noncoding sequences were cloned into these luciferase reporter vectors to analyze the transcriptional activation of luciferase cDNA under the effect of possible enhancer motifs found in these sequences. Positive control vectors (pGL3fos-SV40) were also constructed by the insertion of SV40 strong enhancer into luciferase reporter vectors. We electroporated EL4 (mouse $CD4^+$ SP) cells with pGL3fos-SV40 vectors as a positive control of the luciferase activity. Transcriptional activation of luciferase is detected by the generation of luminescence by the catalytic activity of luciferase. However, due to instrumental limitations in the detection of luminescence, we could not achieve luciferase assays with EL4 cells. But, when we changed the cell line, we observed that efficient transfection of 293 T cells with pGL3fos-SV40 vectors resulted in detectable luminescence generation. Therefore, we concluded that the luminometer we used was not suitable for luciferase assays with cell lines that were inefficiently transfected and for analysis of low enhancer activities.

7 FUTURE WORK

For the analysis of conserved noncoding sequences that we cloned, luminometers that are more suitable for luciferase assays should be used to detect low levels of luciferase activity. Alternatively, other reporter systems can be used for the analysis of transcriptional activity. Destabilized fluorescent proteins may be used as a reporter of transcriptional activity (Cheng and Kain, 1995). Transcriptional activity can be analyzed quantitatively by flow cytometry using a Fluorescence Activated Cell Sorter (FACS) machine. Cells that express fluorescent protein under the control of a *cis*-acting element can be counted by FACS machine. Another advantage of studying *cis*-regulatory elements by fluorescent reporter vectors and FACS machine can be the analysis of differential regulation of gene transcription in different cell populations. In our system, we aimed to study the regulation of transcription of IL-7Rα gene in $CD4^+$ SP T-cells, but analysis in TN cells, memory T-cells, $CD8^+$ SP T-cells that are all expressing IL-7Rα is also necessary and *cis*-regulatory elements that may be selectively active in one of these thymocyte subsets should be identified. Once a *cis*-acting regulatory element is identified, the mechanism by which the element exerts its function should be also assessed. Possible transcription factors that may bind to the regulatory element should also be identified by electrophoretic mobility shift assays or DNA affinity chromatography.

Lastly, identification of new regulatory elements acting on IL-7Rα gene may contribute to the therapeutic studies of immunodeficiency diseases resulting from the loss of IL-7Rα expression. In addition, understanding the regulation of IL-7Rα expression may provide a new approach to the therapeutic potential of IL-7 since IL-7 is promising in that it can be used as an immunomodulator for the treatment of lymphopenia and the enhancement of thymopoiesis in cancer and immunodeficiency.

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APPENDICES

APPENDIX A

CHEMICALS& MEDIA COMPONENTS	SUPPLIER COMPANY
6X Loading Dye	Fermentas, Germany
Acetic Acid	Riedel-de Haén, Germany
Agarose	AppliChem, Germany
Ampicillin	Sigma, Germany
Boric Acid	Riedel-de Haén, Germany
Bradford Reagent	Sigma, Germany
Calcium Chloride ($CaCl_2$)	Merck, Germany
Chloramphenicol	Sigma, Germany
Chloroquine	AppliChem, Germany
Diethylaminoethyl-dextran (DEAE-Dextran)	AppliChem, Germany
Dimethylsulfoxide (DMSO)	Sigma, Germany
Dulbecco's Modified Eagle Medium (DMEM)	Sigma, Germany
Ethylenediaminetetraacetic acid (EDTA)	Riedel-de Haén, Germany
Ethanol	Riedel-de Haén, Germany
Ethidium Bromide	Merck, Germany
Fetal Bovine Serum (FBS)	Biological Industries, Israel

Glycerol	Riedel-de Haén, Germany
HEPES (4-(2-Hydroxyethyl) piperazine-1-ethane sulfonic acid)	AppliChem, Germany
Hydrochloric Acid (HCl)	Merck, Germany
Isopropanol	Riedel-de Haén, Germany
Kanamycin	Sigma, Germany
L-glutamine	Merck, Germany
Liquid nitrogen	Karbogaz, Turkey
Luria Agar	Sigma, Germany
Luria Broth	Sigma, Germany
Magnesium Chloride ($MgCl_2$)	Sigma, Germany
$MgCl_2$ solution (25 mM)	Promega, USA
Na_2HPO_4	AppliChem, Germany
NAH_2PO_4	Merck, Germany
Polyethylenglycol (PEG) -4000	AppliChem, Germany
Penicillin-Streptomycin	Biological Industries, Israel
Piperazine-N,N'-bis(2-hydroxypropane-sulfonic acid) (PIPES)	Sigma, Germany
Potassium Chloride (KCl)	Fluka, Germany
RPMI 1640	Biological Industries, Israel
Sodium Chloride (NaCl)	Riedel-de Haén, Germany
Sodium Hydroxide (NaOH)	Merck, Germany
Tris Base	Fluka, Switzerland
Trypan Blue	Sigma, Germnay
Trypsin-EDTA	Biological Industries, Israel

APPENDIX B

EQUIPMENTS	SUPPLIER COMPANY
5,10,25 ml pipettes	Trp, Switzerland
Autoclave	Hirayama, Hiclave HV-110, Japan Certoclav, Table Top Autoclave CV-EL-12L, Austria
Balance	Sartorius, BP221S, Germany Schimadzu, Libror EB-3200 HU, Japan
Black 96-well Plates	Cole Palmer, USA
Cell counter	Cole Parmer, USA
Centrifuge	Eppendorf, 5415D, Germany Hitachi, Sorvall RC5C Plus, USA Heraeus, Multifuge 3S-R, Kendro Lab. Prod., Germany
CO_2 incubator	Binder , Germany
Deep Freeze	-80°C, Forma -86, Thermo Electron Corp. USA -20°C, Bosch, Turkey
Digital Camera	Spot RT KE Slider, Diagnostic Instruments, USA
Distilled Water	Millipore, Elix-S, France
Electrophoresis	Biogen Inc., USA Biorad Inc., USA
Electroporation cuvettes	Eppendorf, Germany
Electroporator	BTX, ElectroCell Manipulator, ECM 630, Division of Genetronics, USA
Filter membranes	Millipore, USA
Gel Documentation	UVITEC, UVIdoc Gel Documentation System, UK

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 Hood	Heraeus, HeraSafe HS12, Kendro Lab. Prod., Germany
Liquid Nitrogen Tank	Taylor-Wharton, 3000RS, USA
Luminometer	SpectraMax Gemini XS Dual-Scanning Microplate Spectrofluorometer Molecular Devices Inc., USA
Microliter Pipette	Eppendorf, Germany
Microscopes	Olympus CK 40, Japan Olympus CH 20, Japan Olympus 1X 70, Japan
Microwave Oven	Bosch, Turkey
pH Meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Refrigerator	+4°C, Bosch, Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
UV Illuminator	Biorad, UV-Transilluminator 2000, USA
Vortex	Velp Scientifica, Italy

APPENDIX C

MOLECULAR BIOLOGY KITS & ENZYMES

Molecular biology kits

Luciferase Assay System	Promega, USA
Qiagen Midi Plasmid Purification Kit	Qiagen, Germany
Qiaprep Miniprep Plasmid Purification Kit	Qiagen, Germany
Qiaquick Gel Extraction Kit	Qiagen, Germany
Qiaquick PCR Purification Kit	Qiagen, Germany

Enzymes

Calf Intestinal Alkaline Phosphatase& Buffer	Promega, USA
DNA Polymerase I Large (Klenow) Fragment	Promega, USA
Recombinant Luciferase	Promega, USA
T4 DNA Ligase & Ligation Buffer	Promega, USA
Taq polymerase & Buffer	Promega, USA

Restriction enzymes & buffers:

Restriction Enzyme	**Buffer**	**Company**
Hind III	Buffer E	Promega, USA
Pvu II	Buffer B	Promega, USA
Sal I	Buffer D	Promega, USA
Sph I	Buffer K	Promega, USA
Xba I	Buffer D	Promega, USA
Xmn I	Buffer B	Promega, USA

Restriction enzymes & buffers used in double digestions:

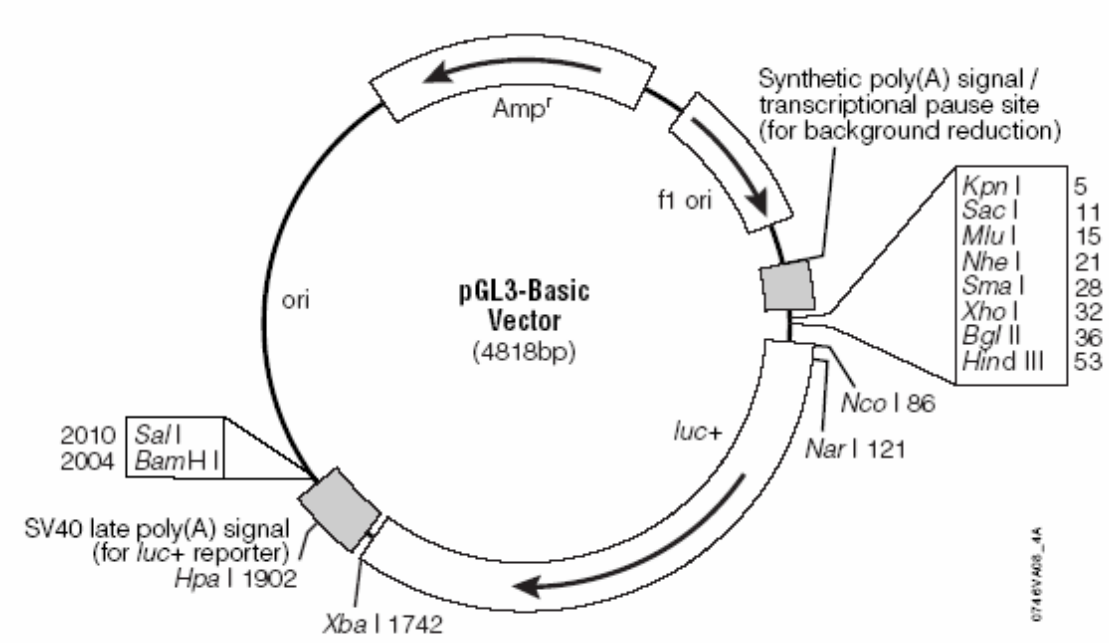
Restriction Enzymes	**Buffer**	**Company**
BamH I + *Nco* I	Buffer E	Promega, USA
BamH I + *Sal* I	Buffer *BamH* I	Fermentas, Germany
Bgl II + *Sal* I	Buffer D	Promega, USA
Hind III + *Nco* I	Buffer E	Promega, USA
Hind III + *Xba* I	Buffer E	Promega, USA
Pvu II + *Sph* I	Buffer B	Promega, USA
Xba I + *Sal* I	Buffer D	Promega, USA

APPENDIX D

VECTORS

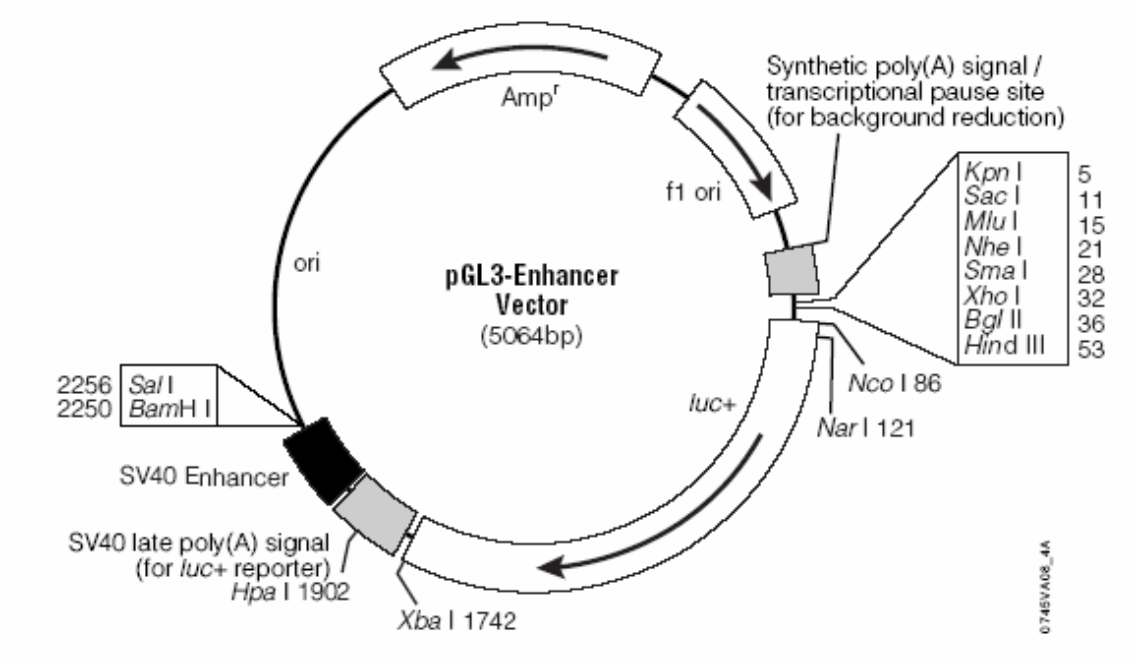
pGL3 Basic and Enhancer	Promega, USA
BAC Clones	Children's Hospital Oakland Research Institute, USA
Δ56-fosCAT	provided by Dr. Batu Erman
pMAX-GFP	Amaxa Biosystems, Israel
psp72-μ170	provided by Dr. Batu Erman

VECTOR MAPS



pGL3-Basic Vector Sequence Reference Points:

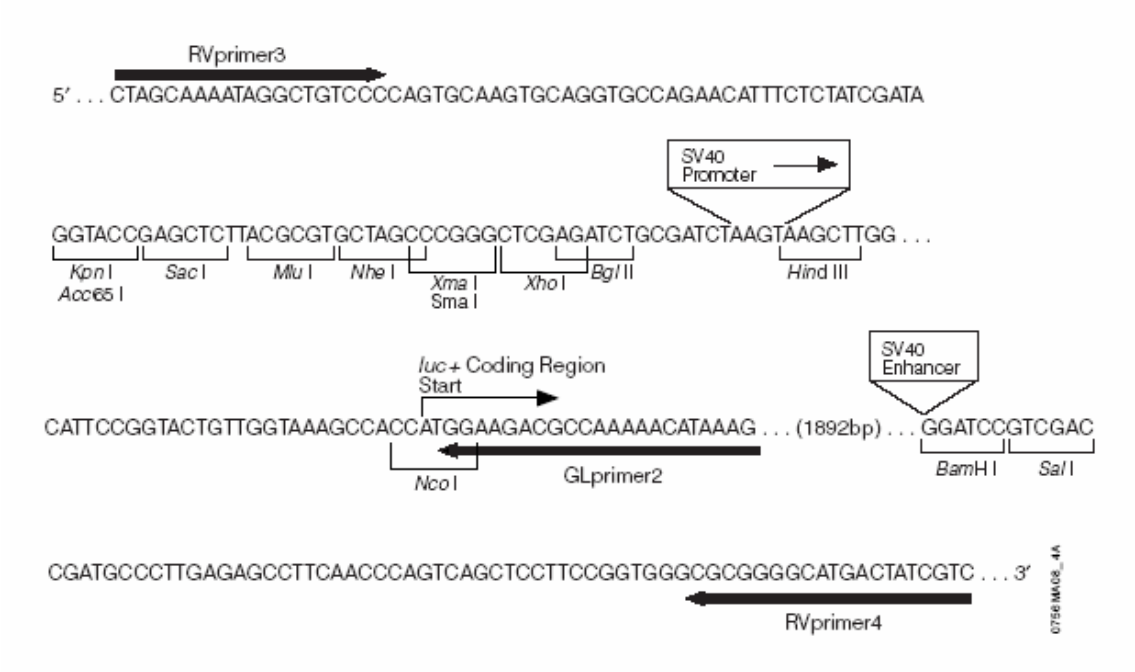
SV40 Promoter	(none)
SV40 Enhancer	(none)
Multiple cloning region	1–58
Luciferase gene (*luc*+)	88–1740
GLprimer2 binding site	89–111
SV40 late poly(A) signal	1772–1993
RVprimer4 binding site	2080–2061
*Col*E 1-derived plasmid replication origin	2318
β-lactamase gene (Ampr)	3080–3940
f1 origin	4072–4527
Synthetic poly(A) signal	4658–4811
RVprimer3 binding site	4760–4779



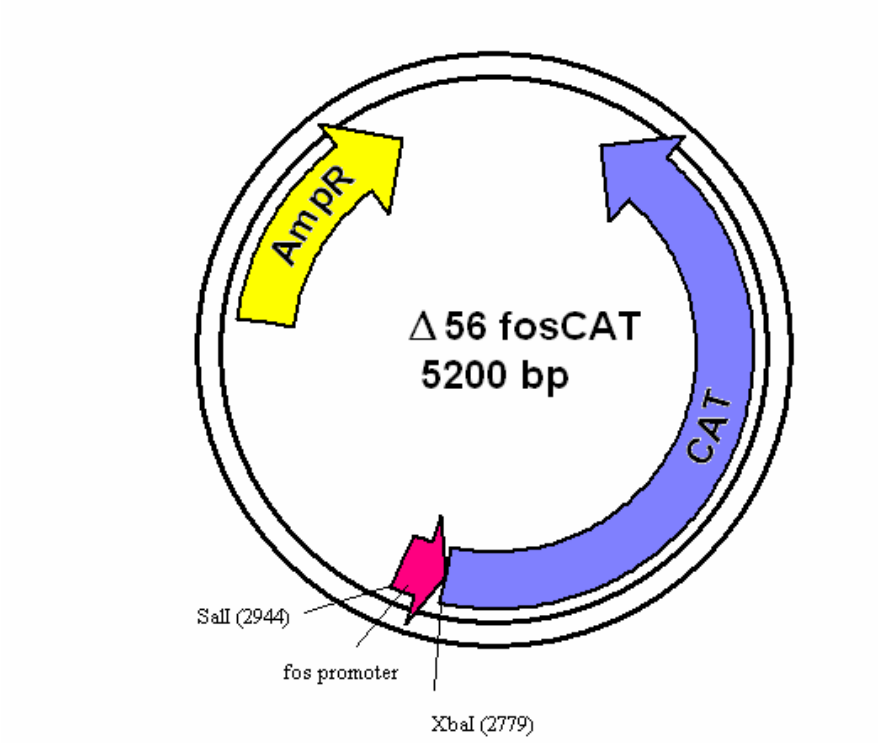
pGL3-Enhancer Vector Sequence Reference Points:

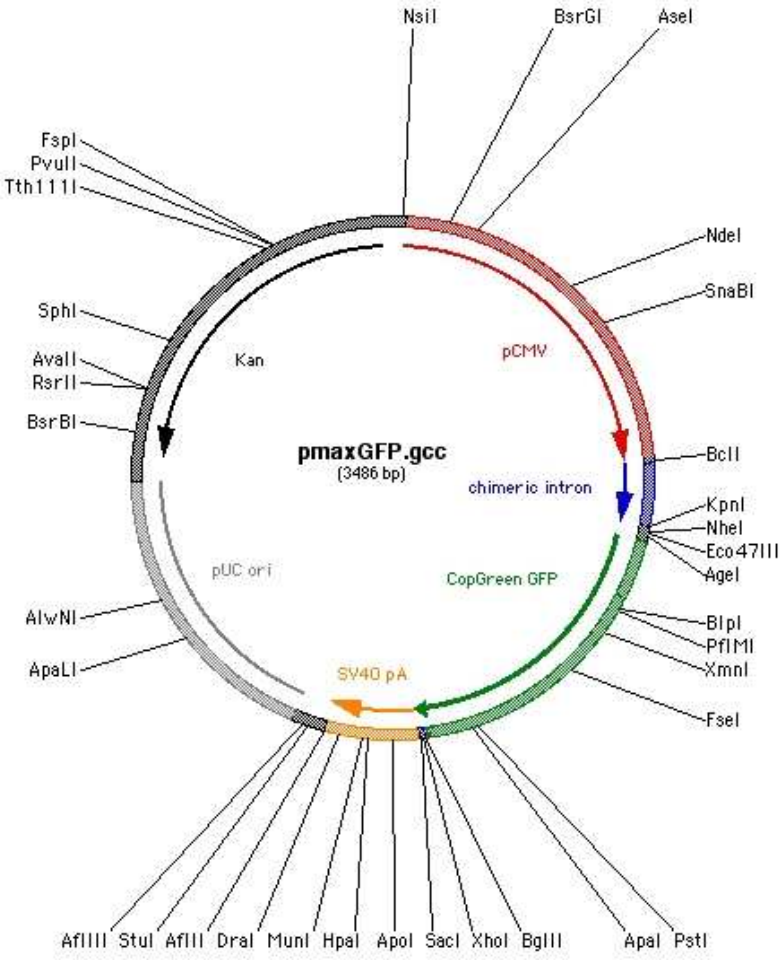
SV40 Promoter	(none)
Multiple cloning region	1–58
Luciferase gene (*luc*+)	88–1740
GLprimer2 binding site	89–111
SV40 late poly(A) signal	1772–1993
SV40 Enhancer	2013–2249
RVprimer4 binding site	2307–2326
*Col*E 1-derived plasmid replication origin	2564
β-lactamase gene (Amp^r)	3326–4186
f1 origin	4318–4773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

Multiple cloning site of pGL3 vectors



Δ56fosCAT vector map has been drawen by pDraw32




NsiI
BsrGI
AseI
FspI
PvuII
Tth111I
NdeI
SnaBI
SphI
AvaII
RsrII
BsrBI
Kan
pCMV
pmaxGFP.gcc
(3486 bp)
chimeric intron
BclI
KpnI
NheI
Eco47III
AgeI
pUC ori
CopGreen GFP
AlwNI
ApaLI
BlpI
PflMI
XmnI
SV40 pA
FseI
AflIII
StuI
AflII
DraI
MunI
HpaI
ApoI
SacI
XhoI
BglII
ApaI
PstI

pSP72-μ170 vector that was used in this study had been previously constructed by the insertion of μ170 enhancer into the *EcoR* V restriction site of pSP72 vector:

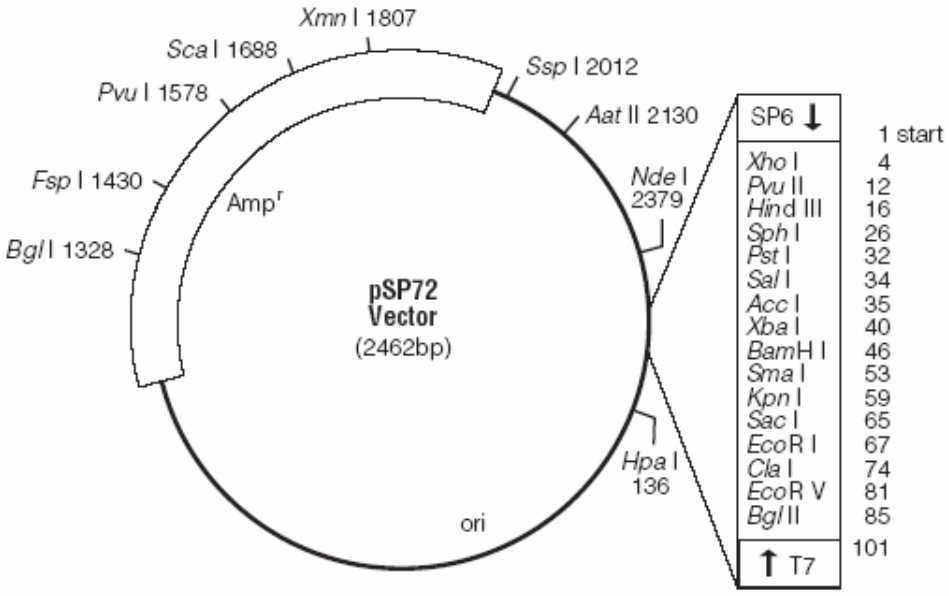


Figure 2. pSP72 Vector circle map and sequence reference points.

pSP72 Vector sequence reference points.

SP6 RNA polymerase transcription initiation site	1
T7 RNA polymerase transcription initiation site	101
SP6 RNA polymerase promoter (–17 to +3)	2446–3
T7 RNA polymerase promoter (–17 to +3)	99–118
multiple cloning region	4–90
β-lactamase coding region	1135–1995

APPENDIX E

OLIGONUCLEOTIDES & DNA MOLECULAR WEIGHT MARKERS

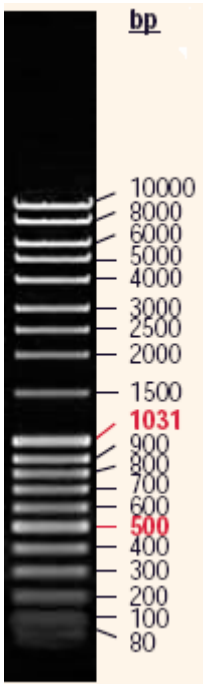
Primers	Sequences
Cap1 Forward	GACGCGTCGACTCAGGAGTTGGCTTATTGATG
Cap1 Reverse	GACGCGTCGACTCAAAATGTCTGCACAAAGG
Cap2 Forward	GACGCGTCGACTGAATATAACCTGCAAAGCACA
Cap2 Reverse	GACGCGTCGACAGTGATGCCCTGAGGAAGAA
Cap3 Forward	GACGCGTCGACGCTATAGAAAGACACAGCCCTTC
Cap3 Reverse	GACGCGTCGACGAAGGGAACCTCACATGGAA
Cap4 Forward	GACGCGTCGACAAAACGATGACCTTTTGAGGA
Cap4 Reverse	GACGCGTCGACGATTTTGGAGGGGGCTTTTA
Cap5 Forward	GACGCGTCGACTCTGCCTCCTGACCCAAGTA
Cap5 Reverse	GACGCGTCGACCAGAGCAACCAATACCAAACA
Cap6 Forward	GACGCGTCGACGTCAGAAGGGGATGAAATGG
Cap6 Reverse	GACGCGTCGACCAGGAGGTTTGGCTCCTATTC
Cap7 Forward	GACGCGTCGACCCTGTCTTCCTTTGCTGTCC
Cap7 Reverse	GACGCGTCGACAACACCGTGGGGAAAGGTAT
Cap8 Forward	GACGCGTCGACTGGTTTTGTTTTCTAGGCTTAAT
Cap8 Reverse	GACGCGTCGACCAAAAATTAGGCTAGTGCTATGCAG

DNA molecular weight markers

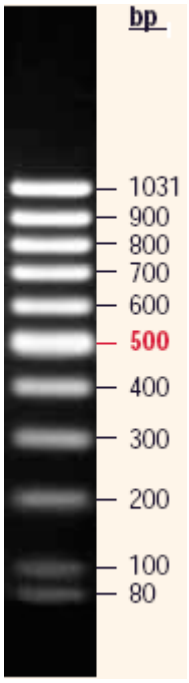
Mass Ruler DNA Ladder High Range	Fermentas, Germany
Mass Ruler DNA Ladder Mix Range	Fermentas, Germany
Mass Ruler DNA Ladder Low Range	Fermentas, Germany
Puc mix marker 8	Fermentas, Germany
Gene Ruler 1 kb	Fermentas, Germany

Agarose gel photographs and molecular weights of the bands of the DNA markers:

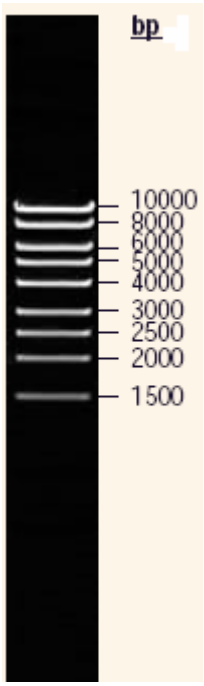
Mass Ruler Mix Range



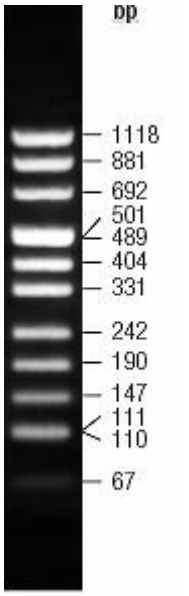
Mass Ruler Low Range



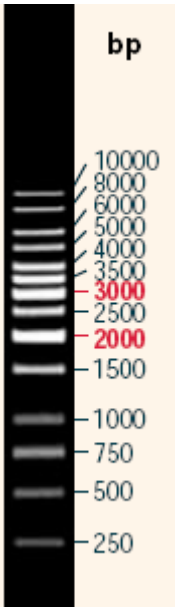
Mass Ruler High Range



Puc mix marker 8



Gene Ruler 1 kb



APPENDIX F

BUFFERS & SOLUTIONS

✓ 2X HEPES-buffered saline

0.8 g of NaCl, 0.027 g of $Na_2HPO_4.2H_2O$ and 1.2 g of HEPES are dissolved in 90 ml of distilled H_2O. pH is adjusted to 7.05 with 0.5 M NaOH and the solution is completed to 100 ml with distilled water. The buffer is filter-sterilized.

✓ 5X Tris-Borate-EDTA (TBE) buffer**:**

54 g of Tris base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA at pH 8.0 are dissolved in 1 L of distilled H_2O.

✓ 10X Tris-EDTA (TE), pH 7.6

100 mM of Tris-Cl (pH: 7.6) and 10 mM of EDTA are prepared in 20 ml distilled H_2O.

✓ 10X STBS solution

3.02 g of Tris base, 8 g of NaCl, 0.38 g of KCl and 0.107 g of $Na_2HPO_4.2H_2O$ are dissolved in 90 ml of distilled H_2O. pH is adjusted to 7.4 and the solution is completed to 100 ml with distilled H_2O and filter-sterilized.

✓ Agarose gel

Agarose gels are prepared as 1% w/v. 1 g of agarose is dissolved in 100 ml of 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide is included in the solution.

✓ $CaCl_2$ Solution for competent cell preparation

60 mM $CaCl_2$, 15% glycerol; 10 mM piperazine-N,N'-bis (2-hydroxypropane-sulfonic acid) (PIPES); at pH:7 is prepared in 500 ml distilled $H_2O$and autoclaved

✓ $CaCl_2$ solution (2.5 mM)

11 g of $CaCl_2.6H_2O$ is dissolved in a final volume of 20 ml distilled H_2O.

✓ Chloroquine (5.16 mg/ml)

52 mg of chloroquine is dissolved in 10 ml of distilled H_2O and filter-sterilized.

✓ DEAE-Dextran (10 mg/ml)

0.1 g of DEAE-Dextran is dissolved in 10 ml of distilled H_2O and filter sterilized.

✓ $MgCl_2$-$CaCl_2$ Solution

0.1 g of $MgCl_2.6H_2O$ and 0.1 g of $CaCl_2.H_2O$ are dissolved in 10 ml of distilledH_2O and filter-sterilized.

✓ Phosphate-buffered saline (PBS)

8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 are dissolved in 800 ml of distilled H_2O. pH is adjusted to 7.4 with HCl and the buffer is completed to 1 L with distilled H_2O.

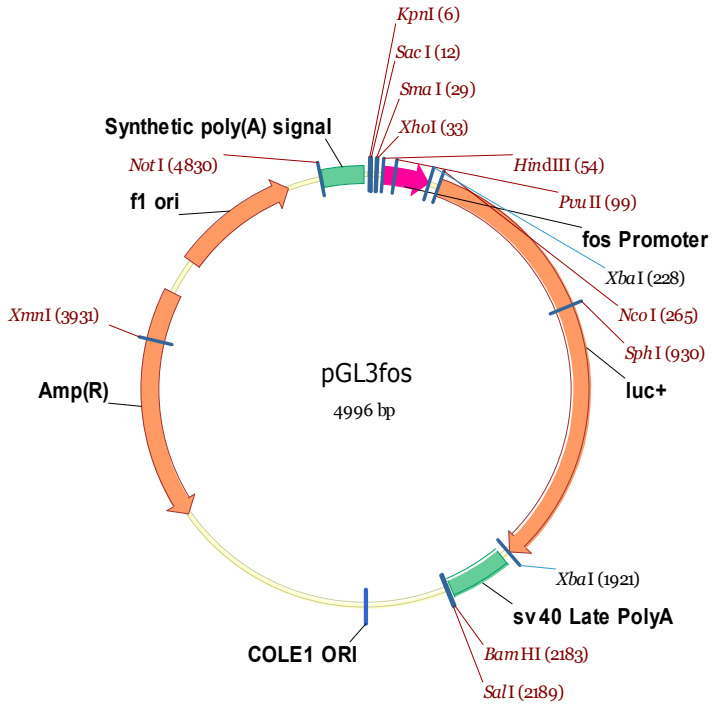
✓ Trypan blue dye (0.4% w/v)

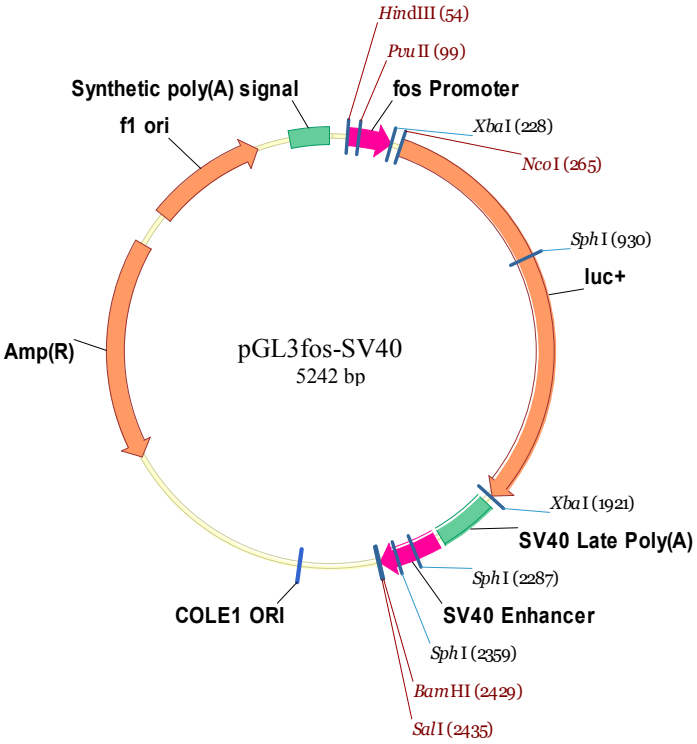
40 μg of trypan blue is dissolved in 10 ml of PBS.

APPENDIX G

VECTORS CONSTRUCTED

Maps of the vectors constructed are drawn by Vector NTI Program.

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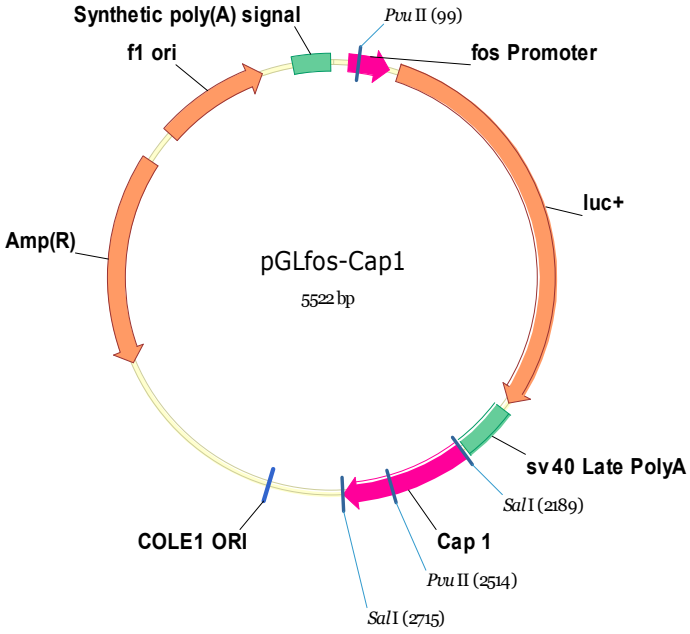
f1 ori
XbaI (228)
NcoI (265)
SphI (930)
luc+
Amp(R)
pGL3fos-SV40
5242 bp
XbaI (1921)
SV40 Late Poly(A)
SphI (2287)
COLE1 ORI
SV40 Enhancer
SphI (2359)
BamHI (2429)
SalI (2435)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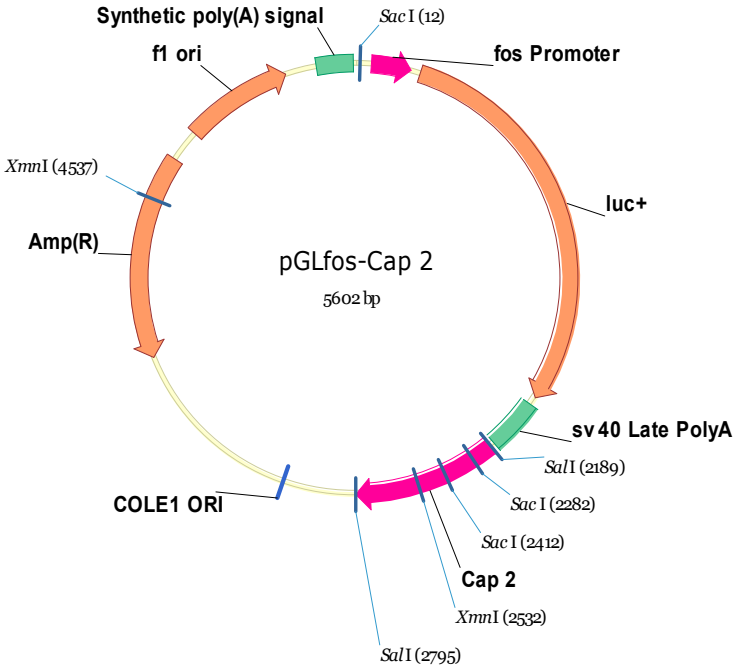
PvuII (99)
f1 ori
fos Promoter
luc+
Amp(R)
pGLfos-Cap1
5522 bp
sv40 Late PolyA
SalI (2189)
COLE1 ORI
Cap 1
PvuII (2514)
SalI (2715)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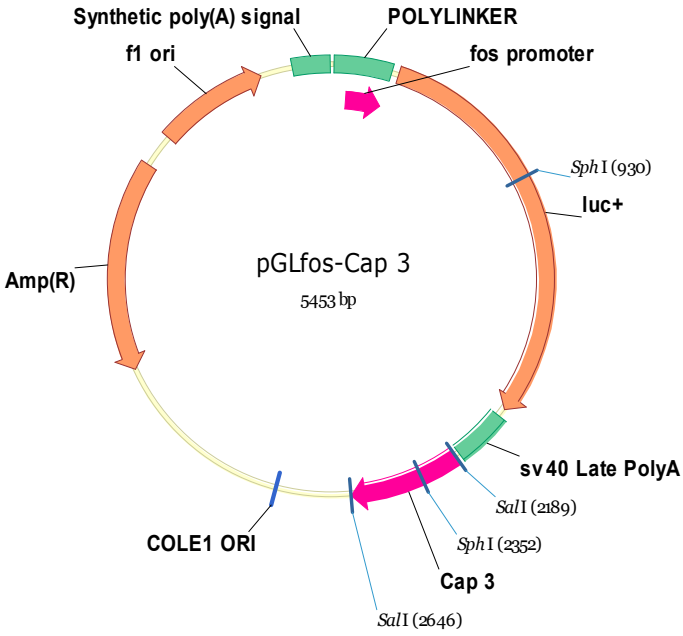
fos Promoter
XmnI (4537)
luc+
Amp(R)
pGLfos-Cap 2
5602 bp
sv40 Late PolyA
SalI (2189)
Sac I (2282)
Sac I (2412)
COLE1 ORI
Cap 2
XmnI (2532)
SalI (2795)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fos promoter
SphI (930)
luc+
Amp(R)
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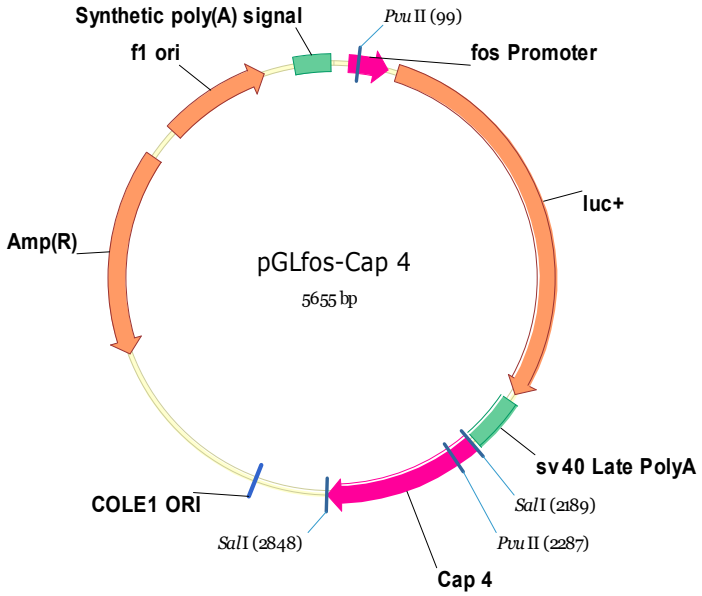
sv40 Late PolyA
SalI (2189)
SphI (2352)
COLE1 ORI
Cap 3
SalI (2646)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fos Promoter
luc+
Amp(R)
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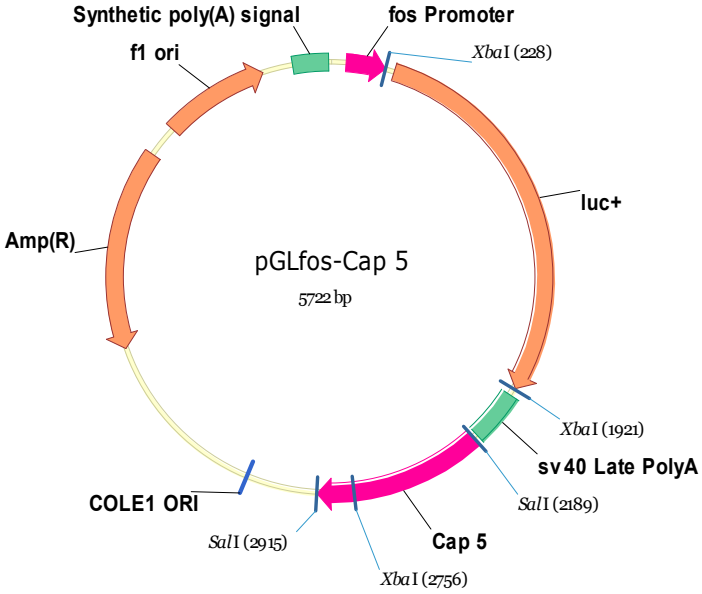
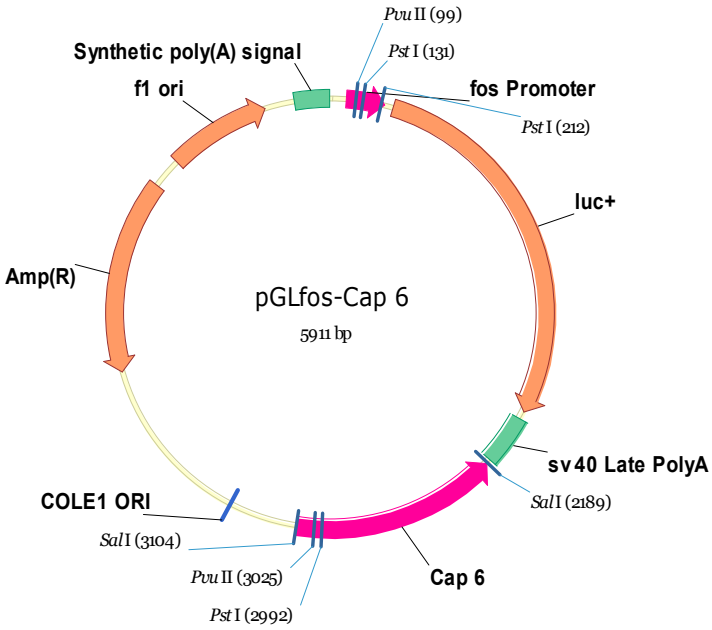
sv40 Late PolyA
SalI (2189)
COLE1 ORI
SalI (2848)
PvuII (2287)
Cap 4


Synthetic poly(A) signal
fos Promoter
f1 ori
XbaI (228)
luc+
Amp(R)
pGLfos-Cap 5
5722 bp
XbaI (1921)
sv40 Late PolyA
SalI (2189)
COLE1 ORI
SalI (2915)
Cap 5
XbaI (2756)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fos Promoter
PstI (212)
luc+
Amp(R)
pGLfos-Cap 6
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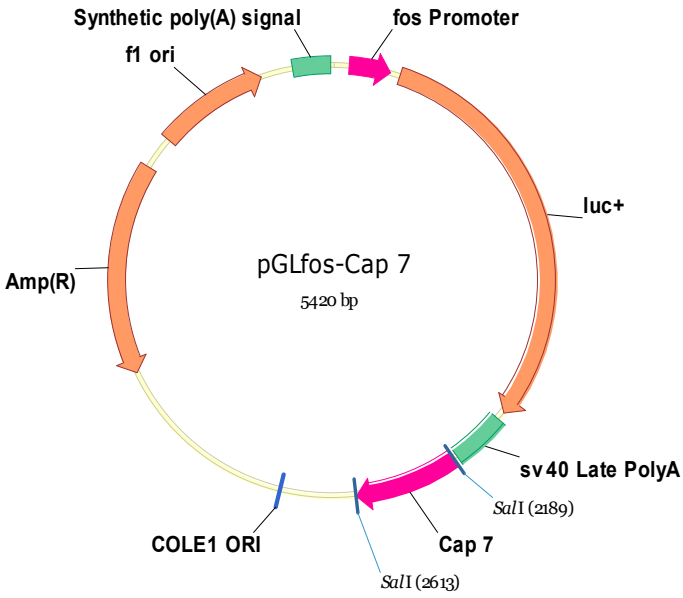
sv40 Late PolyA
SalI (2189)
COLE1 ORI
SalI (3104)
PvuII (3025)
Cap 6
PstI (2992)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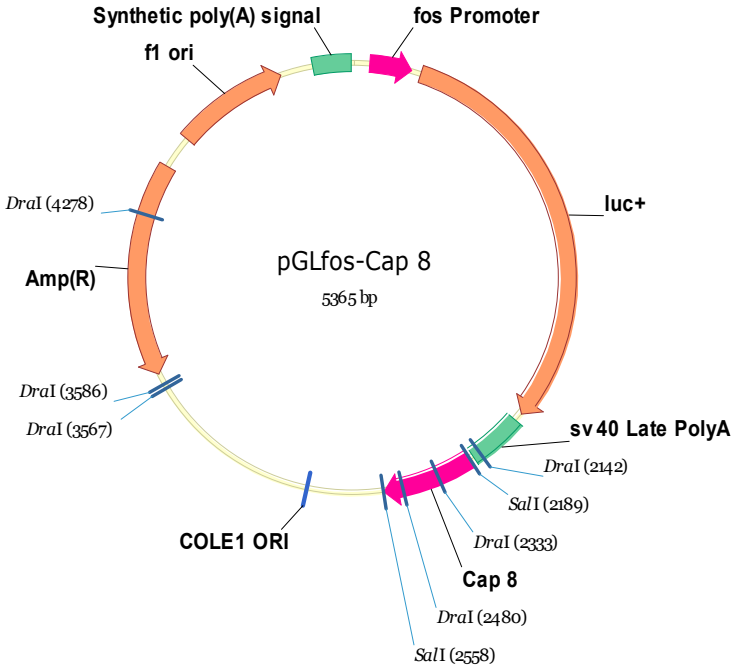
fos Promoter
f1 ori
luc+
Amp(R)
pGLfos-Cap 7
5420 bp
sv40 Late PolyA
SalI (2189)
COLE1 ORI
Cap 7
SalI (2613)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fos Promoter
f1 ori
luc+
DraI (4278)
pGLfos-Cap 8
5365 bp
Amp(R)
DraI (3586)
DraI (3567)
sv40 Late PolyA
DraI (2142)
SalI (2189)
DraI (2333)
COLE1 ORI
Cap 8
DraI (2480)
SalI (2558)

Appendix H

Sequences inserted into pGL3 Basic vector

> fos promoter

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ACTACTCCAACCGCGACTGCAGCGAGCAACTGAGAAGACTGGATAGAGCCG
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> Cap 1

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GA

> Cap2

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> Cap 3

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> Cap4

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> Cap5

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> Cap 6

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> Cap7

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AAG

> Cap 8

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