IDENTIFICATION OF UPSTREAM CONSERVED NONCODING SEQUENCES FOR THE ANALYSIS OF TRANSCRIPTIONAL ACTIVATION ON IL-7 RECEPTOR ALPHA GENE

by

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ABSTRACT

Interleukin-7 (IL-7) is a key cytokine in the development of B- and T-lymphocytes. Responsiveness of lymphocyte populations to IL-7 is controlled by the temporal and cell specific expression of the IL-7 receptor (IL-7R). Although there are studies on the transcriptional regulation of IL-7R expression, these findings are insufficient to explain the tight regulation on IL-7R expression.

In this study, we identified conserved upstream sequences at the mouse IL-7R alpha locus. We aligned murine and human genomic sequences using a pairwise global alignment approach and found seven noncoding genomic regions with more than 85 percent conservation. We amplified the conserved sequences from a specific bacterial artificial chromosome and constructed luciferase reporter vectors representing these mouse genomic sequences. Here we investigate a reporter gene strategy to identify the transcriptional activation properties of these mouse sequences in a mouse T-lymphoma cell line.

We evaluated the efficiency of luciferase assay system in a human kidney fibroblast cell line and a mouse CD4 single positive T-lymphocyte cell line. Due to the distinct expression properties of IL-7R, the present study can be expanded for cells representing the different stages of B- and T-lymphocyte development. Analysis of the transcriptional responses of different cell lineages to the conserved sequences will facilitate the studies on the investigation of the tight regulation on IL-7R expression.

ÖZET

İnterlökin-7 (IL-7) B- ve T-lenfositlerin gelişiminde anahtar rol alan bir sitokindir. Lenfosit populasyonlarının IL-7'ye duyarlılığı, IL-7 reseptörünün (IL-7R) zamana ve hücre tipine bağlı ekspresyonu ile kontrol edilir. IL-7R geninin transkripsyonel kontrolü ile ilgili bir takım çalışmalar yapılmış olsa da, edinilen bulgular bu geninin üzerindeki sıkı kontrolü açıklamak için yeterli değildir.

Bu çalışmada, fare IL-7R alfa lokusunda, transkripsyon başlangıç noktasından önde bulunan, korunmuş diziler aranmıştır. Fare ve insan genomunun bu bölgedeki dizileri ikili genel hizalama yöntemine göre karşılaştırılmış olup, yedi tane yüzde 85'in üzerinde korunmuş kodlamayan dizi bulunmuştur. Bu diziler polimeraz zincir reaksyonu ile, spesifik bakteriyel yapay kromozomundan çoğaltılıp lusiferaz haberci plazmidlerine klonlanmıştır. Klonlanan korunmuş dizilerin fare T-lenfoma hücrelerindeki transkripsyonel aktivasyon özelliklerini araştırmak için, lusiferaz genine dayanan bir genetik haberci stratejisi tasarlanmıştır.

Belirlenen lusiferaz yöntemi, insan böbrek fibroblast hücrelerinde ve fare CD4 pozitif T-lenfoma hücrelerinde denenmiştir. Bu çalışma, IL-7R geninin farklı ekspresyon özelliklerine dayanarak genişletilmelidir. B- ve T-lenfosit gelişiminin farklı evrelerindeki hücrelerin korunmuş dizilere olan transkripsyonel duyarlılıklarının incelenmesi, IL-7R geninin ekspresyonu üzerindeki kontrolü araştıran çalışmaları hızlandıracaktır.

to my family

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ABBREVIATIONS

293T	Human kidney cell line
aa	Amino acid
BAC	Bacterial artificial chromosome
Box1	Cytoplasmic membrane-proximal domain of IL-7R protein
bp	Base pair
BSA	Bovine serum albumin
CD3	Accessory molecule of TCR
CD4, CD8	TCR co-receptors
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
CLP	Common lymphoid progenitor
DMEM	Dulbecco's Modified Eagle Medium
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EL-4	Mouse T-lymphoma cell line
GABP	GA binding protein
GFP	Green fluorescent protein
HCS	Hematopoietic stem cell
HSA	Heat stable antigen
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IL	Interleukin
IL-7Rα	Interleukin-7 receptor alpha
ISP	Intermediate single positive
JAK	Janus kinase
kb	Kilobase pairs
Kpl 7-13	Conserved noncoding regions

MHC	Major histocompatibility complex
NCBI	National Center for Biotechnology Information
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3 kinase
PMT	Photo multiplier tube
PPBSF	pre-pro-B cell-growth stimulating factor
RAG	Recombination-activating genes
RLU	Relative light unit
rpm	Revolution per minute
SCF	Stem cell factor
SH	Src homology domain
SOCS	Suppressor of cytokine signaling
SP	Single positive
STAT	Signal transducer and activator of transcription
SV 40	Simian virus 40
TCR	T cell receptor
TSLP	Thymic stromal lymphopoietin
UCSC	University of California at Santa Cruz
V_H	Ig heavy chain variable gene segment
γc	Common γ chain

1 INTRODUCTION

1.1 Interleukin-7 Signalling

Communication of immune cells is mediated by either cell-to-cell interactions or by local production of soluble factors. Cell-to-cell interactions involve molecules such as the T cell receptor (TCR), major histocompatibility complex (MHC), co-receptor molecules, integrins, accessory molecules and co-stimulatory molecules. On the other hand, cytokines are usually soluble, small-protein signalling molecules acting on cells at close proximity or on cells at a distance. Cytokines are a part of the extracellular signalling network that controls the development of immune cells, inflammation, defense against viruses and regulation of their own signalling. Receptors for cytokines are grouped into four families (Roitt *et al.*, 2001). The largest of the four families is the type I cytokine receptor family that contains the receptors for cytokines such as IL-2, IL-4 and IL-7 (Ozaki and Leonard, 2002).

1.1.1 Interleukin-7

Interleukin-7 (IL-7) is a critical cytokine in the survival and development of lymphocytes. IL-7 is a 25 kDa glycoprotein that is produced by stromal cells, monocytes, and some epithelial cells (Hofmeister et al., 1999). Stimulation of bone marrow precursor cells with cloned IL-7 gene identified IL-7 as a growth promoting factor (Namen *et al.*, 1988). Later on, IL-7 or IL-7R deficient mice showed a severe reduction in the number of lymphocytes that denoted a major role to IL-7 in lymphoid development (Freeden-Jeffry *et al.*, 1994; Peschon *et al.*, 1994). Temporal expression of

the IL-7 receptor carefully controls the responsiveness of lymphoycte populations to IL-7. As lymphocytes differentiate from bone marrow precursors to functional mature cells, they express the receptor for IL-7 at distinct stages, indicating that IL-7 survival signals are tightly regulated. This does not come as a surprise because misregulated survival signals can lead to the uncontrolled growth of lymphocytes.

1.1.2 Interleukin-7 receptor

IL-7 receptor is a heterodimer involving a common gamma chain (γc) and the IL-7 receptor alpha chain (IL-7Rα or CD127). The common γc is shared by other cytokine receptors for IL-2, IL-4, IL-9, IL-15 and IL-21 (Ozaki and Leonard, 2002). It is shown that γc chain alone does not bind to IL-7 (Park *et al.*, 1990). Also, it is demonstrated that IL-7 binds to IL-Rα and γc complex with a considerably higher affinity than IL-7Rα alone (Noguchi *et al.*, 1993). In addition, chimeric receptor chains are created, which consist of the intracellular domains of IL-Rα or γc with the extracellular domain of an inducible receptor; and expression of a reporter gene flanking to a cytokine responsive promoter has been examined (Ziegler 1995). This study indicated that both chains are necessary for reporter gene expression.

The IL-7Rα gene consists of eight exons and is found on chromosome 5 in human and chromosome 15 in mouse. IL-7Rα is particularly expressed on the cells in the lymphoid lineage. Common lymphoid progenitor cells (CLP) -which give rise to T, B and natural killer (NK) cells-, developing B- and T-lymphocytes, mature T cells, dendritic cell precursors and macrophages derived from bone marrow have been shown to express IL-7R (Figure 1.1). Furthermore, it is stated that, IL-7Rα is expressed on normal human intestinal epithelial cells, T cell lymphomas and several other nonlymphoid cancer cells and cell lines (Akashi *et al.*, 1998; Cosenza *et al.*, 2002).

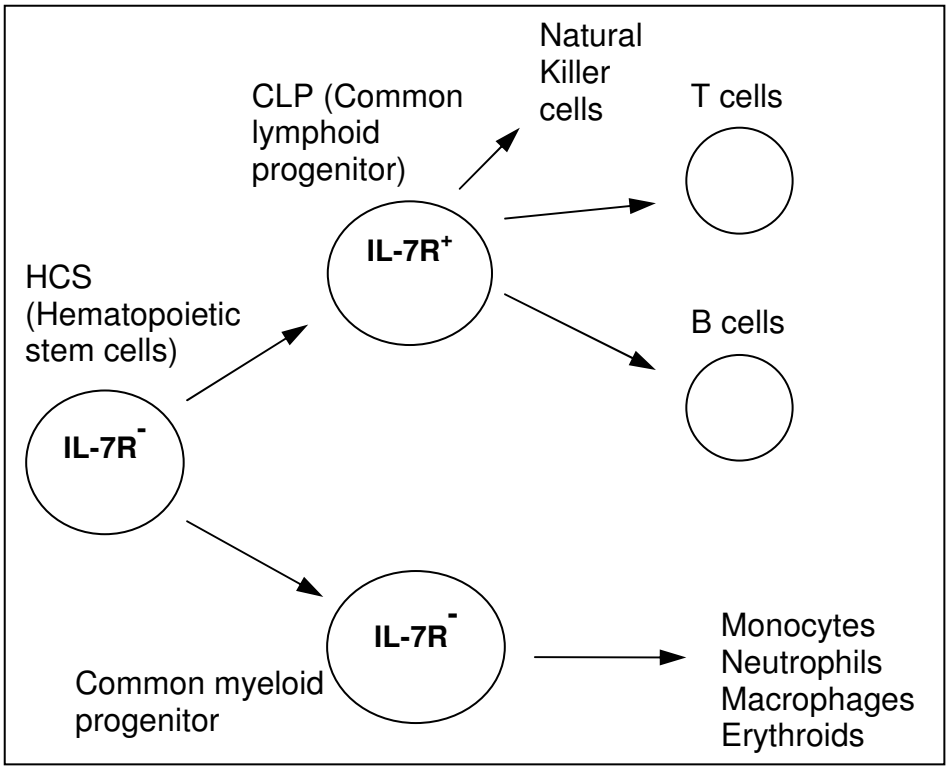


Figure 1.1: Lineages of cells that differentiate from bone marrow precursors

IL-7Rα consists of 439 amino acids and has a molecular weight 49.5 KDa. IL-7Rα carries the characteristic features of type I cytokine receptor family members which all have two fibronectin-like domains, four conserved cysteine residues and a trp-ser motif. It is a membrane glycoprotein with a single 25 aa transmembrane domain. The cytoplasmic domain of IL-7Rα contains a region with acidic residues (region-A), a serine rich region (region-S) and a region with three tyrosine residues (region-T) conserved between human and mouse (Jiang *et al.*, 2005). Moreover, a cytoplasmic membrane-proximal domain named as Box1 is conserved among the type I cytokine receptor family (Murakami *et al.*, 1991). IL-7Rα does not have a kinase domain and it is considered that A, S, T and Box1 regions contribute to the interactions with the intracellular adaptor and nonreceptor kinase molecules.

1.1.3 Interleukin-7 signalling pathways

Signals from the IL-7 receptor are essential for lymphoid development and homeostasis. In brief, IL-7 induces the heterodimerization of IL-7Rα and γc chains, a γc chain associated cytoplasmic kinase called JAK3 phosphorylates JAK1 that is bound to the α chain and the α chain itself, providing the accessibility of STAT transcription factors (Qin *et al.*, 2001). Then, phosphorylated STATs dimerize and translocate into the nucleus resulting in the activation of cytokine specific genes (Foxwell *et al.*, 1995). Furthermore, the activation of PI3 kinase has been found to be essential for the IL-7 mediated survival and proliferation of human T cell precursors, indicating that JAK kinase activation of STAT transcription factors may not be the only downstream effects of IL7R signaling (Pallard *et al.*, 1999).

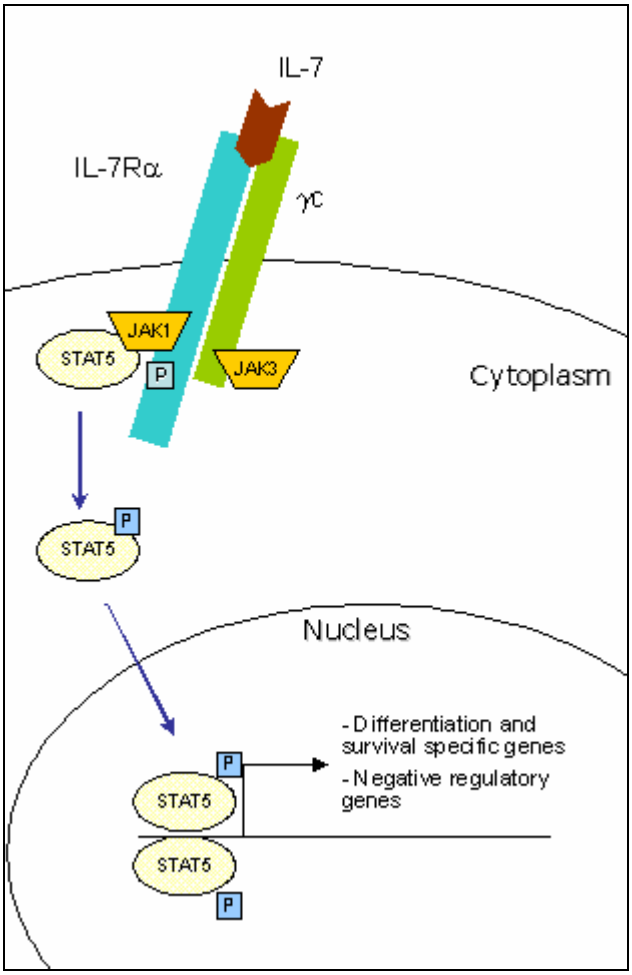


Figure 1.2: General representation of interleukin-7 activated signal transduction pathway (adapted from Kang and Der, 2004).

1.1.3.1 JAK/STAT pathway

1.1.3.1.1 JAK3

JAK3, which is a protein tyrosine kinase that is associated with the carboxy-terminal region of γc, is accepted as the initial step in the signal transduction cascade from the IL-7 receptor. JAK3-deficient mice exhibit defects similar to the γc deficient mice. Mice with JAK3 deficiency show impaired lymphoid development which gives the evidence of the role of JAK3 in transducing the γc-dependent signals (Suzuki *et al.*, 2000). It is proposed that, JAK3 binds to γc by its amino-terminal region including two JAK homology domains (O'Shea *et al.*, 2002).

1.1.3.1.2 JAK1

JAK1 is associated with the IL-7Rα chain and is activated by IL-7 induction. Jak1 deficient mice showed impaired thymic development and gave no response to IL-7 (Rodig *et al.*, 1998), suggesting that this kinase is an essential component in IL-7 signalling. Pyk2, a tyrosine kinase, has been shown to be linked with JAK1 and IL-7Rα. It is demonstrated that IL-7 induced the phosphorylation and enzymatic activity of Pyk2 which has been shown to have a role in survival of a thymocyte cell line (Benbernou *et al.*, 2000). Previously it was stated that, JAK1 interacts with IL-2Rβ chain resulting in the tyrosine phosphorylation of the p85 subunit of PI3 kinase (Migone *et al.*, 1998). However IL-7 induced PI3 kinase activation of JAK1 has not been shown.

1.1.3.1.3 STAT5a/b

STAT family members of transcription factors are activated by a number of cytokine receptors. The SH2 domain of STAT5 docks to a phosphorylated tyrosine residue on IL-7Rα and phosphorylation of this SH2 domain makes dimerization of STAT5 possible. STAT5a and STAT5b are the two isoforms, which show 96%

sequence identity, can form homo or heterodimers upon phosphorylation induced by IL-7 in T cells (Rosenthal *et al.*, 1997). STAT5 is an anti-apoptotic transcription factor and it regulates the expression of many caspases and Bcl-2 family members (Debierre-Grockiego, 2004). When STAT5a, STAT5b and combined STAT5a-b deficient mice were examined, these mice appeared to have impaired thymocyte development or deficiencies in the number of peripheral B and T cells (Teglund *et al.*, 1998). This indicates that activation of STAT5 by IL7 signals plays a role in B and T cell development. Supporting this finding, it was found that phosphorylation of STAT5 was present in immature DN thymocytes, was downregulated in DP thymocytes and was reestablished gradually after positive selection (Van De Wiele *et al.*, 2004). The development of T cells inside the thymus proceeds through a series of stages. The initial stage stands for $CD3^-CD4^-CD8^-$ double-negative (DN) immature thymocytes, followed by a $CD4^+CD8^+$ double-positive (DP) stage and, finally a single-positive (SP) stage containing $CD4^+$ or $CD8^+$ mature T cells (Fry and Mackall, 2002), (Figure 1.4). The presence of phospho-STAT5 exactly coincides with the presence of functional IL7 receptor on the surface of these developing thymocytes, where DN thymocytes express surface IL7R, DP do not, and positively selected SP thymocytes re-express the IL7R. Furthermore, it is demonstrated that inhibition of STAT5b caused an increase in the number of DN thymocytes while the number of DP and γδ thymocytes decreased (Pallard *et al.*, 1999). This finding indicates that IL7R is important in the differentiation of thymocytes beginning at a very early DN stage.

The suppressor of cytokine signalling (SOCS) protein family members contain a characteristic SH2 domain which provides competition with STATs for binding to JAKs. It is shown that, T cells stimulated with IL-7 had an increased expression of SOCS1 (Starr and Hilton, 1998) and T cells of SOCS1 overexpressing mice were arrested at the DNI stage (Trop *et al.*, 2001). Moreover, STAT5 activation is inhibited in SOCS1 transgenic mice (Fujimoto *et al.*, 2000). These results indicate that there is a feed-back regulation in which IL-7 downregulates JAK1 activation by upregulating SOCS1.

1.2 Effects of Interleukin-7 signalling on B- and T-Lymphoid Cells

B cell development proceeds in the bone marrow from a common lymphoid progenitor (CLP). CLP cells are characterized by the expression of IL-7Rα and c-kit which is the receptor for stem cell factor (SCF). Expression of the surface marker, B220 and low levels of heat stable antigen (HSA) is the marker for the pre-pro-B cell which is the first identifiable progenitor committed to the B lineage (Figure 1.3). A period of proliferation and beginning of immunoglobulin heavy chain rearrangement take place during the transition to the pro-Bcell stage. At the early pre-B-cell stage, heavy chain rearrangement is completed and successfully rearranged cells expand in response to IL-7 and other factors. At the late pre-B-cell stage, IL-7Rα expression disappears and is no longer observed on B cells at later stages (Fry and Mackall, 2002).

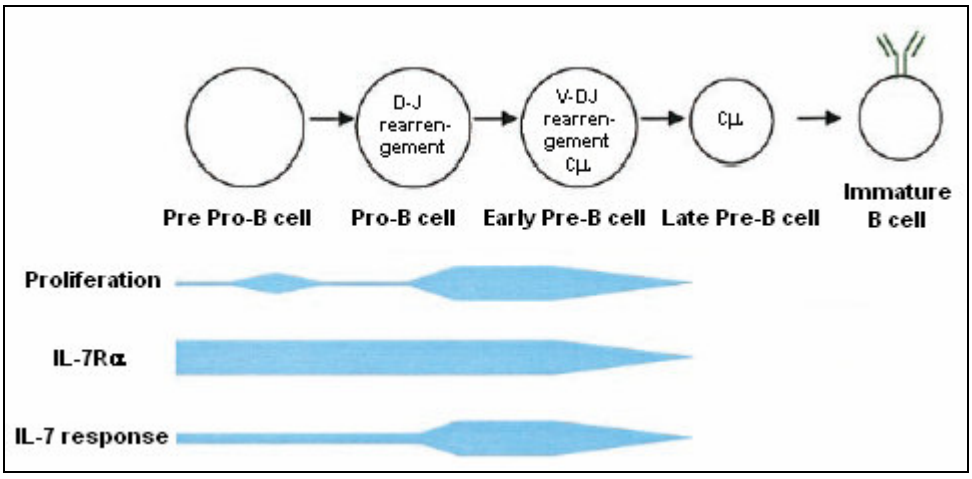


Figure 1.3: B-cell development in relation to IL-7Rα expression and IL-7 responsiveness (adapted from Fry and Mackall, 2002).

Signals from IL-7 receptor are required for B-cell development in mice. It is observed that in IL-7 deficient mice, transition from pro-B-cell stage to pre-B-cell stage is blocked (von Freeden-Jeffry *et al.*, 1995). On the other hand, in IL-7R deficient mice, the block in B-cell development occurred earlier, at the pre-pro-B-cell stage. (Peschon *et al.*, 1994) This is explained by the presence of a second molecule named as Thymic stromal lymphopoietin (TSLP) that also uses IL-7Rα to signal and compensates the loss of IL-7 up to a level at the pre–pro–B-cell stage (Ray *et al.*, 1996) In addition to these

findings, pre-pro-B cell growth-stimulating factor (PPBSF), which is a heterodimer of IL-7 and the variant β-chain of hepatocyte growth factor is demonstrated to stimulate proliferation and differentiation *in vitro* (Mckenna *et al.*, 1998).

More recently, it was proposed that IL-7 regulated the chromosome accessibility of immunoglobulin heavy chain (IgH) locus in pro-B-cells. It is observed that IL-7 induces hyperacetylation of histones and therefore promotes the nuclease accessibility of the largest family of V_H genes and activates these segments for potential recombination by RAG (Chowdhury and Sen, 2001). Compared to pro-B-cells, Chowdhury and colleagues found decreased IL-7R expression and lower levels of activated STAT5 in pre-B-cells. Moreover, they demonstrated that the termination of IL-7 signalling promoted allelic exclusion at certain V_H gene segments in pre-B-cells (Chowdhury and Sen, 2003). These studies also showed the recombinational reactivation of VH genes with exogenous IL-7 in mature B cells whose VH genes have already been allelically excluded.

IL-7 signalling also promotes VDJ recombination at the TCRγ locus in mice. Rearrangement of the TCRγ locus is severely repressed in IL-7Rα deficient mice (Maki *et al.*, 1996). Later on, it was shown that STAT5 overexpression in IL-7R deficient thymocyte precursors resulted in increased levels of acetylated histones associated with the TCRγ locus and unrearranged germline transcripts which are the indicators of TCRγ locus accessibility. Furthermore, mice deficient in IL-7 showed partially restored γδ thymocyte development in response to STAT5 expression (Ye *et al.*, 2001), which suggests that STAT5 has a role in controlling the TCRγ locus accessibility.

CLP stage is the first stage that expression of IL-7Rα can be detected. During thymocyte development IL-7Rα levels are tightly regulated. While high levels of expression is found on DN thymocytes, DP cells do not express detectable amount of IL-7R (Figure 1.4). In later stages, mature SP cells and peripheral T cells express higher levels of IL-7R on their surface (Munitic *et al.*, 2004). The development of T cells inside the thymus proceeds through a series of stages. The first stage stands for $CD3^-$ $CD4^-CD8^-$ double-negative (DN) immature thymocytes, followed by a $CD4^+CD8^+$ double-positive (DP) stage and, finally a single-positive (SP) stage containing $CD4^+$ or

$CD8^+$ mature T cells. The DN, DP, and SP stages respectively represent 5%, 80%, and 15% of the thymocyte pool (Fry and Mackall, 2002). Investigations on IL-7 (Moore *et al.*, 1996) and IL-7Rα (Peschon *et al.*, 1994) deficient mice revealed that thymic cellularity is more severely reduced in IL-7Rα deficient mice and the differentiation of DN thymocytes is only partially inhibited in IL-7 deficient mice compared to IL-7Rα deficient mice. The reason for that is explained by the importance of TSLP as in the B cell development.

IL-7 signalling maintains the survival and expansion of DN thymocytes by upregulating bcl-2 family members. It is stated that double negative DNII (Figure 1.4) cells purified from IL-7 deficient mice have increased apoptosis and decreased levels of bcl2 gene expression (von Freeden-Jeffry *et al.*, 1997) In addition, forced expression of bcl-2 transgene in IL-7Rα deficient mice caused an increased number of thymocytes and the alleviation of some of the defects of IL7R deficiency (Maraskovsky *et al.*,1997).

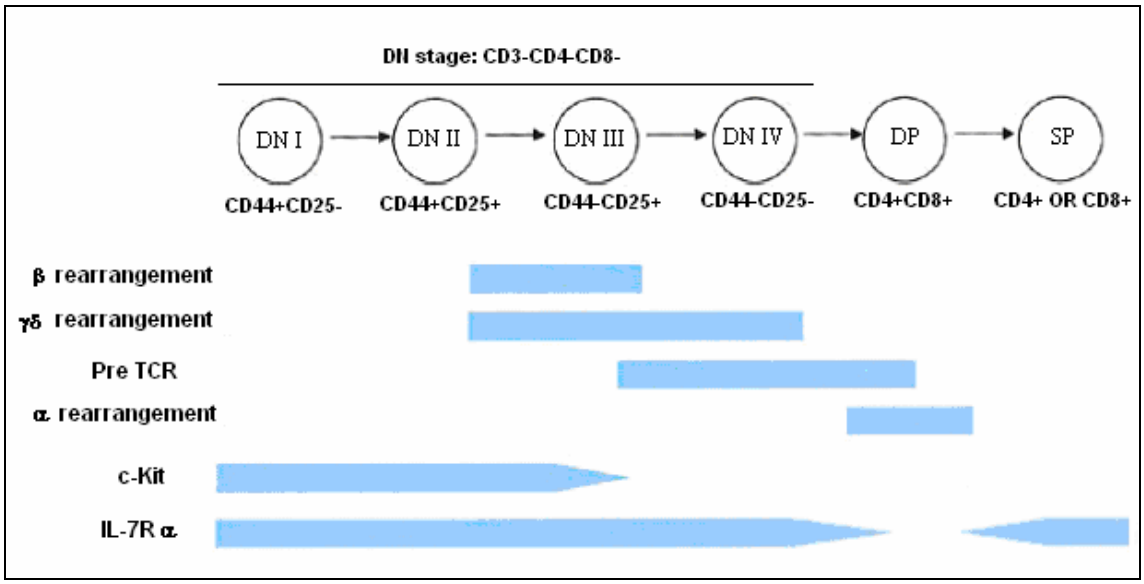


Figure 1.4: Developmental stages and IL-7Rα expression of T-lymphocytes (adapted from Fry and Mackall, 2002).

In a series of experiments, IL-7Rα transgenic mice were used to explain the loss of IL-7Rα expression in normal DP thymocytes. TCF-1, LEF-1, and RORγt transcription factors that promote the transition into the DP stage were downregulated in IL-7Rα transgenic mice, suggesting that IL-7R downregulation is required for transition

into DP stage (Yu *et al.*, 2004). In IL7R transgenic mice, antigen specific negative selection at DP stage was found to be unaffected by the continuous expression of IL-7Rα, however it was demonstrated that number of thymocytes greatly reduced depending on age. This result is explained by the fact that expanding DP thymocytes which express IL-7Rα on their surface decreased the availability of IL-7 to DN cells in thymus due to competition (Munitic *et al.*, 2004). Therefore, tight regulation of IL-7Rα is crucial to maintain essential IL-7 levels for the precursor DN cells.

IL-7 is strictly required for co-receptor reversal at the transition from $CD4^+CD8^-$ intermediate stage into $CD4^-CD8^+$ SP stage. DP thymocytes have the potency to become either $CD4^+$ or $CD8^+$ single positive cells (Figure 1.4). According to the co-receptor reversal model of thymocyte development, $CD4^+CD8^+$ DP thymocytes cease CD8 expression and become $CD4^+CD8^-$ intermediate thymocytes. At this intermediate stage, if TCR signals are sustained, cells differentiate into CD^+4 SP cells; otherwise cells turn into $CD8^+$ SP cells (Singer, 2002). Recently, the significance of cytokine signalling in co-receptor reversal has been demonstrated by showing that IL-7 receptor signals are required for up-regulation of Bcl-2 gene expression to maintain thymocyte viability, enhancement of CD4 gene silencing, up-regulation of glucose transporters on the cell surface and also for functional maturation of intermediate thymocytes into $CD8^+$ SP T cells (Yu *et al.*, 2003).

In peripheral organs, naïve T cells require survival signals from TCR-MHC-peptide interactions and from IL-7 receptor. High levels of IL-7R expression is observed in naïve $CD4^+$ SP and $CD8^+$ SP T cells. Besides the survival function, IL-7 signals are required for homeostasis of naïve T cell population meaning that number of peripheral naïve T cells is under control. Upon antigen stimulation, T cells turn into activated effector cells and IL-7R expression is reduced. At the same time, expression of other cytokine receptors such as IL-2, IL-4 and IL-15 is upregulated which are responsible for differentiation and expansion of effector T cells. After the elimination of antigen, memory T cells are formed and IL-7R expression is upregulated again. IL-7 signals induce survival and maintain homeostatic turnover of memory T cells (Bradley *et al.*, 2005).

1.3 Regulation of IL-7 Receptor-α Gene

As mentioned in the previous chapter, development of B- and T-lymphocytes proceeds through a series of stages. These stages of lymphocytes differ from each other by chromosomal rearrangement status and surface expression of characteristic proteins. For example, IL-7 signalling is essential for the survival of B-lymphocytes at early stages of development (Figure 1.3). But the cells at the pre-B-cell stage loose their responsiveness to IL-7 signalling as a result of disappearing of IL7-R expression on their surfaces. Moreover, during T-lymphocyte development, IL-7R expression is missing at certain stages (Figure 1.4).

The influence of IL-7 signalling on lymphoid cells can not be explained by IL-7 due to the fact that the regulation of IL-7 production is poorly understood (Alpdogan and van den Brink, 2005). In fact, which lymphoycte population is responsive to IL7 is carefully controlled by the temporal expression of the IL7 receptor. This indicates the presence of a tight regulation on IL-7R gene expression at distinct stages of lymphocyte development.

An ETS family transcription factor, PU.1 has been shown to control IL-7R expression in early B cell progenitors. Two PU.1 binding sites have been identified at the IL-7R gene locus. It is shown that PU.1 defective lymphoid progenitor cells failed to express IL-7Rα gene (DeKoter *et al*., 2002). PU.1 is not expressed after lymphoid progenitors are fully committed to the T cell lineage. Furthermore, a GGAA binding protein (GABP) is identified to bind the PU.1 binding motif in T cells (Xue *et al*., 2004). However, these findings were not sufficient to clarify the mechanism for the tight regulation of IL-7R expression at distinct T-lymphocyte stages.

1.4 Influence of Cis-regulatory Regions on Gene Transcription

Complexity of organisms is mostly referred to complexity and organization of gene expression rather than gene number. Temporal and spatial patterns of gene expression, variable alternative splicing and stability of mRNAs, post-translational modification and differential targeting of proteins are some considerations that reflect the complexity of organisms. Transcriptional initiation requires binding of specific proteins to promoter sequences as well as to a variety of regulatory elements frequently located far from the transcription initiation site. These regulatory regions often contain multiple copies of protein binding sites (Rutherford, 2000). Nardone and colleagues summarize these findings by stating that "The modular architecture of proteins and DNA is likely to be responsible for the complexity, versatility, flexibility and robustness of organisms and for their continued ability to evolve and adapt" (Nardone *et al.*, 2004).

Achievements in genome sequencing in recent years allowed comparative sequence analysis of vertebrate genomes (Nardone *et al.*, 2004). Results of bioinformatic studies and biological analyses showed that conserved noncoding sequences (CNS) are where transcriptional cis-regulatory elements reside in the genome. These cis-regulatory elements may be situated either upstream or downstream of the regulated gene. Although the size of cis regulatory sequences can be only a few hundred basepairs, these regulatory elements may be located at tens to hundreds of kilobases away from the transcriptional start site (Mortlock *et al.*, 2003).

Functional conservation of regulatory elements between species and reflection of this conservation into DNA sequences are the assumptions for utilizing comparative bioinformatic tools. Several comparative genomics programs are available online. AVID is a global alignment program (Bray *et al*,. 2003) available in the VISTA web site (www-gsd.lbl.gov/vista/) (Mayor *et al.*, 2000). Global alignment programs generate a single alignment across the entire length of two sequences. On the other hand, local alignment programs such as BLASTZ that is available in PipMaker web site (http://pipmaker.bx.psu.edu/pipmaker), find short high-quality alignments between two sequences. When using AVID-VISTA, a few parameters are required for the alignment.

Window length and minimum similarity to be visualized are set by the user. Setting a large window length may ignore some short CNSs. Conversely, smaller window lengths may cause higher background.

Genomic comparison of closely related species (e.g. mouse and rat) often gives a high degree of similarity. On the other hand, choosing very distant organisms (e.g. chicken and human) will result in only a few highly conserved sequences. The optimal way is comparing moderately related organisms such as mouse and human depending on the purpose. In addition, comparison of multiple species increases the power of the technique, further refining the boundaries of CNS regions (Thomas *et al.*, 2003).

As mentioned above, comparative softwares are valuable tools for identifying CNSs. The exact function of these conserved elements can be evaluated and verified by biological experiments. DNase I hypersensitivity assay, targeted disruption of putative regulatory sequences, chromatin immunoprecipitation, gel mobility shift assay and a variety reporter gene assays are some of the methods often used for identifying functional regulatory sequences (Nardone *et al.*, 2004).

1.5 Reporter Genes

In recent years chemi- and bioluminescence measurements have become extremely popular and been used to determine the amount of a specific unknown in a sample. Experimentally, the amount of light output is proportional to the amount of the luminescent material in the sample of interest. Chemiluminescence is the light emission upon a chemical reaction. Similarly, bioluminescence is a type of chemiluminescence in which an enzyme catalyzes the chemical reaction that emits light (Kricka, 1995). More recently, bioluminescence methods have become very important tools for gene expression and gene regulation studies (Wood, 2004).

1.5.1 Luciferases

Enzymes that catalyze a reaction yielding visible light are called luciferases as a generic term. The reaction involves the formation of an electronically excited intermediate and return of it to the ground state resulting in the emission of a photon light. Luciferases catalyze the oxygenation of substrates known as luciferins, creating high energy peroxidic intermediates. These intermediates spontaneously decompose and generate electronically excited products that emit a photon in the range of visible light upon decaying (Wilson and Hastings, 1998). In the case of firefly luciferase (an enzyme cloned from the *Photinus pyralis* species of fireflies), this return to the ground state results in the emission of a yellow-green light at the wavelength of 562–570 nm with maximum intensity (Shimomura *et al.*, 1977).

The structure of Luciferase enzymes are diverse among different organisms. These enzymes catalyze diverse reactions and use a wide range of substrates. However, involvement of oxygen is common for all luciferase reactions (Hastings, 1983). The emission of light occurs by different molecular mechanisms in different luciferase enzymes. Therefore, it is thought that rather than evolve from a common ancestoral enzyme, current day luciferases evolved independently in different species (Wilson and Hastings, 1998). For example, luciferase cDNA cloned from the dinoflagellate, *Gonyaulax polyedra*, does not show any sequence similarities to other luciferases in databases (Li *et al.*, 1997).

Among the bioluminescent organisms, insects are the most diverse group. Currently ~2500 species are described to have luminescence (Viviani, 2002). Collembola (springtails), Diptera (flies), and Coleoptera (beetles) have been shown to have luminescence (Herring, 1978). Except the firefly luciferases, which have been extensively studied, the mechanisms and properties of many insect luciferases are still poorly characterized. The firefly luciferase is the most studied and best characterized model among other insect luciferases (Viviani, 2002). Between 1950s and the 1980s, biochemical studies required collecting samples from natural firefly collectors. However, cloning of the luciferase cDNA from the North American firefly, *Photinus pyralis*, and expression of the gene in E.coli provided an alternative source of the enzyme (De Wet *et al.*, 1985). Later on, many laboratory organisms around the world

have been transfected and as a consequence of expression of the firefly luciferase, they began to emit the specific yellow-green luminescence (Baldwin, 1996).

1.5.1.1 Firefly luciferase reaction

Photinus pyralis luciferase, which has a molecular weight of 62 kDa, requires ATP, O_2 and luciferin as substrates (Figure1.5). In the first reaction enzyme-luciferyl adenylate complex is formed. In the second reaction, CO_2, AMP, oxyluciferin and light is produced as a result of an oxidative decarboxylation of lucyferyl adenylate complex (De Wet *et al.*, 1987). Figure 1.6 shows the chemical structure of beetle luciferin and the bioluminescent reaction catalyzed by firefly luciferase. In case of excess substrates, a flash of light is produced that is proportional to the quantity of enzyme in the reaction.

$$\text{Luciferase} + \text{Luciferin} + \text{ATP} \xleftrightarrow{Mg^{2+}} \text{Luciferase-Luciferly-AMP} + \text{Ppi}$$

$$\text{Luciferase- Luciferyl-AMP} + O_2 \rightarrow \text{Luciferase} + \text{Oxyluciferin} + CO_2 + \text{AMP} + h\nu$$

Figure 1.5: Reaction catalyzed by firefly luciferase.

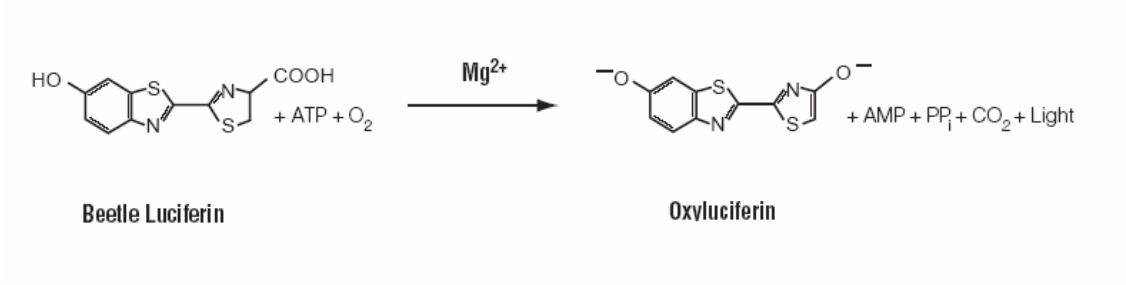


Figure 1.6: Chemical structure of beetle luciferin and bioluminescent reaction catalyzed by firefly luciferase.

1.5.2 Reporter gene systems

Genetic reporters are widely used in cell biology to study gene expression. It is also possible to study cellular events such as receptor activity, signal transduction and protein interactions which are coupled to gene expression (Ausubel *et al.*, 2002). Reporter vectors usually consist of a reporter gene encoding for a protein whose presence or activity due to expression can be detectible. A promoter activator binding site or an enhancer sequence under interest can be inserted into the multiple cloning site of a reporter vector. Analysis of the reporter gene expression indicates the transcription promoting ability of the sequence under interest. Commonly, reporter vectors are transiently transfected; however expression of the reporter protein should not have any effects on physiology of the host cell and show no toxicity. Moreover, in a good reporter system, the expression or activity of the reporter molecule should be sensitively detectible. Many systems provide measurement within several logs of activity (Alam and Cook, 1990).

Expression of most eukaryotic genes is controlled by complex regulational mechanisms. Cis-acting sequences and trans-acting elements have a major role in recruiting certain transcription activators which specifically recognize these sequences. Specific binding of transcription factors to promoter or enhancer elements which are generally found upstream of genes mediate the initiation of transcription. Direct methods for expression analysis involve the measurement of mRNA synthesized using northern blots or similar techniques. Such techniques are often labor intensive and time consuming compared to reporter systems (Ausubel *et al.*, 2002).

1.5.2.1 Reporter genes other than luciferase

In vitro reporter systems can either use isotopic or nonisotopic methods. Isotopic assays for reporter gene activity involves the chloramphenicol acetyltransferase (CAT) and human growth hormone (hGH) reporter systems. CAT is a prokaryotic enzyme which catalyzes the transfer of acetyl groups from acetyl-coenzyme A to chloramphenicol. Lysates of the cells that are transiently transfected with CAT vectors are incubated with [C^{14}]chloramphenicol. Acetylated and unacetylated chloramphenicol

molecules are separated by thin layer chromatography for qualitatively estimating the CAT activity on X-ray films (Glover, 1985). CAT assay has very low signal-to-noise ratios due to low competing activities of the prokaryotic CAT enzyme with most eukaryotic cells. However, CAT assays are time consuming and require radioactive substrates which are relatively expensive and have hazardous risk. In addition, CAT assays have lower sensitivity compared to nonisotopic methods that have been recently developed (Ausubel *et al.*, 2002). hGH is another isotopic genetic reporter which is normally secreted from the somatotrophic cells in the anterior pituitary gland. Common use of hGH as a reporter gene is for normalizing the transfection efficiency as an internal control (Selden *et al.*, 1986). However, hGH requires hazardous radioimmunoassay for quantitation and has relatively low sensitivity than the other secreted reporter protein SEAP.

Nonisotopic assays for reporter gene activity involves the β-galactosidase, β-glucuronidase (GUS), secreted alkaline phosphatase (SEAP) and firefly luciferase reporter assays. β-galactosidase is one of the most widely used *in vitro* and *in vivo* genetic reporters. The β-galactosidase enzyme which is encoded by *E.coli lacZ* gene catalyzes the hydrolysis of several β-galactosides. X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside is the most commonly used substrate for reporter assays (Fowler and Zabin, 1983). In addition to being a reporter gene alone, β-galactoside is commonly used as a normalizing factor when expressed in cells together with the luciferase gene (Martin *et al.*, 1996). Similar to β-galactosidase, principle of GUS assays is the hydrolysis of various β-glucuronides. GUS reporter gene is very commonly used to study gene expression in plants due to low endogenous GUS expression in most plants compared to mammalian cells (Bronstein *et al.*, 1994). Another reporter gene SEAP is a form of human placental alkaline phosphatase gene but lacks the membrane anchorage domain. Different from other reporter genes, SEAP is secreted to the medium. This property of SEAP allows performing the assays without disruption of the cells and gene expression kinetics can be studied. However, SEAP reporter assay has a narrow dynamic range and low sensitivity than other commonly used assays. To overcome these disadvantages, there are some modification and enhancement studies for this method such as two step SEAP assay which utilizes a substrate that is linked to

luciferin. In the second step, released luciferin serves as the substrate for firefly luciferase which has a greater sensitivity (Berger *et al.*, 1988).

1.5.2.2 Luciferase reporter gene system

Cloning of the luciferase gene from *Photinus pyralis* created a nonisotopic genetic reporter system (De Wet *et al.*, 1985). Firefly luciferase requires ATP, Mg^{2+}, O_2 and luciferin to catalyze a bioluminescent reaction. Luciferase protein has a half life of three hours in cultured cells due to its sensitivity to degradation by proteases. The short half life in the cytoplasm makes of the luciferase protein suitable for studying inducible systems. Reaction of luciferase with the substrates generates a flash of light which decays rapidly in one second. Therefore a luminometer equipped with an autoinjection device is required. The detected light is presumed to be an indirect estimate of luciferase gene transcription. Although luciferase produces a flash of light in the classical method addition of Coenzyme-A in the reaction mixture increases the duration of signal nearly constant up to one minute due to favorable interaction of luciferase with luciferyl- CoA that eliminates the need for a luminometer with an autoinjection device (Wood, 2004).

Measurement of luminescence is performed by photomultiplier tubes (PMTs) that are the photon detection devices commonly found in luminometers (Turner, 1985). Luminometers are either current-measuring or photon-counting according to their processing of the luminescent signal. A current-measuring luminometer measures the signal in a way that it produces an electrical current due to striking of the photons to the PMT. On the other hand, a photon-counting luminometer counts each photon that reach to the PMT. Photon-counting luminometers show a great sensitivity therefore very low levels of light can be detected. However these instruments have a limited dynamic range compared to current-measuring luminometers. While low light levels can be measured sensitively, at high levels of luminescence, the instrument is saturated which makes the linear range of detection narrower. On the other hand, there are difficulties with current producing luminometers in that low levels of light is hard to be detected sensitively and consistency is lost. Indeed, lower limit of detection of current producing luminometers may be undesirably high compared to photon-counting luminometers (Wampler, 1985).

Firefly luciferase as a reporter protein can be measured with a very broad linearity and with very high sensitivity, down to just femtogram levels in a sample. Using a single reporter in an assay is a quick way of acquiring data for gene expression in cells. However, cells have complex physiology and therefore data acquired from a single reporter assay may not be reliable all the time. So, dual assays are developed which improved the reliability of experimental results (Wood, 2004). Significant variability lowers the reproducibility between samples in an experiment due to the complex nature of cells. In addition, variability may come from instrumental reasons due to static electricity and humidity across the plate (Faridi *et al.*, 2003). To overcome these undesired variables, dual assays are used which leads to more accurate comparisons between samples. Moreover, cytotoxicity of the reporter protein may cause down regulation of expression. Using a dual assay allows the normalization of the measurement of reporter gene activity independent of cell physiology and call viability (Hirose *et al.*, 2002).

2 PURPOSE OF THE STUDY

As mentioned in the Introduction, lymphoid development proceeds through a series of stages in which the bone marrow precursors differentiate into functional lymphocytes. IL-7 is a critical cytokine for both developing and mature lymphocytes. It provides survival and proliferation signals whose loss can not be compensated by other cytokines and misregulated survival signals can lead to the uncontrolled growth of lymphocytes. At some stages of development, lymphocytes are responsive to IL-7 signals and at some stages, they are not. Responsiveness of cells to IL-7 is strictly controlled by the regulated expression of IL-7 receptor. Studies for explaining the tight control on IL-7R expression revealed that a number of transcription factors have the ability to bind IL-7R promoter region. However, these findings are still insufficient to clarify the temporal and cell specific surface expression of IL-7 receptor.

In this study, we aimed to evaluate the possible role of upstream conserved noncoding sequences in the expression of IL-7R gene. For this reason, we determined several conserved sequences by the comparative analysis between mouse and human genomic sequences. To investigate the transcriptional activation capabilities of these sequences, we cloned them into luciferase reporter gene plasmids for further transfections and reporter assays. An IL-7R+ mouse $CD4^+$ T-lymphoma cell line was selected to examine the transcriptional activities of cloned sequences.

Details of identification and cloning of upstream conserved sequences are presented in this thesis together with the luciferase reporter assays performed with a T-lymphoid and a non-lymphoid cell line. Next steps involve the examination of cloned sequences with different cell lines which represent the distinct developmental stages of lymphocyte development by using fluorescent protein reporter systems. Identifying an

enhancer region acting on IL-7R gene expression will contribute to the understanding of IL-7 signalling in lymphocyte development.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Chemicals used in the study are listed in Appendix A.

3.1.2 Molecular Biology Kits

Molecular biology kits used in the study are listed in Appendix B.

3.1.3 Equipment

Equipment used in the study is listed in Appendix C.

3.1.4 Enzymes

Enzymes and their reaction buffers that are used in restriction digestion, amplification, ligation and other processes are listed in Appendix D.

3.1.5 Plasmids, cells, oligonucleotides and molecular weight markers

Plasmids, cells, oligonucleotides and DNA markers that are used in the study are listed in Appendix E. Maps of plasmids that are used and constructed are given in Appendix F.

3.1.6 Softwares and online programs

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

3.1.7 Growth media

3.1.7.1 Bacterial growth media

3.1.7.1.1 Solid media

40 g of Luria Agar was dissolved in 1 L of distilled water and autoclaved at 121°C for 15 minutes. If necessary, ampicillin or kanamycin at a final concentration of 100 µg/ml; or chloroamphenicol at a final concentration of 12.5 µg/ml were added into the medium for selection. Then, approximately 15 ml of the medium was poured into each sterile Petri plate.

3.1.7.1.2 Liquid media

20 g of Luria Broth was dissolved in 1 L of distilled water and autoclaved at 121°C for 15 minutes. If necessary, appropriate antibiotics were added into the liquid medium for selection.

3.1.7.2 Mammalian growth media

3.1.7.2.1 Growth media for adherent cells

293T human kidney fibroblast cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) culture medium which was supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10% fetal bovine serum, 100 unit/ml penicillin and 100 unit/ml streptomycin.

3.1.7.2.2 Growth media for suspension cells

EL4 mouse T-lymphoma cells were grown in RPMI 1640 cell culture medium which was supplemented with 5 % fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin and 100 unit/ml streptomycin.

3.1.7.2.3 Freezing medium

DMSO was added into fetal bovine serum at a concentration of 10% v/v and stored at 4°C.Freezing medium

3.1.8 Buffers and solutions

Standard buffers and solutions used in the study were prepared according to the protocols in Sambrook *et al*., 2001.

- 5X Tris-Borate-EDTA (TBE) Buffer: 54 g Tris base, 27.5 g boric acid and 20 ml 0.5 M EDTA at pH 8.0 were dissolved in 1 L of distilled H_2O.

- Agarose gel: For 1% w/v agarose gel preparation, 1 g of agarose was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide is included in the solution.

- Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g of KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 were dissolved in 800 ml distilled H_2O. pH was adjusted to 7.4 by drop wise addition of concentrated HCl and the buffer was completed to 1 L with distilled H_2O.

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

- Trypan blue dye (0.4% w/v): 40 µg of trypan blue was dissolved in 10 ml PBS.

- 0.1% or 1% Triton X-100 Lysis Buffer: 60mM Na_2HPO_4, 40 mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$ and 0.1% or 1% Triton X-100 at pH 7.

3.2 Methods

3.2.1 Culture growth

3.2.1.1 Culture of bacterial cells

3.2.1.1.1 Liquid culture

The *E.coli* cells were grown overnight (16-18 hours) at 37°C shaking at 270 rpm in Luria Broth. Selective antibiotics were added to the media depending on the application.

3.2.1.1.2 Solid culture

The *E.coli* cells were grown overnight in Luria Agar overnight (16-18 hours) at 37°C. Cells were either spreaded or streaked on to solid agar plates. Antibiotics were added where necessary.

3.2.1.2 Culture of Mammalian cells

3.2.1.2.1 Culture of adherent cells

293T cells were grown in 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 unit/ml Streptomycin and 100 unit/ml Penicillin. Cell cultures were washed with PBS, trypsinized and splitted in every 2-3 days by 1/10dilutions.

3.2.1.2.2 Culture of suspension cells

EL4 cells were grown in 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 unit/ml Streptomycin and 100 unit/ml Penicillin. Cell cultures were splitted in every 2-3 days by 1/10dilutions.

3.2.1.3 Frozen stock preparation

E.coli cells were grown overnight at 37°C and stored in Luria Broth containing 15% glycerol. Cells were frozen in liquid nitrogen and stored at -80°C.

EL-4 and 293T cells at mid to late growth phase were resuspended in freezing medium (10% DMSO in fetal bovine serum) and stored at -80°C for 48 hours. Then, the cells were stored in liquid nitrogen tank. After thawing, cells were immediately washed with medium to get rid of DMSO.

3.2.2 Construction of vectors

3.2.2.1 Transformation of bacteria

Competent cells were prepared with *E.coli* DH5α strain as described in Ausubel *et al.*, 2002. Δ56fosCAT, pGL3 Basic, pGL3 Enhancer, pGL3 Control and pMaxGFP plasmids were transformed to competent *E.coli* cells according to Sambrook *et al.*, 2001. Transformed cells were spreaded on Luria Agar plates containing appropriate antibiotics. Luria Agar was supplemented with ampicillin as the selective marker for cells transformed with Δ56fosCAT and pGL3 plasmids. Kanamycin was used in the case of pMaxGFP.

3.2.2.2 Isolation of plasmid DNA

Plasmid isolations were done either by following the alkaline lysis protocol in Sambrook *et al.*, 2001 or with the Qiaprep Spin Miniprep and Qiagen Midi kits. *E.coli* cells were picked up by toothpick and the cells were grown overnight at 37°C overnight prior to plasmid isolation. Chloramphenicol was added to medium when growing DH10 cells carrying BAC clones. Other antibiotics were supplemented to the medium as mentioned in the previous section. BAC vector (RP23-365P6), Δ56fosCAT, pGL3 Basic, pGL3 Enhancer, pGL3 Control and pMaxGFP plasmids were isolated. Concentration of each plasmid was determined by spectrophotometry at 260 nm.

3.2.2.3 Construction of pGL3fos

3.2.2.3.1 Restriction enzyme digestion

Amount of restriction enzymes did not exceed 10% of the reaction mixture. Appropriate buffers were used for each enzyme according to the supplier information for all digestions. All digestion reactions were carried out at 37°C for 1-2 hours.

Isolated pGL3 Basic plasmid was linearized by Hind III digestion. Δ56fosCAT plasmid was double digested with Xba I and Sal I to extract the fos promoter. Double digestion conditions were followed from the supplier web site (www.promega.com).

Linearized pGL3 Basic vector was dephosphorylated at the 5' protruding end by bovine intestinal alkaline phosphatase (CIAP). CIAP was added directly to the restriction enzyme reaction mixture after the completion of reaction. Unit of CIAP for a reaction was determined according to the supplier instructions and moles of DNA to be dephosphorylated. Dephosphorylation reactions were performed at 37°C for 30 min.

3.2.2.3.2 Agarose gel electrophoresis

Agarose gels were prepared by polymerization of agarose in 0.5X Tris-Borate-EDTA (TBE) buffer by heating 1-3 minutes in a microwave oven and cooling 20-30 minutes at room temperature. 0.5X TBE buffer was also used as the electrophoresis buffer. 6X loading dye was mixed with the DNA samples at a 1:5 ratio. DNA samples were run on 1% agarose gels at 100-110 Volts for 40-60 minutes

Linearized pGL3 Basic and double digested Δ56fosCAT samples were applied to 1% agarose gel. Linearized pGL3 Basic and fos promoter fragment were extracted from agarose gel by Qiaquick Gel Extraction Kit according to the supplier's instructions.

3.2.2.3.3 Blunt ending of linearized pGL3 Basic vector

5' overhangs of pGL3 Basic vector and fos promoter were made blunt by DNA Polymerase I Large (Klenow) fragment according to supplier's manual (Promega). Blunt ending reactions were done at 24°C for 30 min. Blunt ended DNA fragments were purified by Qiaquick PCR purification kit according to the instruction manual.

3.2.2.3.4 Blunt end ligation

Blunt ended fragments were ligated by T4 DNA ligase at 3:1 insert to vector molar ratio. Different from the supplier information (Promega), we added 50% PEG 4000 to the 20 ml of ligation mixture. Ligation was carried out at 16°C for 18 hrs. Vector only ligations were also performed to evaluate the self circularization. 10µl of the ligation mixture was transformed into *E.coli* and plasmid isolations were performed as described in section 3.2.2.2.

3.2.2.4 Construction of pGL3fos Enhancer

Constructed pGL3fos and pGL3 Enhancer plasmids were double digested with BamH I and Nco I restriction enzymes and linearized pGL3fos vector was dephosphorylated with CIAP as described in section 3.2.2.3.1. Digestion products were run on agarose gel and purified from gel as described in section 3.2.2.3.2. Linearized pGL3fos and extracted SV40 enhancer + luciferase cDNA fragment that have sticky ends were ligated by T4 DNA polymerase at 3:1 insert to vector molar ratio. Ligation mixtures were transformed into *E.coli* and plasmid isolation was performed from colonies as described in Section 3.2.2.2.

3.2.2.5 Construction of pGL3fos-Kpl vectors

3.2.2.5.1 Identification of conserved noncoding regions

Mouse, human and rat genomic sequences representing the IL-7R gene upstream region were downloaded from UCSC genome browser (http://www.genome.ucsc.edu). Exon files were generated by the Spidey program available at the NCBI web site (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). The sequences were aligned by AVID and similarity plot was generated by VISTA program both are available at the VISTA server (http://www-gsd.lbl.gov/vista/). 50 bp window length and 85% minimum similarity parameters were set to VISTA. (see details in section 4.1)

3.2.2.5.2 Amplification of mouse conserved noncoding sequences

Conserved noncoding sequences were amplified from RP23-365P6 BAC vector by PCR. Reaction volumes and final concentrations of PCR components were determined according to supplier's recommendations (Promega). Primers were designed to amplify the mouse genomic regions that were identified as conserved noncoding sequences (CNS) using the Primer3 program (http://frodo.wi.mit.edu/primer3). A Sal I site was added to the 5' end of each primer (Appendix E).

PCR thermal cycle conditions:

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

3.2.2.5.3 Insertion of PCR products into pGL3fos vector

PCR products (Kpl7-Kpl13) and pGL3fos vector were digested with Sal I in order to get compatible sticky ends. PCR products were purified by Qiaquick PCR purification kit. Linearized pGL3fos vector was dephosphorylated by CIAP as in section 3.2.2.3.1 and extracted from 1% agarose gel. Then, PCR products were ligated into the linearized pGL3fos vector. Ligations with only pGL3fos were performed to evaluate the self circularization of the vector. Ligation products were transformed into *E.coli*. Confirmation of the inserts into the vector was done by either restriction digestion followed by plasmid isolation or colony PCR.

3.2.3 Confirmation of vector constructs

3.2.3.1 Colony PCR

Colony screening of pGL3fos-Kpl constructs were initially confirmed by colony PCR method aiming to amplify the fragment inserted. Colonies were picked up from Luria Agar-ampicillin plates and swirled in 8.5 µl sterile dH_2O in 20 µl PCR tubes and

streaked on replica plates. Cells were lysed for 8 minutes at 95°C. Same conditions with the first amplification of CNS regions from the BAC clone were used. Colony PCR results were analyzed on 1% agarose gel.

3.2.3.2 Confirmation by restriction enzyme digestions

According to the reaction conditions recommended by the supplier (Promega), diagnostic restriction enzyme digests performed for constructed vectors. The results of these digestions were analyzed on 1% agarose gel. We performed Hind III-Nco I double digestions in order to cut the fos promoter back from the pGL3fos vector. We also digested the pGL3fos vector by Xba I with the expectation that two large fragments would appear on agarose gel. Because a site for this enzyme was in the vector backbone and another site was generated after blunt end ligation.

We performed Pvu II-Sph I double digestions for the constructed pGL3fos Enhancer vector. Pvu II has a site in fos promoter and Sph I has a site in luciferase cDNA and two proximal sites in SV40 enhancer sequence. On agarose gel, we expected to see three relatively large fragments and a short band in the length of 72 bp.

In addition, a second confirmation for the pGL3fos-Kpl vectors was performed. Colonies which were determined to be positive for carrying the insert were analyzed by diagnostic digestions. Plasmid DNAs were isolated from positive colonies and digested with enzymes which have at least one site in the insert sequence and along the pGL3fos vector backbone. Same vectors were also digested with Sal I in order to excise the cloned fragment. All these restriction sites are indicated in the vector maps in appendix-F. The results of these diagnostic digestions were analyzed on 1% agarose gel.

3.2.4 Mammalian cell transfections

3.2.4.1 Electroporation

pGL3fos Enhancer, pGL3 Control and pmaxGFP plasmids were used in transfection of EL-4 cells by electroporation. 1-2x10^7 EL-4 cells at late growth phase were washed once with serum free medium. The cells were then resuspended in 500µl serum free medium and incubated 15 minutes after addition of 20 µg plasmid DNA. The cells transferred to electroporation cuvettes. Different electroporation conditions in the ranges of 200 to 400 V, 800 to 1800 µF and 25 to 400Ω were applied to EL-4 cells. Pulsed cells were incubated for an additional 15 minutes and transferred into 60 mm culture dishes. Cells were either harvested for luciferase activity or visualized for GFP expression.

3.2.4.2 Calcium phosphate mediated transfections

293T cells were transfected with pGL3fos Enhancer, pGL3 Control and pmaxGFP plasmids by calcium phosphate mediated transfection according to the conditions in Sambrook *et al.*, 2001. 293 T cells at 70% confluency were trypsinized and seeded in 60 mm dishes 24 hours prior to transfection. And the medium was refreshed 60 minutes before the transfection. 100 µl 2.5 M $CaCl_2$, 25 µl plasmid DNA (~25 µg) was completed to 1 ml with 0.1X TE buffer (pH 7.6) and mixed with 1 ml 2X HEPES-buffered saline. The solution was incubated for 1 minute at room temperature and 100 µl of the solution was added to 1ml of the culture medium. The cells were incubated for 48 hours prior to analysis.

The effect of the amount of plasmid DNA on transfection efficiency was investigated. Five different amounts of plasmid DNA were transfected. Each 1 µg, 3 µg, 6.5 µg and 10 µg of pGL3 Control plasmid DNAs were used for calcium phosphate mediated transfection of 293T cells according to the conditions stated above. In addition, the effect of number of cells for each transfection was also analyzed. 0.25x10^6,

$0.5x10^6$, $1x10^6$, $3x10^6$ and $5x10^6$ 293T cells were seeded 24 hours prior to transfection with 6.5 μg pGL3fos Enhancer plasmid.

3.2.5 Luciferase reporter gene assays

Luciferase reporter assays were performed according to manufacturer's instructions with some modifications (Promega). Suspension cells were pelleted at 1000 rpm, washed with PBS and resuspended in 400 μl lysis buffer. Adherent cells were lysed directly in 60mm dishes. Either reporter lysis buffer (RLB) (Promega), 0.1% or 1% Triton X-100 buffers were used for lysing the cells. Cell lysis was performed by incubating the lysate 10-15 minutes with pipetting up and down. A single freeze-thaw cycle was done in some of the assays. Lysed cells were centrifuged at 12000 rpm and the supernatant was assayed for luciferase activity. Luciferase assay reagent was equilibrated to room temperature for 30 minutes. To measure the luciferase activity, 50 μl cell lysate was mixed with 100μl luciferase assay reagent in 96 well black plates and luminescence was measured immediately in the luminometer.

3.2.6 Protein content determination by Bradford assays

Protein standards were prepared with Bovine Serum Albumin (BSA) by diluting 10 mg/ml BSA stock. 0.1-1 mg/ml BSA standards were prepared. 5 μl of each standard was mixed with 250 μl Bradford reagent (Sigma Aldrich) in 96-well plates. A microplate spectrophotometer was used to measure the optical density at 595 nm. To determine the protein concentration of cell lysates, 1-5 μl cell lysate was used and measurements were done by the same way with standards.

4 RESULTS

As mentioned in Section 1.5.2, we aimed to use a reporter gene assay system to detect the transcriptional activation properties of various conserved noncoding sequences in the IL-7 gene locus. First we identified these conserved sequences using the computer programs AVID-VISTA (www-gsd.lbl.gov/vista). Next we obtained seven putative enhancer sequences by PCR from a specific BAC clone and cloned them into a modified firefly luciferase reporter plasmid. We modified the reporter vector by inserting a weak promoter sequence into it. Then we also constructed a control reporter vector containing the weak promoter and a strong enhancer sequence to assess the transcriptional ability of the promoter.

We investigated the achievability of luciferase assay system for a human kidney fibroblast cell line due to its high transfection efficiency and high expression capability. We transfected these cells with luciferase control vectors as well as with a green fluorescent protein (GFP) carrying plasmid for visual detection of transfections. Since we needed an IL-7R expressing cell line in order to examine the transcriptional activity of the cloned sequences, luciferase control and GFP vectors were also introduced into a $CD4^+$ single positive T-lymphoma cell line. For both cell lines, we evaluated the transcription efficiency at various conditions and analyzed the luciferase activity of strong regulatory sequences.

4.1 Comparative Analysis of Genomic Sequences

4.1.1 Determination of genomic regions

The IL-7R gene is located on mouse chromosome-15, human chromosome-5 and rat chromosome-2. IL-7R gene and upstream neighbouring genes or gene predictions were displayed in NCBI Entrez Gene web site (www.ncbi.nlm.nih.gov/entrez).

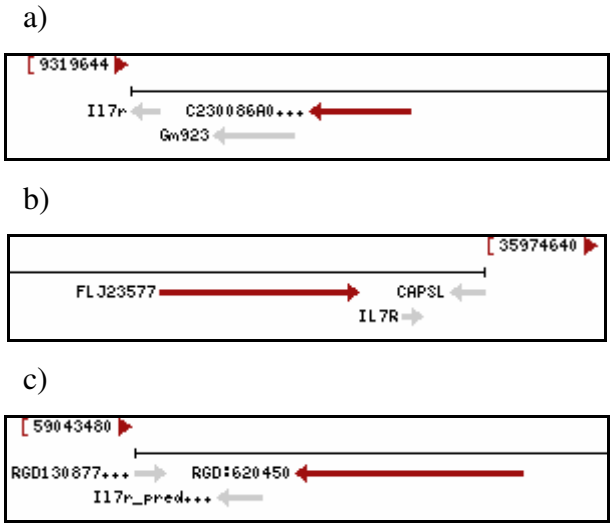


Figure 4.1: Genomic locations of IL-7R and upstream neighbouring genes or gene predictions. C230086A09 and Gm923 on mouse chromosome-15 (a), FLJ23577 on human chromosome-5 (b) and RGD:620450 (Kpl2) on rat chromosome-2 (c) are all homologous to Klp2 gene.

According to NCBI Entrez Gene (Fig 4.1), IL-7R upstream genes or gene predictions all show homology to Kpl2 gene which is first cloned and annotated in rat (Ostrowski *et. al.* 1999). Thus, IL-7R gene locus shows conservation between mouse, human and rat by means of location, orientation and homology of upstream genes.

4.1.2 Retrieval of genomic sequences

Mouse, human and rat IL-7R gene loci were displayed on UCSC Genome Browser (http://www.genome.ucsc.edu). For mouse and human, May-2004 genome assemblies were used. For the rat, the available genome assembly was June-2003.

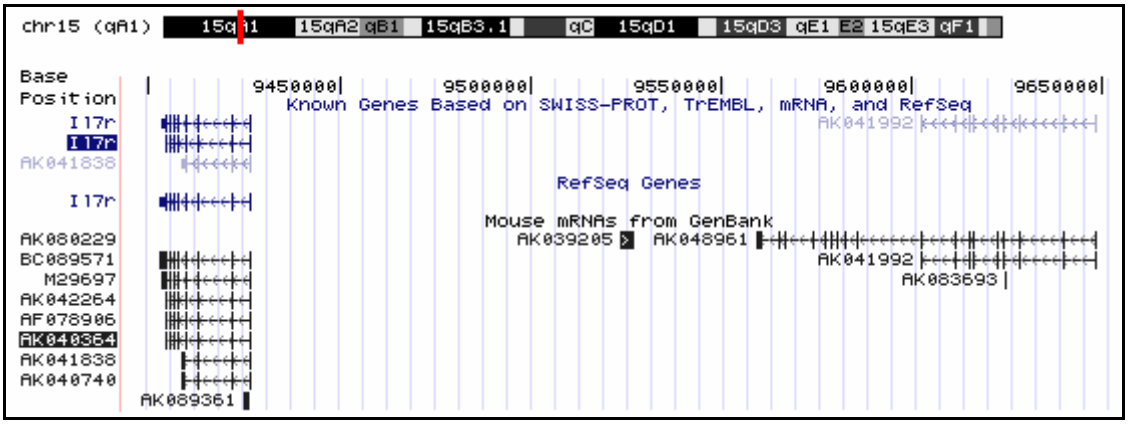


Figure 4.2: Mouse IL-7R gene and upstream region including Kpl2 homologous gene AK041992 and its variant mRNA AK048961.

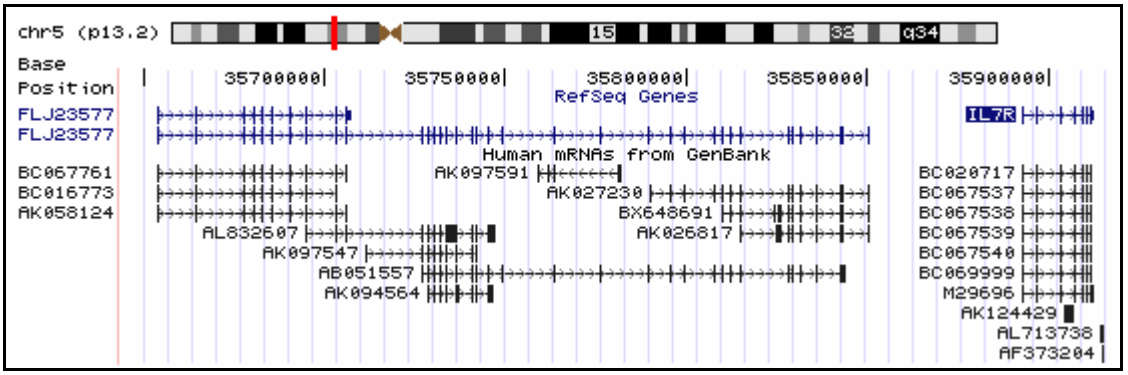


Figure 4.3: Human IL-7R gene and upstream region including Kpl2 homologous gene FLJ23577.

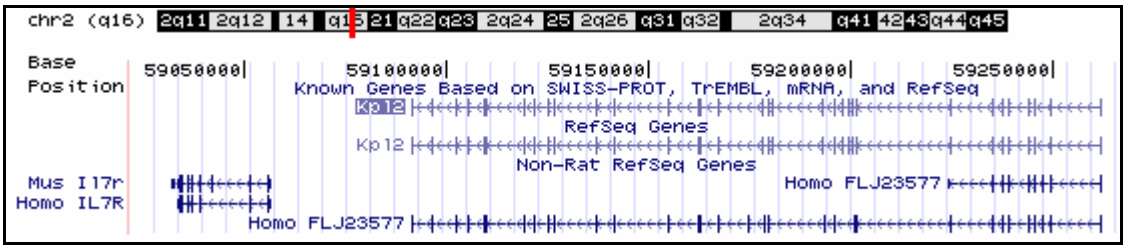


Figure 4.4: Rat genomic region corresponding to mouse and human IL-7R genes and upstream region including Kpl2 gene.

According to Figure 4.4, Kpl2 gene is located in the upstream region of IL-7R on rat choromosome-2. FLJ23577 gene (Fig 4.3) is the homolog of Kpl2 in human chromosome-5. In mouse chromosome-15, AK048961 cDNA (Fig 4.2) and gm923 prediction (Fig 4.1.a) are Kpl2 homologs. Gm923 is a gene model predicted computationally with no experimental data; which is located between Kpl2 homolog AK048961 and il7r gene. AK048961 and gm923 occupy together a region that corresponds to the location of Kpl2 in rat genome.

Boundaries of genomic regions to be used in comparative analysis were determined. Genomic regions spanning from 5'end of IL-7R gene to 10Kb upstream of Kpl2 homolog genes were obtained from UCSC genome browser as in Table4.1. When taking sequences, direction of IL-7R gene was accepted as the positive direction. Therefore, reverse complement of the genomic sequences was taken if IL-7R gene is in opposite direction to the chromosome it is located.

Organism	Chromosome number	Starting point	Ending point	Direction taken
Mouse	15	9,402,535	9,658,035	Reverse
Human	5	35,643,746	35,912,678	Forward
Rat	2	59,032,272	59,271,440	Reverse

Table 4.1: Boundaries and directions of genomic sequences taken from UCSC genome browser.

4.1.3 Alignment of genomic sequences

Genomic sequences were aligned by AVID (www-gsd.lbl.gov/vista) which is a pairwise global alignment program (Bray *et al.*, 2003). Following the alignment, conserved genomic sequences were visualized by VISTA that is a software used for visualizing global DNA sequence alignments of arbitrary length (Mayor *et al.*, 2000).

Genomic region at mouse chromosome-15 that is spanning from 9,402,535 bp to 9,658,035 bp was selected as the base sequence for global alignment. Since the chromosomal location of sequences was not an input of AVID-VISTA, genomic region

between 9,402,535 bp - 9,658,035 bp corresponded to 1 bp - 255500 bp in the output of the alignment. Therefore, in the rest of the study, locations of sequences were given relative to 1 bp-255500bp aligned mouse sequence.

Usually, exons of coding or predicted genes show high level of similarity compared to noncoding sequences. VISTA accepts an exon file which determines the location of coding regions. Thus, the program does not consider exons as conserved noncoding regions (CNS). An exon file was prepared by the use of NCBI Spidey program. The previously selected mouse genomic region (1 bp - 255500 bp) was given to Spidey (www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/) as an input. Additionally, sequences of mouse IL-7R and AK048961 mRNAs and gm923 predicted mRNA were entered to Spidey for determination of exon locations. Table 4.2 shows the exon places of these mRNAs on the selected mouse genomic region.

Since an exon file was created, VISTA did not report the exons as CNS regions. However, vertebrate noncoding genomic sequences usually contain lots of repeat regions that should be eliminated. VISTA optionally detects repeat sequences such as LINEs and SINEs and does not utilize these repeats as CNS regions.

Kpl2 Exons (bp)	Gm923 Exons (bp)	IL-R Exons (bp)
9909 - 10066	112306-112374	231536-231733
18164-18214	112818-112945	236202-236340
19895-19903	113026-113164	245077-245234
20145-20159	125691-125828	248527-248684
20381-20385	129739-129890	250290-250458
20904-20909	134225-134405	251310-251403
29505-29759	147963-148116	252787-252862
34121-34261	149595-149861	253211-255410
35718-35782	152667-152805	
41838-42024	154913-155142	
42959-43147	164906-165012	
46539-46726	165620-165802	
55286-55454	169511-169603	
72240-72459	172418-172557	
75256-75407	172660-172828	
77562-77619	178058-178297	
79096-79148	178446-178568	
80817-80920	184506-184880	
83687-83943		
91399-91510		
93009-93166		
97142-98822		

Table 4.2: Exon locations of mouse mRNAs relative to mouse genomic sequence selected for comparative analysis.

Mouse genomic region at chromosome-15 and corresponding human genomic sequence were aligned by AVID and visualized by VISTA (Fig 4.5). Window length was set as 50 bp. Another parameter was the minimum percent similarity that was determined as 85%. Therefore, non-exon sequences longer than 50 bp and having at least 85% similarity between the two genomes were reported as CNSs.

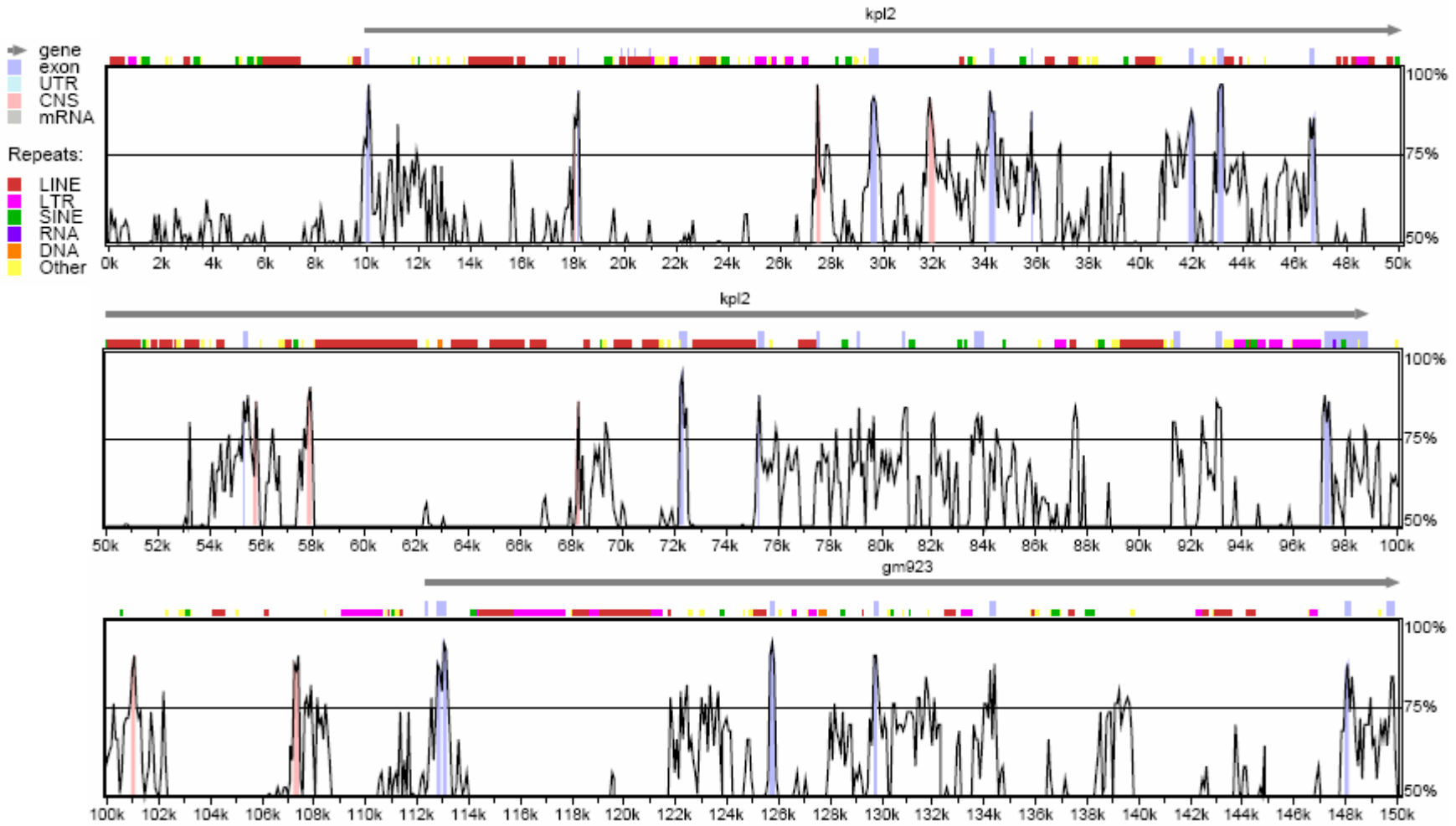


Figure 4.5: VISTA plot of mouse-human genomic sequences alignment with a window length of 50 bp. Regions showing 85% or higher similarity are displayed in pink and considered CNS.

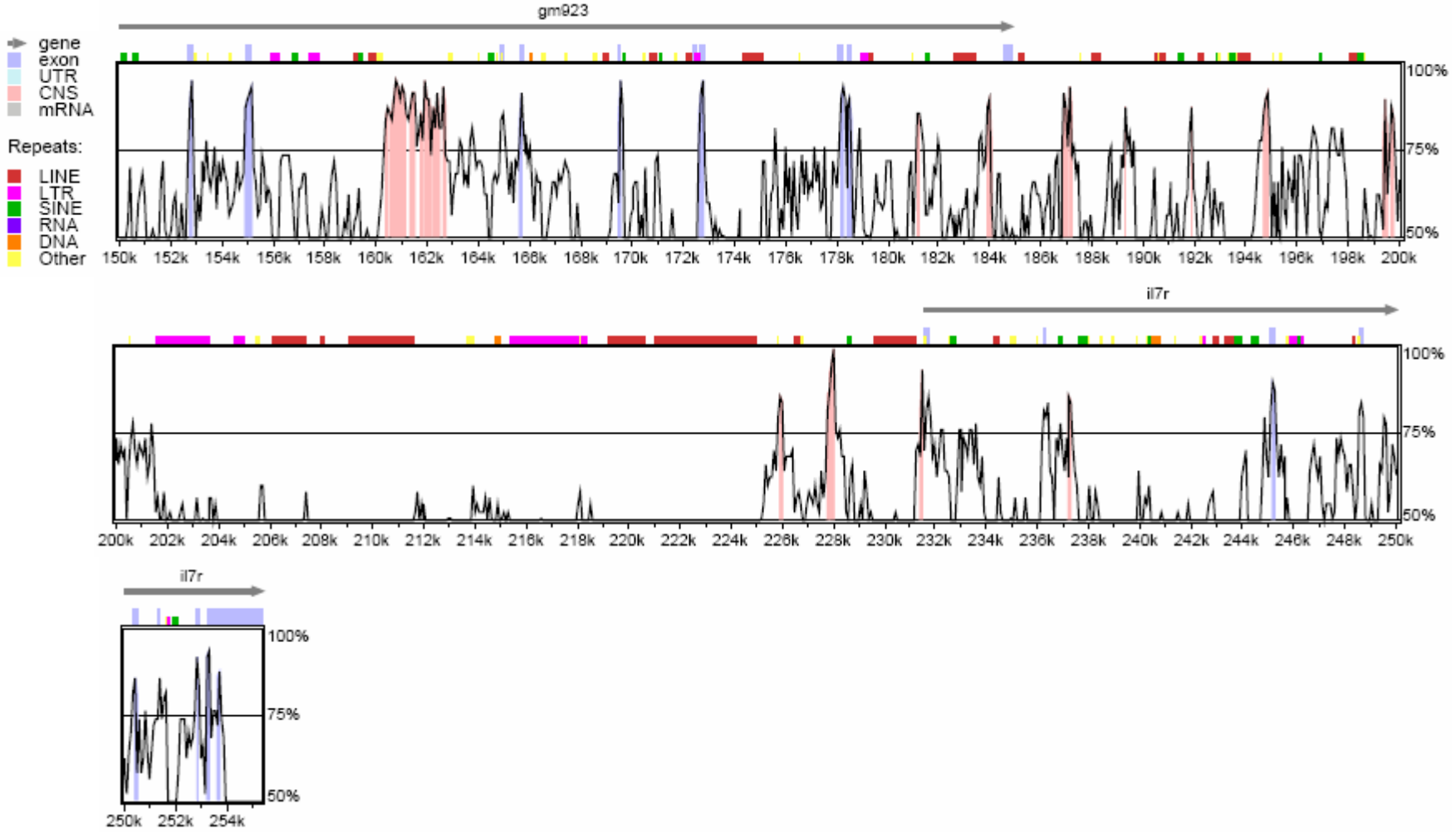


Figure 4.5 (continued): VISTA plot of mouse-human genomic sequences alignment with a window length of 50 bp. Regions showing 85% or higher similarity are displayed in pink and considered CNS.

In addition to the similarity plot, exact locations of conserved regions which have higher similarity than 85% were reported. Table 4.3 shows the borders of conserved regions spanning 40 kb upstream from the first exon of IL-7R gene. Additionally, the last noncoding sequence was found in the second intron of IL-7R. Noncoding sequences were named as CNS I-X. Some of the conserved sequences correspond to IL-7R gene exons. Thus, these exon regions were not considered for further study.

	Interval	Length (bp)	Percent similarity	Status
CNS I	191846- 191900	55	89.1%	noncoding
CNS II	194658- 194858	201	86.6%	noncoding
CNS III	199372- 199431	60	86.7%	noncoding
CNS IV	199440- 199488	49	87.8%	noncoding
CNS V	199713- 199774	62	83.9%	noncoding
CNS VI	225927- 225995	70	82.9%	noncoding
CNS VII	227818- 227877	60	86.7%	noncoding
CNS VIII	227882- 228047	167	91.6%	noncoding
CNS IX	231436- 231505	70	91.4%	noncoding
CNS X	237230- 237279	50	86.0%	noncoding
Exon 3	245148- 245217	70	87.1%	coding
Exon 5	250395- 250444	50	86.0%	coding
Exon 7	252787- 252857	71	90.1%	coding
Exon 8	253213- 253276	64	85.9%	coding
Exon 8	253664- 253715	52	88.5%	coding

Table 4.3: Intervals of conserved sequences relative to mouse genomic region of interest.

Mouse-Rat and rat-human alignments were done for the same genomic region (data not shown). It is verified that, all the noncoding conserved sequences form CNS I to CNS X show also 85% or higher conservation in both mouse-rat and rat-human alignments.

4.1.4 Determination of BAC clone

For laboratory analysis of the identified conserved noncoding sequences, the specific mouse bacterial artificial chromosome (BAC) clone was required. Therefore, BAC clone database on UCSC genome browser was searched. RP23-365P6 BAC clone

was found to fully cover all the identified CNS sequences (Figure 4.6). For PCR amplification of CNS regions, RP23-365P6 BAC clone was used as template.

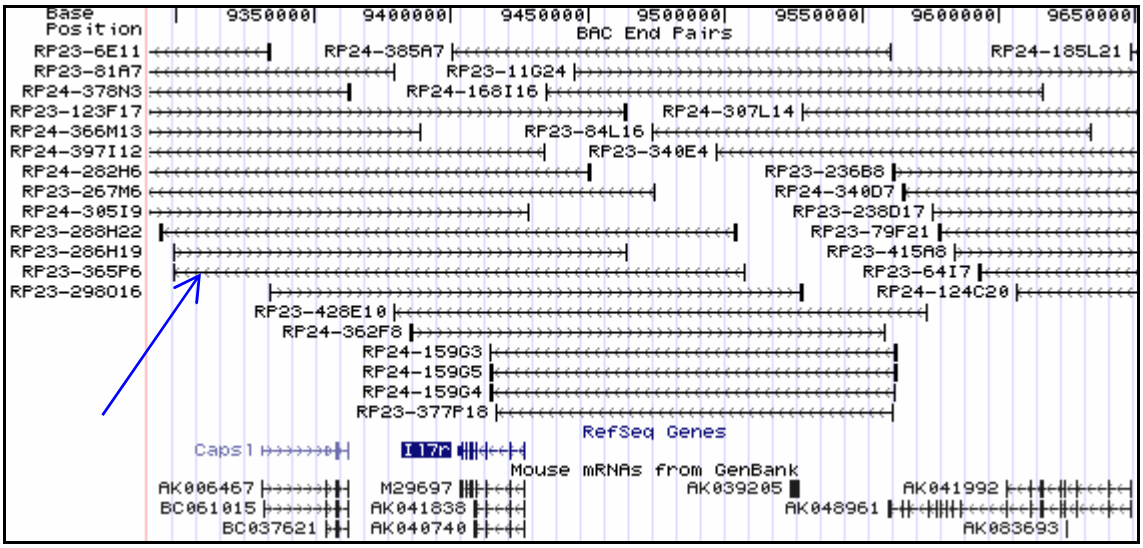


Figure 4.6: Mouse RP23-365P6 BAC clone (indicated with arrow) covers IL-7R gene and its 40 Kb upstream region.

4.2 Cloning of Conserved Noncoding Sequences

4.2.1 PCR amplification of CNS regions

Primers for amplification of CNS sequences from BAC clone were determined using the Primer3 program. Some primers were designed to amplify more than one CNS. Primers were selected to amplify additional ~200-300 bp sequences at both 5' and 3' ends of CNS regions. Sal I site was included into 5' end of primer sequences for ligation of PCR products into Sal I site of PGL3fos plasmid. Table 4.4 shows the boundaries of amplified fragments relative to aligned mouse genomic sequence. Since the identified conserved noncoding sequences are at the upstream region of IL-7R gene and Kpl2 gene is also found upstream of IL-7R, amplified fragments were named as Kpl1, Kpl2, ….., Kpl7.

Primers	PCR fragment	Interval	Length	Covered CNSs
Kpl7F & Kpl7R	Kpl7	191602-200032	430	CNS I
Kpl8F & Kpl8R	Kpl8	194402-194969	567	CNS II
Kpl9F & Kpl9R	Kpl9	199149-199883	834	CNS III, IV, V
Kpl10F & Kpl10R	Kpl10	225061-226299	1238	CNS VI
Kpl11F & Kpl11R	Kpl11	227544-228299	755	CNS VII, VIII
Kpl12F & Kpl12R	Kpl12	231102-231494	492	CNS IX
Kpl13F & Kpl13R	Kpl13	237036-237577	541	CNS X

Table 4.4: Boundaries of amplified fragments relative to aligned mouse genomic region. First column indicates the primers used for PCR and the last column shows the amplified conserved noncoding sequences.

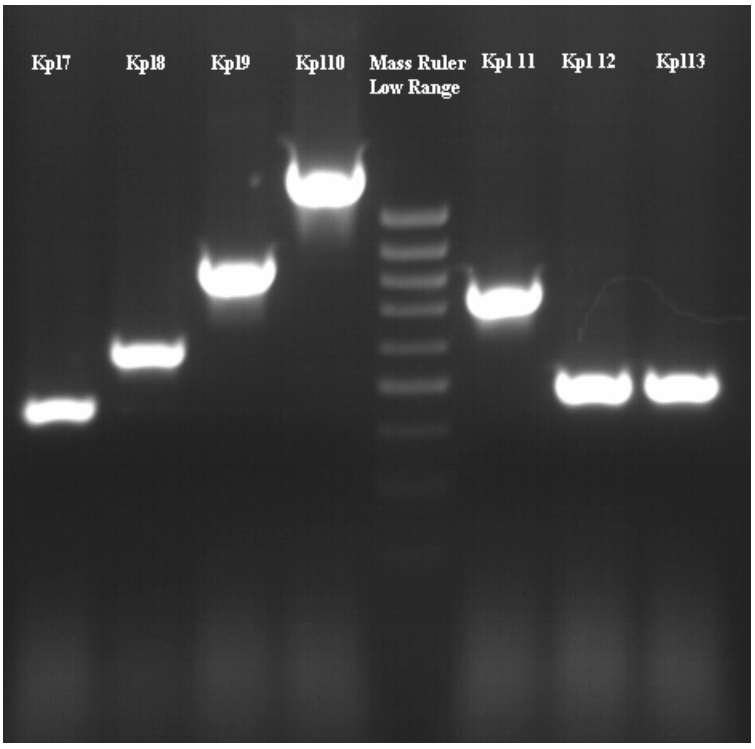


Figure 4.7: Analysis of PCR products containing CNS regions.

Genomic sequences containing CNS regions were amplified by PCR. RP23-365P6 BAC clone was used as template DNA. Figure 4.7 shows the amplified fragments on 1% agarose gel. It should be noted that the observed fragment lengths relative to the molecular weight marker, correlate with the expected fragment sizes indicated in Table 4.4.

In order to analyze the possible enhancer activity of the amplified CNS regions experimentally, a luciferase reporter vector with a weak promoter was required. Therefore, promoter of fos gene was cut out from the available source vector Δ56fosCAT. Following section describes the blunt end ligation of fos promoter into pGL3 Basic luciferase reporter vector.

4.2.2 Construction of pGL3fos vector

Δ56fosCAT was double digested with Xba I and Sal I. Double digestion yielded a fragment which corresponds to fos promoter with al length of 165 bp.

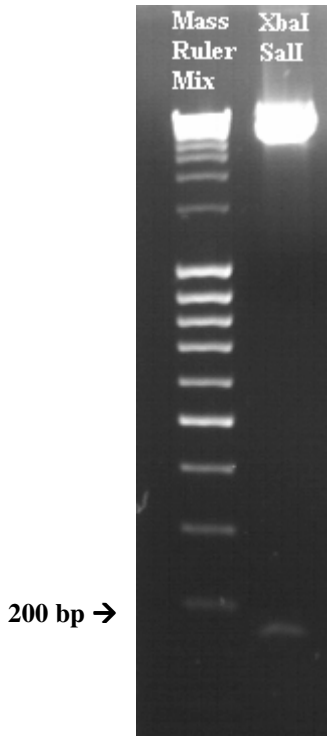


Figure 4.8: Xba I-Sal I double digestion of Δ56fosCAT plasmid.

The 165 bp fragment was extracted form agarose gel. The resultant 5' protruding Xba I and Sal I ends of the fos promoter were made blunt by Klenow enzyme.

pGL3 Basic vector was linearized by Hind III digestion and extracted from agarose gel. Similar to fos promoter, Klenow enzyme was used for blunt ending the 5' protruding ends of linearized pGL3 Basic vector.

Blunt-end ligation of fos promoter and pGL3 Basic was performed. When a Hind III blunt end is ligated to Sal I blunt end, a Hind III site is regenerated. However, when a Hind III blunt end is ligated to Xba I blunt end, an Xba I site is generated. Therefore, if the fos promoter was ligated in positive direction, the resultant vector construct must carry a Hind III site at the 5' end of fos promoter and an Xba I site at the 3' end.

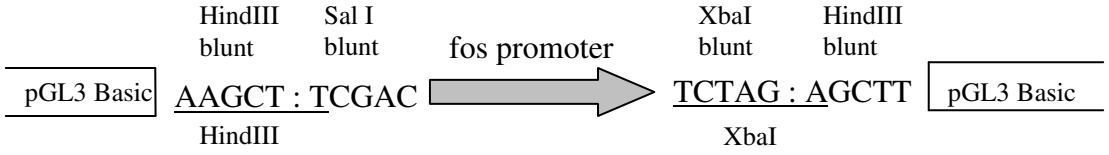


Figure 4.9: Blunt end ligation of fos promoter and pGL3 Basic vector. Hind III and Xba I sites are created on the construct vector are underlined.

Ligation mixture was transformed into *E.coli* DH5α and spreaded on Luria Agar-ampicillin plates. Resultant colonies were grown in Luria Broth-ampicillin medium and plasmid isolation was performed.

For screening of constructs, plasmids isolated from each colony were double digested with Hind III and Nco I restriction enzymes. This digestion should give a 211 bp fragment if the promoter was ligated in the positive direction. If the plasmid was a product of self ligation or the insert was reverse oriented, only a 33 bp fragment was formed by the double digestion and it could not be observed in 1% agarose gel. Colonies that gave 211 base pair fragment were accepted as positive.

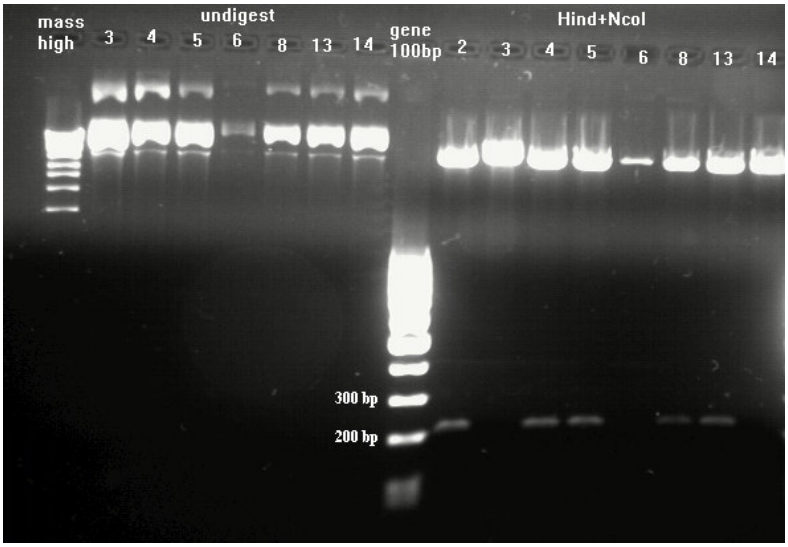


Figure 4.10: Analysis of Hind III-Nco I double digest of isolated plasmids. The 211 bp fragment contains fos promoter that is cut out from pGL3fos vector.

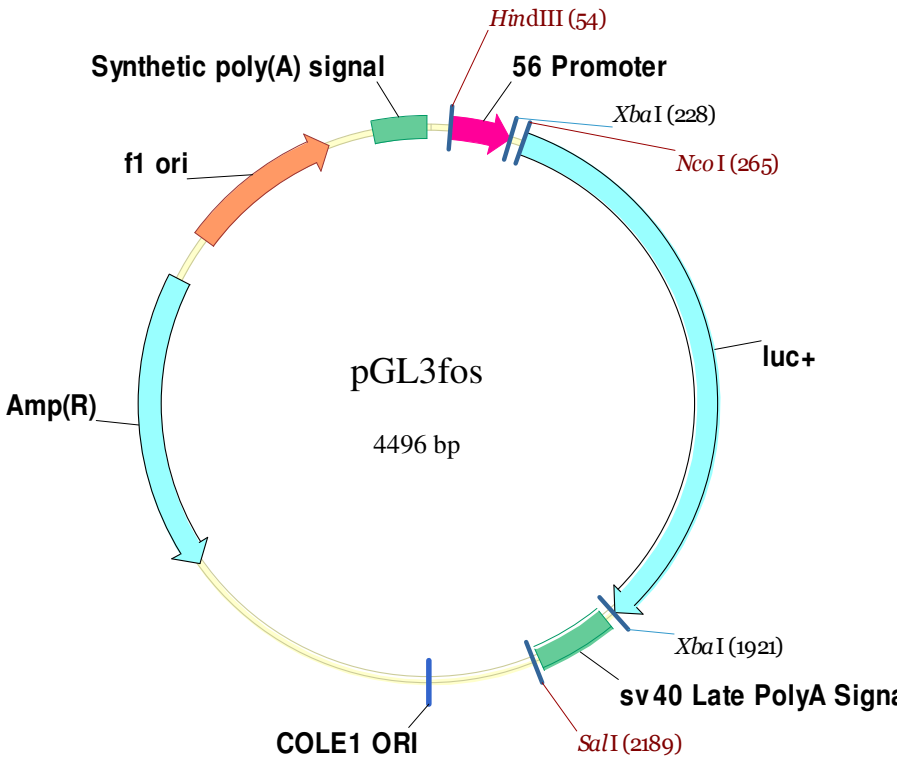


Figure 4.11: Map of constructed pGL3fos vector. Restriction sites: Xba I, Hind III and Nco I are used for diagnostic digests.

Additionally, since an Xba I site was generated at the 3' end of fos promoter upon blunt-end ligation, isolated plasmids were also digested with Xba I for further confirming the insertion of fos promoter into pGL3 Basic. This digestion should give

3303 and 1693 bp fragments if the fos promoter was ligated in positive direction. On the other hand, if promoter was ligated in reverse direction, 3138 and 1858 bp fragments should be observed. Self ligation should give only one band in the length of pGL3 Basic.

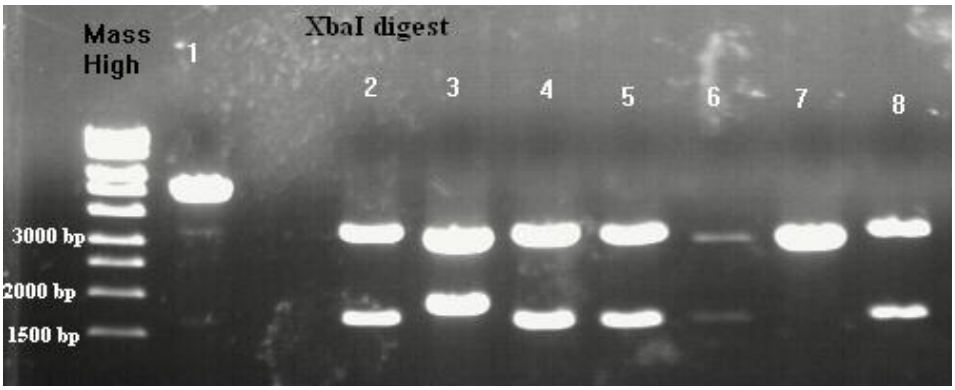


Figure 4.12: Analysis of Xba I digests of isolated plasmids.

According to figures 4.10, 4.11 and 4.12, presence of insert in positive direction was confirmed for plasmids isolated from colonies 2, 4, 5 and 8. Colony 3 seemed to have the insert in negative direction whereas colony 1 did not have the insert. In addition, colony 6 might have the insert however; its plasmid yield was low.

4.2.3 Insertion of PCR fragments into pGL3fos vector

Amplified PCR fragments and pGL3fos vector were digested with Sal I in order to create compatible sticky ends. PCR fragments containing CNS regions were ligated with the constructed pGL3fos. Ligation mixtures were transformed into E.coli DH5α cells and spreaded on Luria Agar- ampicillin plates. Colonies were screened in order to verify the insertion of the PCR fragments. For this purpose, plasmid DNAs were isolated and digested with Sal I. Following the analysis on agarose gel, colonies that gave fragments in the length of inserted PCR products were confirmed.

However, plasmid isolation and restriction digestion is a time consuming procedure for screening several ligations, initial confirmation for most ligations were

done by colony PCR method. Colony PCR products were visualized on agarose gel and colonies that gave fragments at the size of inserts were accepted as positive.

After plasmid isolation of positively selected colonies, another confirmation step was performed. Apart from the Sal I sites located at both ends of ligated inserts, restriction enzyme sites inside the cloned PCR fragments were determined. These selected restriction sites are also represented in PGL3 Basic vector by at least one site. Each isolated plasmid was digested with those restriction enzymes as indicated in Table 4.5. It should be noted that Kpl12 fragment does not have an available restriction site. Expected fragments in case of inserts were ligated in forward direction are given in Table 4.5. However, since directional cloning was not aimed, some of the inserts were ligated in reverse direction.

Vector	Restriction enzyme sites	Expected Fragment Length (bp)
pGL3fos-Kpl7	Pvu II	3231 2201
pGL3fos-Kpl8	Xmn I	3573 1912 84
pGL3fos-Kpl9	Xba I	3426 1693 717
pGL3fos-Kpl10	Sma I	3395 2845
pGL3fos-Kpl11	Nco I	3328 2429
pGL3fos-Kpl12	-	-
pGL3fos-Kpl13	Hind III	3277 2266

Table 4.5: Restriction enzyme sites inside the cloned sequences. Last column shows the expected fragment lengths when the vector constructs in the first column were digested with indicated restriction enzymes. Fragment lengths correspond digestion products in the case of ligation in forward direction.

A) B)

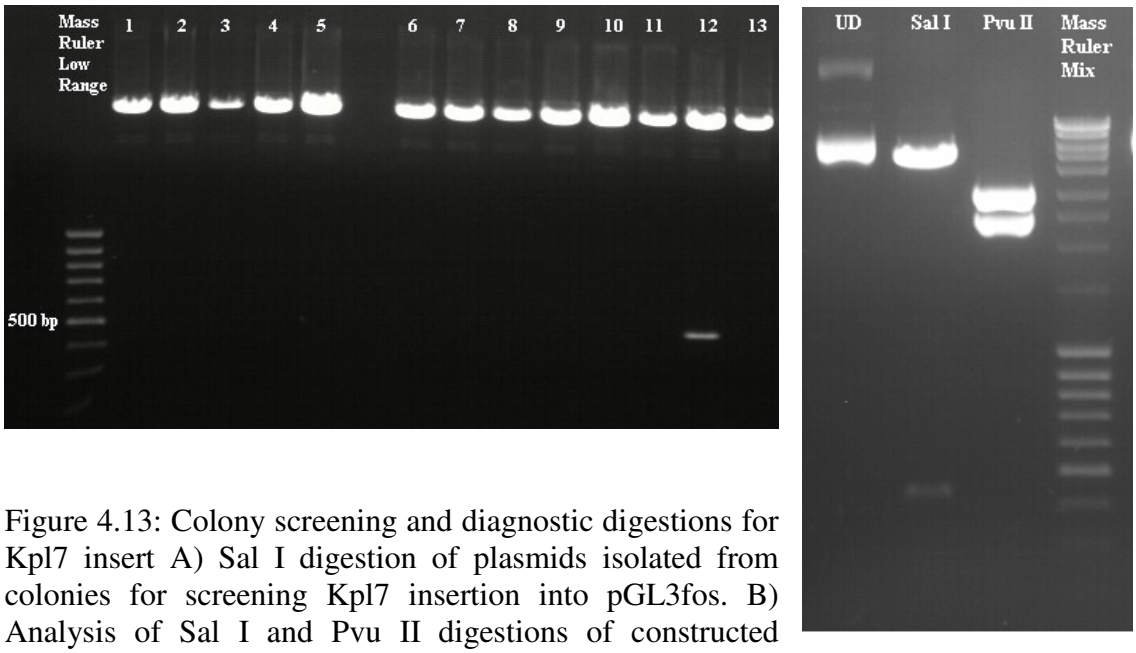
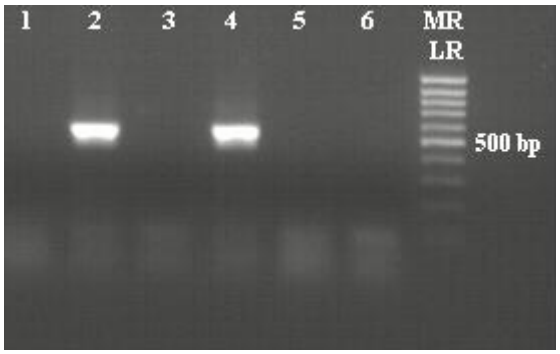


Figure 4.13: Colony screening and diagnostic digestions for Kpl7 insert A) Sal I digestion of plasmids isolated from colonies for screening Kpl7 insertion into pGL3fos. B) Analysis of Sal I and Pvu II digestions of constructed pGL3fos-Kpl7 vector. UD represents undigested plasmid.

Transformed colonies were screened for Kpl7 insertion into pGL3fos vector by Sal I digestion of isolated plasmids. Colony number 12 gave a fragment in the length of Kpl7 (Figure 4.13.A). After midiprep plasmid isolation, Pvu II digest was performed. The length of resultant fragments indicates that Kpl7 was ligated in reverse direction. As can be seen from pGL3fos-Kpl7 vector map (appendix F), when Kpl7 is ligated in reverse direction, Pvu II digest gives two fragments. 3017 bp and 2415 bp fragments correlate with the bands observed on the third lane in Figure 4.13.B.

A) B)



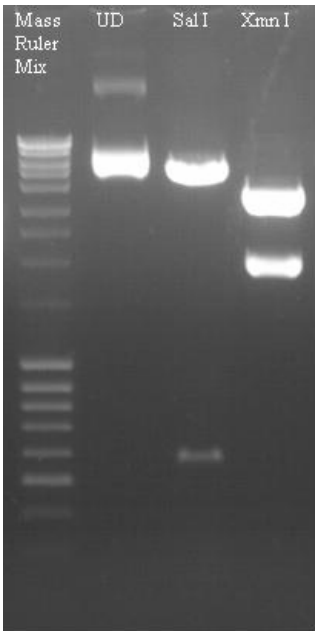
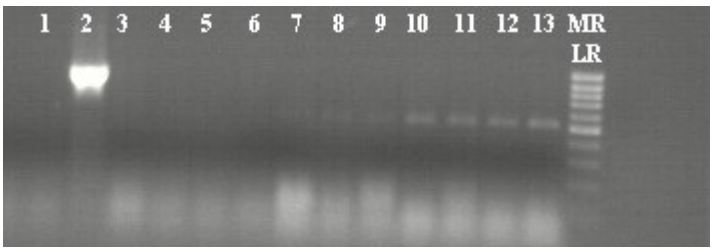
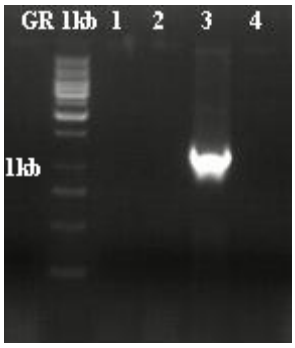


Figure 4.14: Analysis of colony PCR results for screening Kpl8 insertion into pGL3fos (A). Sal I and Xmn I digestions of constructed pGL3fos-Kpl8 vector (B).

In order to screen Kpl8 insertion into pGL3fos vector, colony PCR method is performed for colonies transformed with ligation mixture. Primers designed for amplification from BAC clone were used. Kpl8 insert was amplified from two colonies as can be seen in Figure 4.14.A lanes two and four. Additionally, Sal I digest for isolated plasmid gave a band in the length of Kpl8. Moreover, Xmn I digest gave the expected fragments for pGL3fos-Kpl8 as indicated in Table 4.5 although a 84 bp band could not be observed in 1% agarose gel.





A) B)

Figure 4.15: Colony PCR results for screening Kpl9 (A) and Kpl10 (B) insertions into pGL3fos. Mass Ruler Low Range and Gene Ruler 1kb molecular weight markers are used respectively.

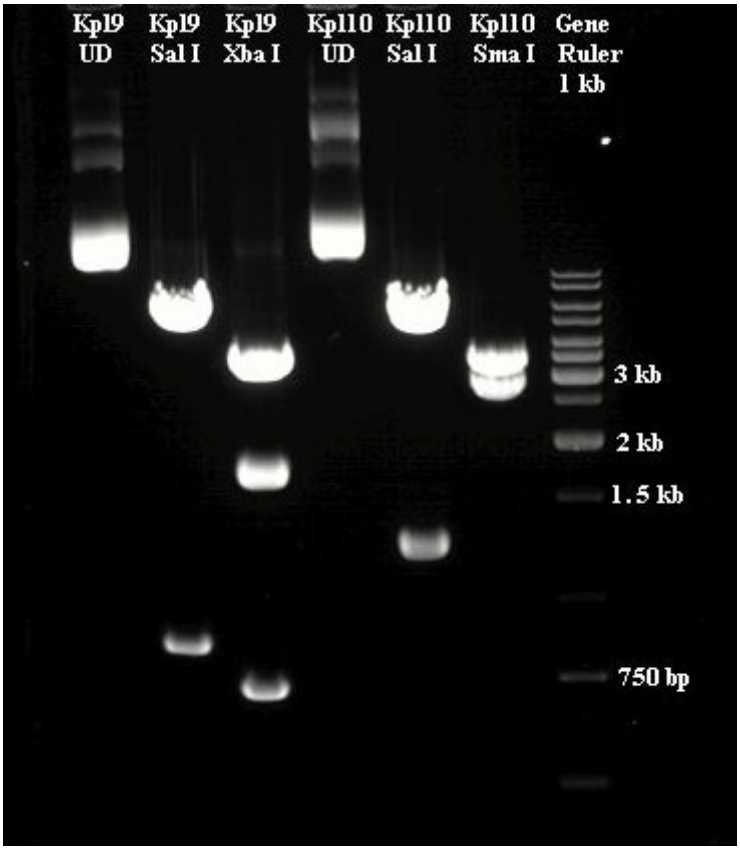


Figure4.16: Restriction enzyme digestion analysis of pGL3fos-Kpl9 and pGL3fos-Kpl10 vectors.

After selection of positive colonies by colony PCR screening (Figure 4.15), pGL3fos-Kpl9 and pGL3fos-Kpl10 vectors are digested with the indicated enzymes in Figure 4.16. Sal I digests gave bands equal to the lengths of Kpl9 and Kpl10 inserts. Xba I digest of Kpl9 and Sma I digest of Kpl10 gave fragment lengths that correlate with Table 4.5. Therefore, it can be concluded that Kpl9 and Kpl10 were successfully cloned into pGL3fos vector.

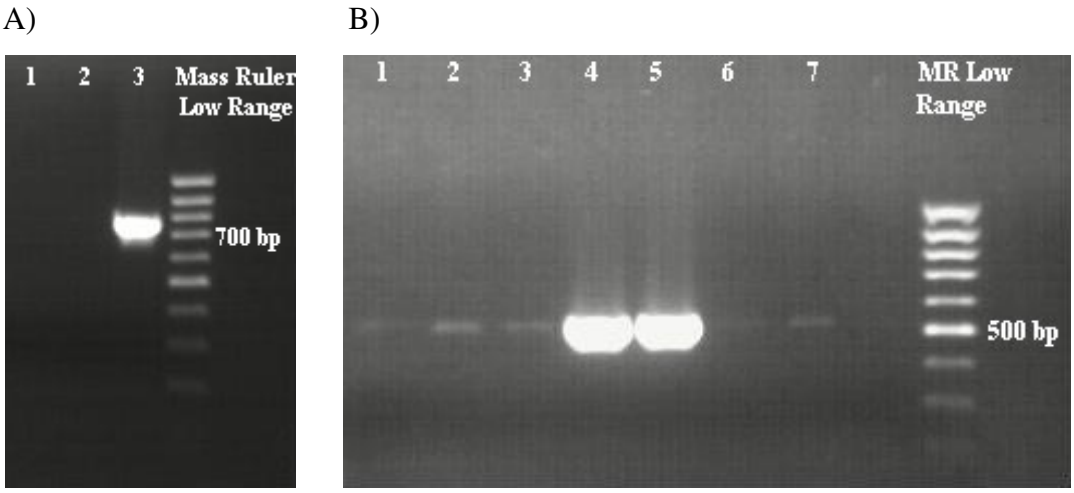


Figure 4.17: Analysis of colony PCR results for Kpl11 (A) and Kpl13 (B) insertions into pGL3fos vector.

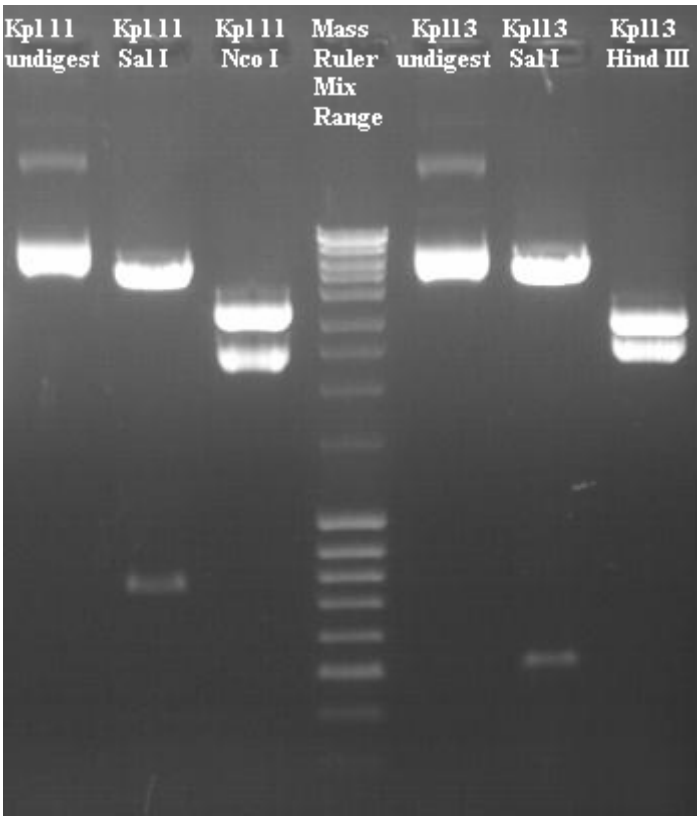
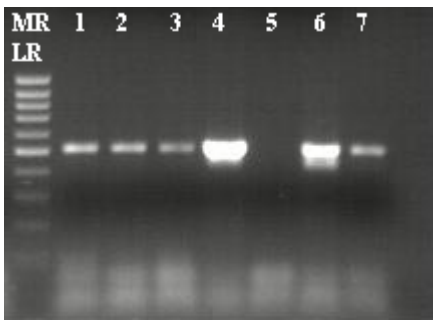
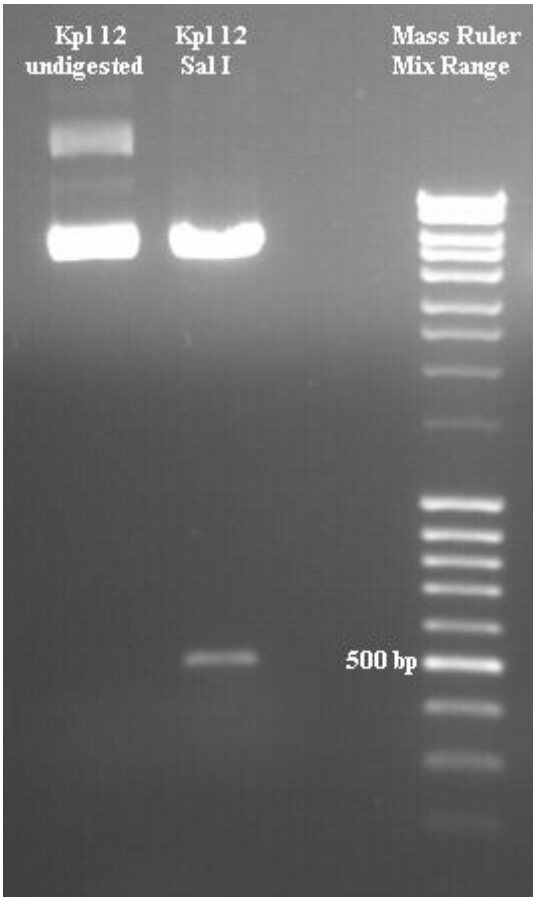


Figure 4.18: Agarose gel electrophoresis for restriction enzyme digestion products of constructed pGL3fos-Kpl11 and pGL3fos-Kpl13 plasmids.

Kpl11 PCR product was ligated with pGL3fos vector. Positive colony was selected by colony PCR (Figure 4.17.A). Insertion of Kpl11 was confirmed by Sal I and

Nco I digestions (Figure 4.18). Sal I digest gave a band in the length of the Kpl11 insert. In addition, Nco I digested the vector into two fragments whose sizes were consistent with the expected results indicated in Table 4.5. Kpl13 fragment was also inserted into pGL3fos plasmid. Hind III digest of pGL3fos-Kpl13 gave two fragments whose lengths were consistent with the expected fragment sizes (2992 bp and 2551 bp) in the case of reverse ligation. Kpl13 PCR fragment was inserted in reverse direction as in the vector map (Appendix F).





A B

Figure 4.19: Colony PCR (A) and Sal I digestion (B) of pGL3fos-Kpl12 vector.

pGL3fos-Kpl12 positive colonies were selected by colony PCR (Figure 4.19 A). Since no available restriction enzyme could be identified in Kpl12 sequence, only Sal I digest was performed for confirmation which cut the Kpl12 fragment back (Figure 4.19.B).

4.3 Transient Transfection of EL-4 and 293TCell Lines with pmaxGFP

Due to the simple analysis of GFP by fluorescence microscopy, pmaxGFP vector was selected for monitoring the transfection efficiency. pmaxGFP encodes the green fluorescent protein (GFP) from *Copepod potellina* species. Although the main goal of this study was evaluating luciferase reporter activity in EL-4 T-lymphoma cells, these cells were also transfected with pmaxGFP to examine the success of transfections. In addition to EL-4 cells, 293T cell line, which is a human embryonic kidney fibroblast cell line, was used. Due to efficient transfection and high expression properties of 293T cells, these cells were transfected with pmaxGFP in order to compare the GFP expression profiles with transfected EL-4 cells.

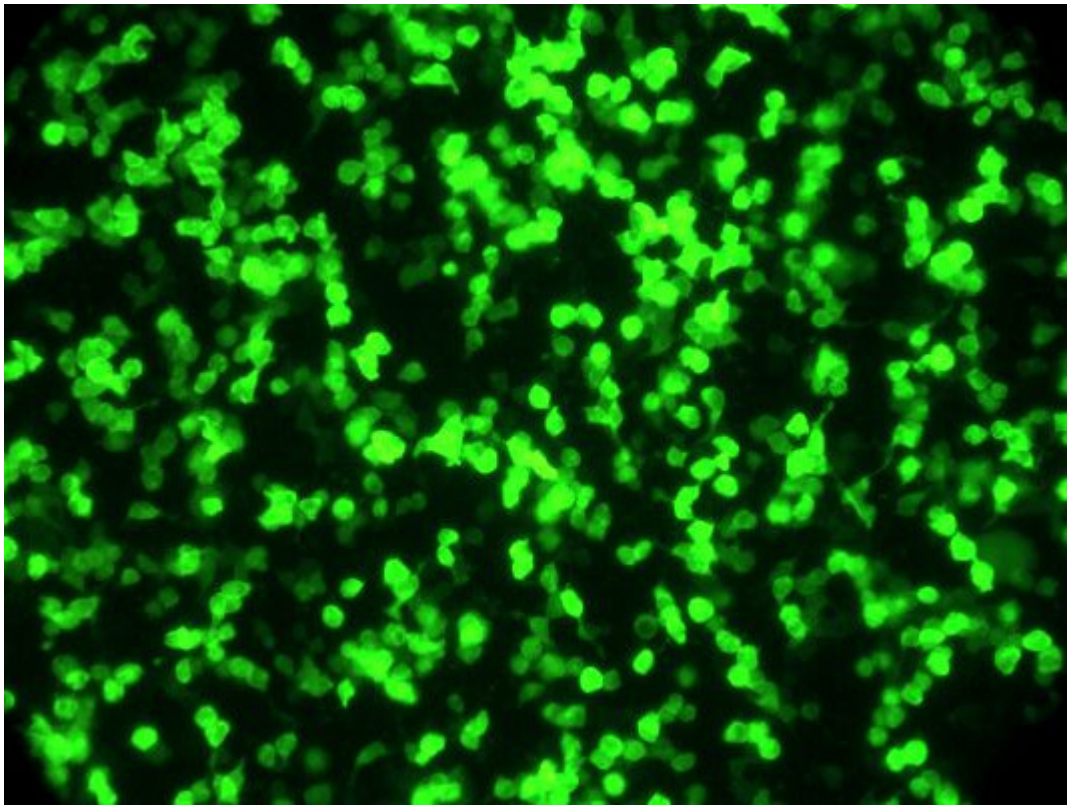


Figure 4.20: pmaxGFP transfected 293T cells visualized by fluorescence microscopy at 200X magnification.

pmaxGFP was introduced into 293T cells by calcium phosphate mediated transfection. $0.5x10^6$ cells were seeded 24 hours prior to transfection and 6.5 µg DNA

was used. 48 hours after transfection, 293T cells were monitored under fluorescence microscope in order to evaluate their transfection efficiency and GFP expression.

EL-4 cells were transfected with pmaxGFP by electroporation. Several electroporation conditions were tested to improve the efficiency. After examining numerous experimental conditions, best transfection efficiency was obtained at 300V, 1200μF and 200Ω. Transfected cells were visualized by fluorescence microscopy.

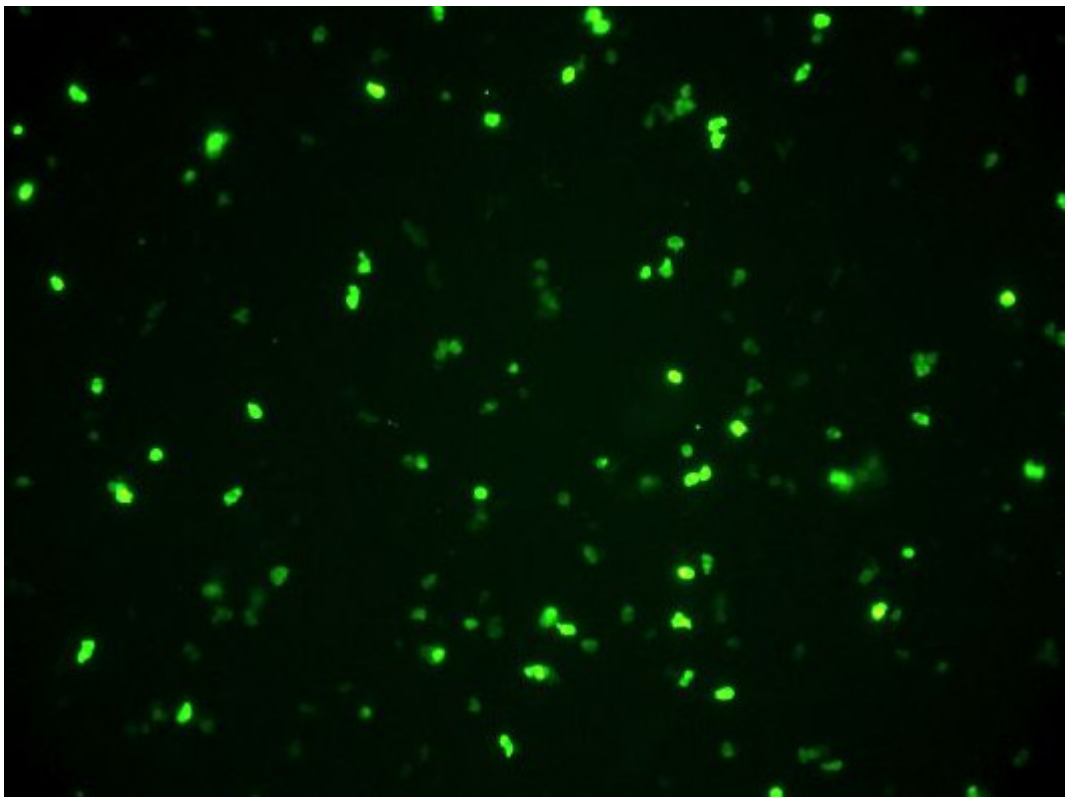


Figure 4.21: pmaxGFP transfected EL-4 cells visualized by fluorescence microscopy at 200X magnification.

Transfections results of the two cell lines (Figures 4.20 and 4.21) were different in terms of percentage of light emitting cells, meaning that 293T cells were more efficiently transfected than EL-4 cells. Transfection efficiency of 293T cells was evaluated as 35-40%, whereas EL-4 cells were transfected at 15-20%. Moreover, transfected 293T cells emitted green light at a greater intensity compared to EL-4 cells. This suggested that 293T cells expressed greater levels of GFP protein than the EL-4 cells did.

4.4 Luciferase Reporter Assays

4.4.1 Construction of pGL3fos-Enhancer Plasmid

Aim of the present study was examining the putative transcriptional activities of various conserved noncoding sequences (CNS) located in IL-7R gene locus. For this purpose, one intronic and six upstream CNSs are cloned into pGL3fos plasmid. pGL3fos was constructed by inserting the weak fos gene promoter into the downstream of luciferase gene in the pGL3 Basic vector.

To evaluate the transcriptional efficiency of fos promoter, we needed a strong enhancer sequence. I selected SV40 enhancer in order to insert into pGL3fos vector and to use a positive control. pGL3 Enhancer vector contains a SV40 enhancer at the downstream of luciferase cDNA. However, suitable restriction enzyme sites could not be found for cutting and ligating only the SV40 sequence. Therefore, SV40 enhancer was excised from pGL3 Enhancer vector together with the luciferase cDNA. The fragment containing both SV40 enhancer and luciferase cDNA was replaced with the luciferase gene of pGL3fos plasmid in order to construct the pGL3fos Enhancer vector.

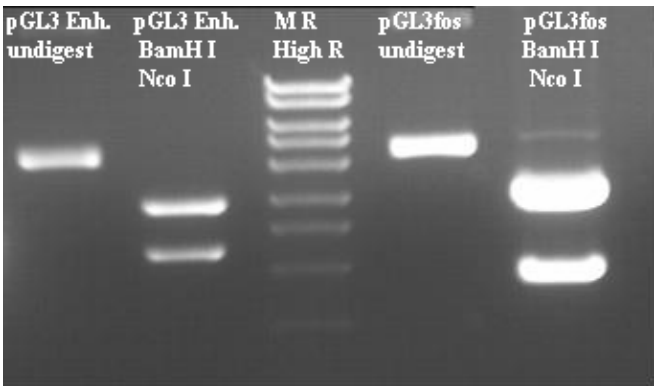


Figure 4.22: BamH I-Nco I double digestion for pGL3 Enhancer and pGL3fos vectors. Mass Ruler High Range was used as molecular weight marker.

Fragment containing SV40 enhancer and luciferase cDNA was extracted from pGL3 Enhancer vector by BamH I-Nco I double digestion (2164 bp fragment in Figure 4.22 lane 2). At the same time, luciferase cDNA was removed from pGL3fos plasmid by the

same double digestion. Then, the fragment containing SV40 enhancer and luciferase cDNA was ligated with the linearized pGL3fos which lacks luciferase cDNA (3078 bp fragment in Figure 4.22 last lane). Construction of pGL3fos Enhancer vector was confirmed by Pvu II-Sph I double digestion. Pvu II has a site in fos promoter, while Sph I has two sites in SV40 enhancer and a single site in luciferase cDNA. Pvu II and Sph I do not have any other restriction sites in the backbone plasmid pGL3 Basic.

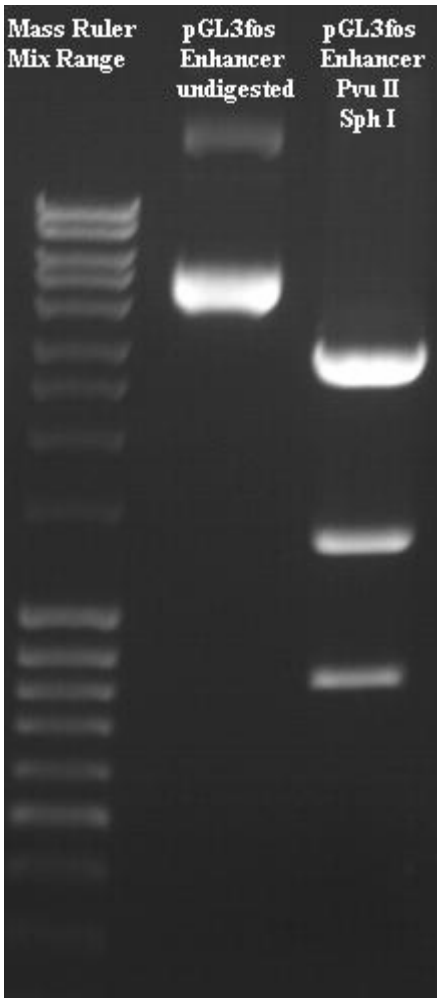


Figure 4.23: Analysis of Pvu II-Sph I double digestion of pGL3fos Enhancer vector.

Construction of pGL3fos Enhancer was confirmed. Expected fragment lengths from Pvu II-Sal I double digestion was 2982, 1357, 811 and 72 base pairs. Except the 72 bp fragment which could not be seen on 1% agarose gel, all bands in the above figure were consistent with expected sizes.

4.4.2 Determining the linear range of luminescence detection

Luciferase reporter activity can be measured by a number of commercially available luminometers. However, linear range of detection may vary among different instruments. For his purpose, purified recombinant luciferase enzyme was used to produce a calibration curve to evaluate the linear range of SpecraMax GeminiXS microplate spectrofluorometer (Molecular Devices Inc.). At various enzyme concentrations, luminescence values were measured.50 ml of each standard was mixed with luciferase assay reagent.

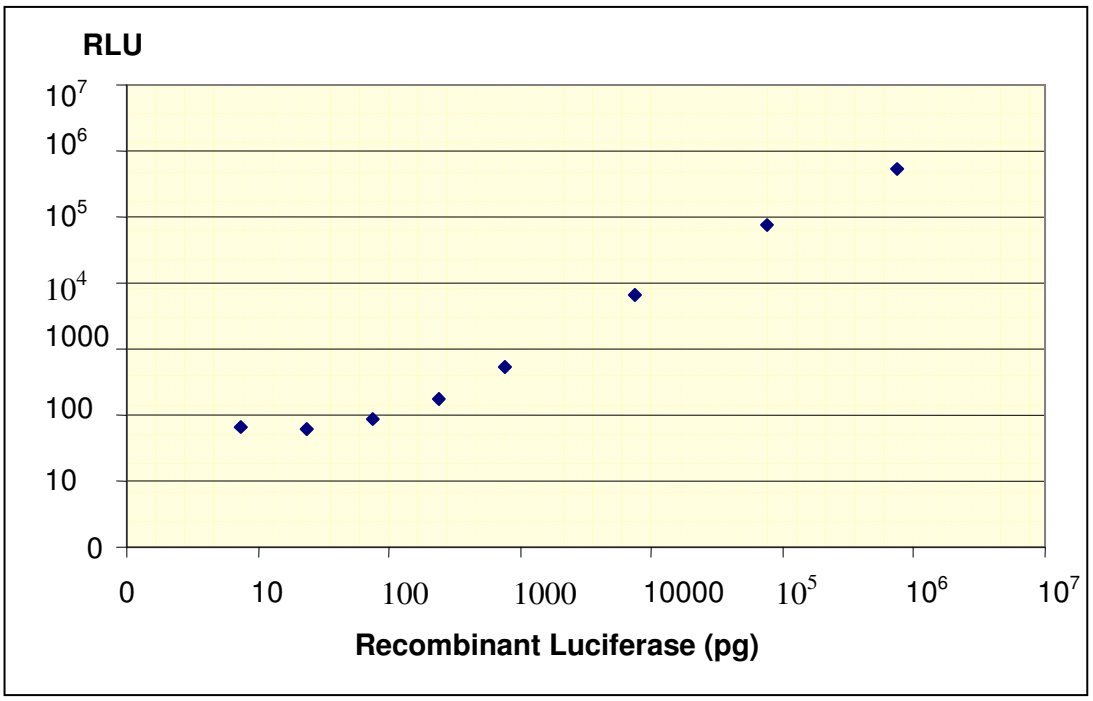


Figure 4.24: Standard curve for determination of linear range of luminescence detection. Note that values were plotted logarithmically.

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As can be seen from the calibration curve in Figure 4.24, measured luminescence levels were in a good linear relationship with the luciferase enzyme quantities that were higher than 75 pg/well. On the other hand, the low end of the curve was not in the linear range of detection. This means that the luciferase amounts lower than 75 pg/well could not be reliably measured. For this purpose, we decided to find out the lower limit of detection of the instrument. Lower limit of detection is defined as the three positive standard deviations added to the average of statistically significant number of blank

measurements (McGown *et al.*, 2000). Blank measurements were done by mixing only the lysis buffer with the luciferase assay reagent. We found the blank value as 51±16 RLU which indicated that the lower limit of detection was 99 RLU at 99% confidence level. This result showed consistency with the standard curve data because 99 RLU approximately corresponds to 75 pg/well luciferase that is the lowest point in the linear range of detection in the standard curve.

4.4.3 Transfection of 293T cell line for luciferase assay

Since 293T cells showed greater transfection efficiency compared to EL-4 cells (Section 4.3), 293T cells were also assayed for luciferase activity measurements. 293T cells were transfected with pGL3 Control vector in order to confirm that luciferase reporter system works well. pGL3 Control vector contains a SV40 promoter and a SV40 Enhancer. These strong transcriptional elements provide the expression of flanking genes in most mammalian cells. So that, pGL3 Control vector was introduced into 293T cells as a positive control. In addition, the constructed pGL3fos Enhancer vector was transfected into 293T cells to examine the transcriptional activity of inserted fos promoter.

Luciferase reporter gene assay was accomplished for 293T cells by calcium phosphate mediated transfection. 24 hours prior to transfection $5x10^6$ cells were seeded in a 60 mm plate. 50 µL of each cell lysate was mixed with 100 µL luciferase assay reagent and the reaction was observed in microplate luminometer. Luciferase reporter activity of cells transfected with various DNA concentrations were examined (Figure 4.25). 293T cells were transfected with different amounts of pGL3 Control vector. However, a significant deviation could not be detected between these conditions.

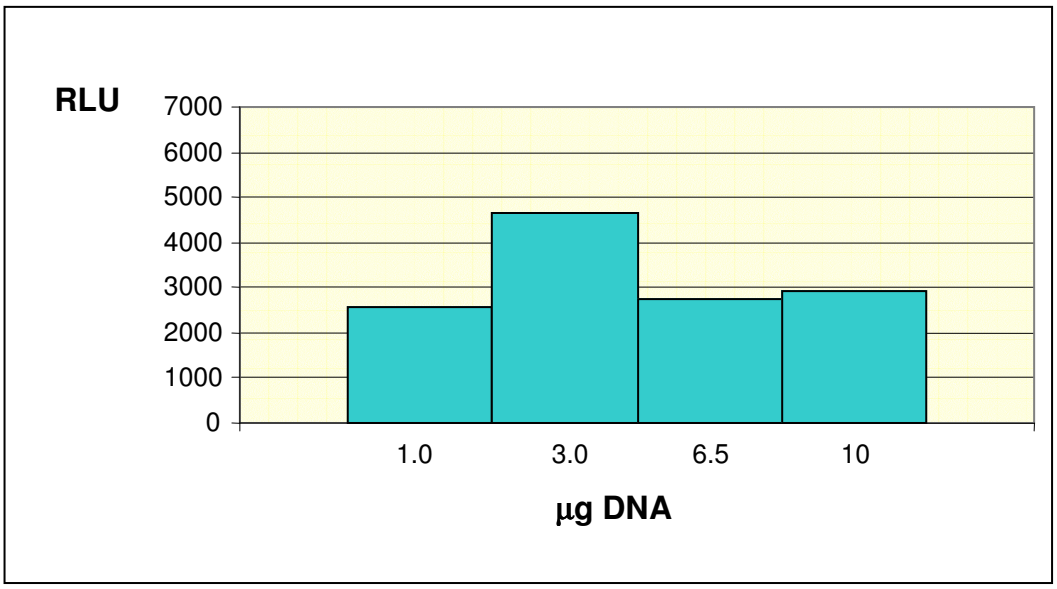

Figure 4.25: Luciferase activity of 293T cells transfected with different amounts of pGL3 Control vector.

In addition to examining the effects of different DNA concentrations, number of cells was titrated from $0.25x10^6$ to $5x10^6$ in 60 mm tissue culture plates. As seen in Figure 4.26 highest luciferase activity (~100000 RLU) was observed in the sample with the lowest seeding density. On the other hand, transfections with higher seeding densities yielded approximately ten fold decrease in luciferase activity.

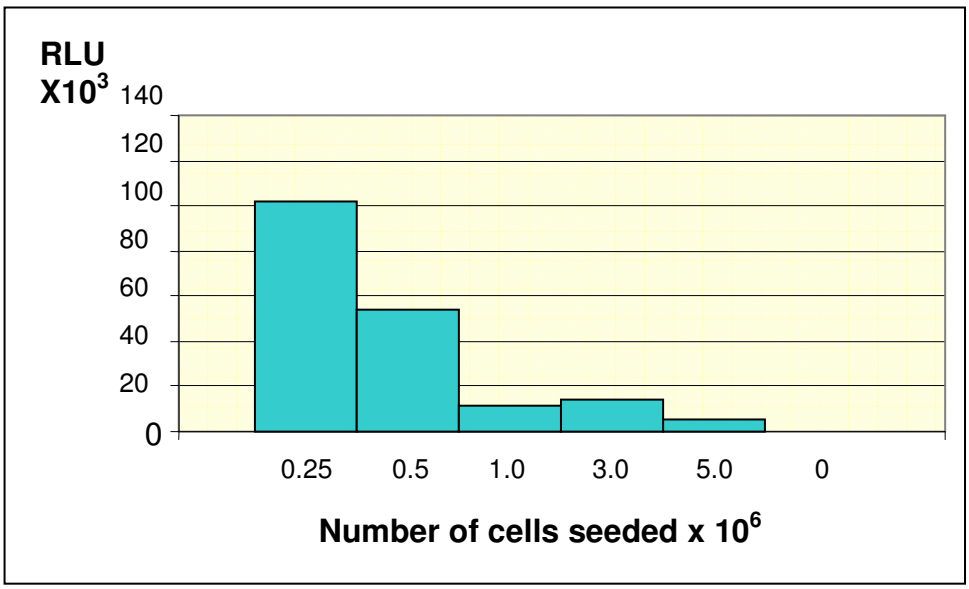

Figure 4.26: Luciferase activity of 293T cells transfected with pGL3 Control vector at different cell densities. X-axis shows the number of cells seeded 24 hours prior to transfection.

As well, the constructed pGL3fos Enhancer vector was transfected into 293T cells to examine the transcriptional activity of inserted fos promoter. $0.25x10^6$ 293T cells were transfected with 6.5 µg DNA. Luciferase activity was measured as 4500 RLU which indicated that fos promoter was functional for transcriptional activity.

4.4.4 Transfection of EL-4 cell line for luciferase assay

Since luciferase reporter gene assay was accomplished for 293T cell line successfully, we investigated the EL-4 T-lymphoma cell line for luciferase activity in response to positive control vector. pGL3 Control and pGL3fos Enhancer vector were transfected into EL-4 cells. Several transfection and cell lysis conditions were tested. Transfections were performed in the electroporation conditions that gave the best transfection efficiencies when the cells were transfected with pmaxGFP. For the lysis of the cells, reporter lysis buffer (RLB) from Promega, 0.1% TritonX-100 or 1% TritonX-100 buffers were used in independent experiments. However, a significant luciferase activity could not be measured. We could not observe a luminescence measurement higher than 99 RLU which is found to be the lower limit of detection of the instrument (Section 4.4.2.2).

We determined protein concentrations in the cell lysates by Bradford assay, and confirmed that cells were lysed successfully. We first prepared a standard curve with different BSA (Bovine serum albumin) concentrations between 0.1-1.4 mg/ml. Then, we measured the total protein concentration in cell lysates. Cell lysis for different transfection experiments and with different lysis buffers gave protein concentrations in the range of 0.3-1.0 mg/ml. This indicated that an inefficient lysis was not the reason for the absence of luminescence.

Transfections of EL-4 cells with the positive control vectors did not yield a significant luciferase activity. For this reason, constructed pGL3fos-Kpl plasmids containing mouse CNS regions were not assayed for transcriptional activity.

5 DISCUSSION

As lymphocytes differentiate from bone marrow precursors to functional mature cells, they express IL-7R at distinct stages, indicating that IL7 survival signals are tightly regulated (Munitic *et al.*, 2004). Although a few transcription factors have been reported to bind to the promoter region of IL-7R gene (Xue *et al.*, 2004; DeKoter *et al.*, 2002), a key factor for the temporal and cell specific regulation has not been identified. In this study we followed a strategy for analyzing the transcriptional regulation of IL-7R gene. We used a bioinformatics approach to identify possible regulatory elements conserved at the upstream region of the IL-7R gene. Then we cloned these conserved noncoding sequences into a reporter vector. After that, we examined the practicability of luciferase reporter gene system in two different cell lines.

5.1 Comparative Analysis

Initiation of transcription requires binding of specific factors to promoter sequences as well as to a variety of regulatory elements frequently located far from the transcription start site (Rutherford, 2000). These cis-regulatory elements may be conserved between different species and may be located at tens to hundreds of kilobases away from the gene (Mortlock *et al.*, 2003). Therefore, we aimed to study the transcriptional activation capability of a number of conserved noncoding sequences (CNS) located several kilobases away at the upstream of IL-7R gene.

First, we determined the homology of IL-7R upstream sequences among different organisms. We found that a neighbouring gene called Kpl2 is located at the upstream of

IL-7R gene in rat genome. Furthermore, the same gene was found to reside at a similar position in mouse, human and in a few mammalian species. Although these sequences could exist with different aliases due to the complexity in nomenclature, all of them have been shown as Kpl2 similar sequences. Therefore, we confirmed the homology of genomic loci to compare.

For performing a comparative analysis, we then determined the organisms whose genomic sequences were to be compared. We aligned the homologous loci between several organisms and monitored the conservation among their genomic sequences (Data not shown). We ended up with a very low conservation between distinctly related organisms (chicken and human) and could observe only a few highly conserved noncoding regions. Conversely, between the closely related species such as human and chimpanzee, conservation was much greater that was unlikely to be considered as the preservation of functional sequences. These results indicated that moderately related organisms were a better choice for comparative analysis in order to reliably explore noncoding conserved sequences.

We decided to perform a global alignment between mouse and human genomic loci containing more than 200 kilobases upstream and intronic sequences of IL-7R gene. Due to the high percentage of unfunctional repeat sequences in vertebrate genomes, we eliminated those sequences in order to prevent false positive consideration. We set the minimum conservation level and window length parameters that define which sequences will be monitored as CNS regions by VISTA. More than twenty noncoding sequences were determined to be conserved among the 255.5 kilobase region. Considering the required labor and time, for primary investigation, we preferred to analyze six of these sequences that were located in a 40 kilobase region at the upstream of IL-7R gene. In addition, one CNS inside the second intron of IL-7R gene was also selected for analysis. Since we planned to further analyze conserved mouse genomic sequences, we determined the positions of CNS regions relative to the aligned mouse genomic region for simplicity.

5.2 Construction of Vectors

As we identified CNS regions in mouse genomic sequence, we amplified these sequences from a BAC clone which corresponds to the sequences of our interest. When designing primers for amplification, a Sal I restriction enzyme site was added to the 5' end of each primer in order to insert the PCR fragments into the Sal I site of the reporter plasmid. In addition to the Sal I site, the primers were extended by five bases at the 5' end to allow the Sal I enzyme to bind to the PCR products efficiently. After ligation of PCR products into the luciferase reporter vector, we applied Sal I digestions in order to confirm the insertion of the amplified fragments in the proper lengths. However, the ligation efficiency was found to be low and required screening a lot of colonies. We decided to increase the insert to vector ratio to increase the efficiency. In addition, we used colony PCR method for the conformation of inserts. This strategy was not only time saving compared to plasmid isolations followed by restriction enzyme digestions, but also eliminated the requirement of using lots of enzyme. After selecting the positive colonies, we performed further confirmation digestions with Sal I and other restriction enzymes. Each of these restriction enzymes was confirmed to have at least one recognition site inside the cloned PCR fragment and through the vector backbone.

The reporter vector which we cloned the amplified CNS sequences into, was prepared by modifying the pGL3 Basic luciferase vector. We inserted a weak promoter into the 5' end of luciferase cDNA in the reporter vector. The reason why we selected a weak promoter was that, a weak promoter would not mask the transcriptional activity of the noncoding conserved sequences to be analyzed. We constructed a positive control plasmid in order to use in luciferase assays. Since the conserved sequences were cloned into the downstream of luciferase cDNA that is controlled by a weak promoter, we needed a strong enhancer to check the transcriptional activity of this promoter. For this reason, we inserted the SV40 strong enhancer sequence into the reporter vector.

5.3 Transfections and Luciferase Assays

We used 293T cell line in the transfection experiments in order to allow a comparison with the EL-4 cell line. EL-4 cell line is a $CD4^+$ single positive T-lymphoma suspension cell line that is known to be IL-7R positive. We evaluated the transfection efficiencies and luciferase reporter activities. 293T cells are adherent human kidney fibroblast cells that have efficient protein expression capacity. Therefore, we transfected both 293T and EL-4 cells with pmaxGFP plasmid by calcium phosphate mediated transfection and electroporation respectively. Monitoring by fluorescence microscopy indicated that EL-4 cells were transfected at a much lower efficiency than 293T cells. Several electroporation conditions were tried and a maximum of 15-20% efficiency has been achieved. When harsher electroporation conditions were applied, viability of the cells severely reduced.

Then, we transfected 293T cells with the constructed pGL3fos Enhancer plasmid in order to detect luciferase activity. Cell lysates were positive for reporter gene activity suggesting that the fos promoter was efficient for transcriptional activation mediated by the strong SV40 enhancer sequence. In addition, we tested the luciferase activity at different luciferase conditions. Titrated DNA concentrations and cell numbers were analyzed. DNA concentration was found not to be critical for produced luciferase activity. On the other hand, number of cells that were seeded 24 hours prior to transfection dramatically affected the luciferase activity. Interestingly, transfection of smaller number of cells resulted in several folds of increase in activity. This indicated that transfection of the reporter plasmid and expression of the reporter gene was more efficient when lower number of cells were used. In contrast, transfection of pGL3fos Enhancer vector into reduced number of EL-4 cells did not gave rise to an increased luciferase activity. This may be because of the distinct properties of calcium phosphate mediated transfection and electroporation methods to introduce DNA into cells. It should also be noted that dissimilar culture growth and physiological characteristics of the two cell lines may cause differences in utilization of the reporter vector.

We performed several electroporation experiments to introduce pGL3 Control and pGL3fos Enhancer vectors in order to evaluate the practicability of luciferase reporter system in EL-4 mouse T-lymphoma cells. However, we could not find a reliable luciferase activity in these cells. We checked the efficiency of lysis by protein determination assay and found that lysis was efficient. All of the luminescence measurements were out of the linear detection range of the luminometer (Section 4.4.2). In addition, all the measurements were under the range of lower limit of detection of the instrument (~75 pg luciferase per well) which is determined by summing the average of significant number of blank measurements with the three standard deviations (average of blanks ± 3 SD). Therefore we can conclude that the luminometer that we used was not sensitive enough for measuring luciferase levels lower than 75 pg/well. However, luminometers that are sensitive up to a few femtograms of luciferase are available. Thus, we need to reexamine the reporter vector constructs by the luciferase assay system with a more sensitive luminometer or by different reporter gene methods.

6 CONCLUSIONS AND FUTURE WORK

In this study we followed a strategy that combined bioinformatic and molecular cell biology approaches to investigate the upstream transcriptional regulation of IL-7R gene. IL-7 signalling is essential for the development and survival of B- and T-lymphocytes. Lymphocytes at distinct stages differentially express IL-7R on their surfaces, which determines their response to IL-7 signalling. Although there are studies on the transcriptional regulation of IL-7R expression, these findings were insufficient to explain the cell specific and temporal expression of IL-7R. For this purpose, we identified conserved noncoding sequences in the IL-7R gene locus that may have transcriptional activities and cloned these sequences into a luciferase reporter vector in order to evaluate their transcriptional capabilities.

We tested the performability of luciferase assay with two cell lines by transfecting them with reporter vectors containing strong activator sequences. Although, the human kidney fibroblast cell line was efficient for the luciferase reporter activity, the mouse $CD4^+$ single positive T-lymphoma cell line did not give a sufficient luciferase activity. To increase the efficiency of the reporter assay, a luminometer with a higher sensitivity can be used. In addition, a reliable normalization vector can be co-transfected into the cells in order to minimize the deviation between samples caused by external and cellular factors (Section 1.5.2.2). Vectors carrying reporter genes such as β-galactosidase or renilla luciferase under the control of weak or moderately strong promoters can be used as internal controls in reporter assays. Moreover, in vivo GFP reporter vectors can be used for the investigation of transcriptional activities of cloned regions by fluorescence activated cell sorting (FACS) analysis. FACS is a very sensitive system that the reporter expression properties of lymphocytes at distinct stages can be comparably observed.

Due to cell specific and temporal expression of IL-7R during the development of B- and T-lymphocytes, the strategy presented here can be expanded for other cell lines. Different regulatory sequences may be activated in lymphocytes at different stages. Therefore, activation capabilities of conserved regions can be investigated in several cell lines which represent the distinct developmental stages. Double negative, double positive, activated or memory single positive cells should be investigated in order to evaluate the possible IL-7R activation derived by the cloned conserved noncoding sequences.

Although several cellular mechanisms involving cytokines have been uncovered, IL-7 signalling will remain as a popular topic for scientists because, many molecular mechanisms still remain to be established. Moreover, numerous studies are being performed on the possible use of IL-7 on clinical applications (Alpdogan and van den Brink, 2005). As IL-7/IL-7R signals are the major components of survival, proliferation and peripheral homeostasis of T-lymphocytes, studies on diseases such as AIDS, lymphopenia and lymphomas, will increasingly be concentrated on IL-7 signalling.

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APPENDIX A

CHEMICALS

Name of the chemical	Supplier
6X Loading Dye	Fermentas, Germany
Acetic Acid	Riedel-de Haén, Germany
Agarose low EO	Applichem, Germany
Ampicillin	Sigma, Germany
Boric Acid	Riedel-de Haén, Germany
Bradford Reagent	Sigma, Germany
Calcium Chloride	Merck, Germany
Chloramphenicol	Sigma, Germany
Dimethylsulfoxide (DMSO)	Sigma, Germany
Distilled water	Milipore, France
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	AppliChem, Germany
Hydrochloric Acid	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	Promega, USA
Na_2HPO_4	AppliChem, Germany
NaH_2PO_4	Merck, Germany
PEG-4000	AppliChem, Germany
Penicillin-Streptomycin	Biological Industries, Israel
Potassium Chloride	Fluka, Germany
RPMI 1640	Biological Industries, Israel
Sodium Chloride	Riedel-de Haén, Germany
Sodium Hydroxide	Merck, Germany
Triton X-100	Promega, USA
Tris	Fluka, Switzerland
Trypan Blue	Sigma, Germnay
Trypsin-EDTA	Biological Industries, Israel

APPENDIX B

MOLECULAR BIOLOGY KITS

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

APPENDIX C

EQUIPMENT

Name of the equipment	Supplier
Autoclave	Certoclav, Table Top Autoclave CV-EL-12L, Austria
	Hirayama, Hiclave HV-110, Japan
Automatic pipette	Pipetus Akku, Hirshmann Laborgerate, Germany
Balance	Sartorius, BP221S, Germany
	Schimadzu, Libror EB-3200 HU, Japan
Black 96-well Plates	Cole Palmer
Cell counter	Cole Palmer, USA
Cell culture disposables	Trp, Switzerland
Centrifuge	Eppendorf, 5415D, Germany
	Hitachi, Sorvall RC5C Plus, USA
CO_2 incubator	Binder,CO_2 incubator, Germany
Deep Freeze	-80°C, Thermo Electron Corp.,Forma -86ULT Freezer USA
	-20°C, Bosch, Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis	Biogen Inc., USA
	Biorad Inc., USA
Electroporation cuvettes	Cole Palmer, USA

Electroporator	BTX ElectroCell Manipulator, ECM 630, Division of Genetronics, USA
Filter membranes	Millipore, USA
Flourescent Microscope	Olympus BX 60, Japan Olympus IX 70, Japan
Gel Documentation	UVITEC, UVdoc Gel Documentation System, UK
Hematocytometer	Hausser Scientific, Blue Bell Pa., USA
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, Germany Memmert, Modell 600, Germany
Laminar Hood	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Luminometer	SpectraMax Gemini XS Dual-Scanning Microplate Spectrofluorometer Molecular Devices Inc., USA
Microliter Pipette	Eppendorf, Germany
Microscopes	Olympus CH20, Japan Olympus CK40, Japan
Microwave Oven	Bosch, Turkey
pH Meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Refrigerator	+4°C, Bosch, Turkey
Shaker	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Thermomixer	Thermomixer Comfort Eppendorf, Germany
UV Illuminator	BioRAD UV-Transulliminator 2000, USA
Vortex	Velp Scientifica, Italy

APPENDIX D

ENZYMES AND REACTION BUFFERS

Name of the Enzyme or Buffer	Company
BamH I	Promega, USA
Buffer B	Promega, USA
Buffer D	Promega, USA
Buffer E	Promega, USA
Buffer E	Promega, USA
Buffer K	Promega, USA
Calf Intestinal Alkaline Phosphatase (CIAP)	Promega, USA
DNA Polymerase I Large (Klenow) Fragment	Promega, USA
Hind III	Promega, USA
Klenow Enzyme Buffer	Promega, USA
Nco I	Promega, USA
Pvu II	Promega, USA
Recombinant Luciferase	Promega, USA
Sal I	Promega, USA
Sma I	Fermentas, Germany

Sph I	Promega, USA
T4 DNA Ligase	Promega, USA
T4 DNA LigaseBuffer	Promega, USA
Tango Buffer	Fermentas, Germany
Taq DNA Polymerase	Promega, USA
Taq DNA Polymerase Buffer	Promega, USA
Xba I	Promega, USA
Xho I	Promega, USA
Xmn I	Promega, USA

APPENDIX E

Plasmids, cells, oligonucleotides and molecular weight markers

Item	Supplier
Δ56fosCAT	NCI, USA
293T cell line	NCI, USA
BAC Clone (RP23-365P6)	Children's Hospital Oakland Research Institute, USA
E.coli DH5α	Sabancı University Biolab-1
EL-4 cell line	NCI, USA
Gene Ruler 1 kb DNA Ladder	Fermentas, Germany
Gene Ruler 100 bp DNA Ladder	Fermentas, Germany
Mass Ruler DNA Ladder High Range	Fermentas, Germany
Mass Ruler DNA Ladder Low Range	Fermentas, Germany
Mass Ruler DNA Ladder Mix	Fermentas, Germany
pGL3 Basic	Promega, USA
pGL3 Control	Promega, USA
pGL3 Enhancer	Promega, USA
pGL3fos	Constructed in lab
pGL3fos Kpl 7-13 vectors	Constructed in lab

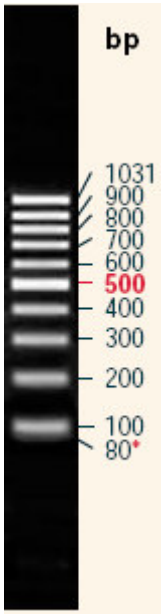
pmaxGFP	Amaxa Biosystems, Israel
Primers	Operon Biotechnologies Inc., USA

Primers:

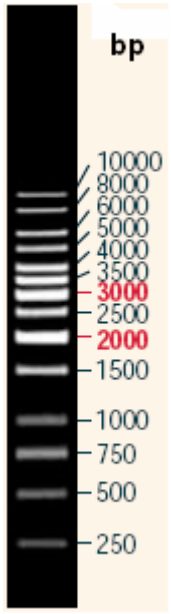
Kpl 7	Forward Primer:	GACGCGTCGACGGCTCCAAAGCTAACCAAAA
	Reverse Primer:	GACGCGTCGACGAACGTGGTCCTCTCACACA
Kpl 8	Forward Primer:	GACGCGTCGACTCGTAAACCTATCGTCTGTTGG
	Reverse Primer:	GACGCGTCGACAGACCCAATAGTTTGCACTGAA
Kpl 9	Forward Primer:	GACGCGTCGACACATTCCTCACAGGGAAGCA
	Reverse Primer:	GACGCGTCGACGCTGATCGAATGCAAAATGA
Kpl 10	Forward Primer:	GACGCGTCGACCCTTCCCCCTCCAGATATAAA
	Reverse Primer:	GACGCGTCGACCTAATCCCGGGTCCAAATCT
Kpl 11	Forward Primer:	GACGCGTCGACCCAAGGAATAAACCCAAGGA
	Reverse Primer:	GACGCGTCGACTCTCCCAAGCAACAAAAGAA
Kpl 12	Forward Primer:	GACGCGTCGACATAGTGCAGCCTTGCCTCTC
	Reverse Primer:	GACGCGTCGACAGAAGCACGCTTGTATGTGC
Kpl 13	Forward Primer:	GACGCGTCGACAAAATCACCCACACTGTTGAAA
	Reverse Primer:	GACGCGTCGACCCAAGACGTTGTTCCTCCAG

DNA molecular weight markers:

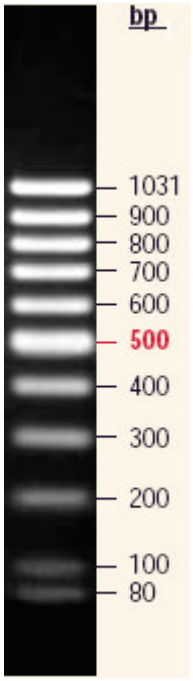
Gene Ruler 100 bp
DNA Ladder



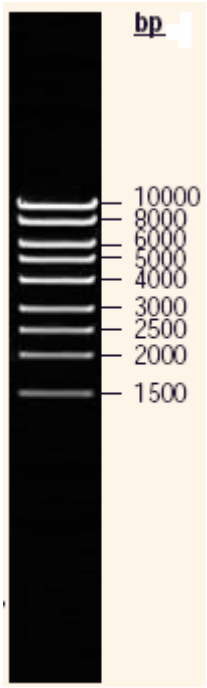
Gene Ruler 1 kb
DNA Ladder



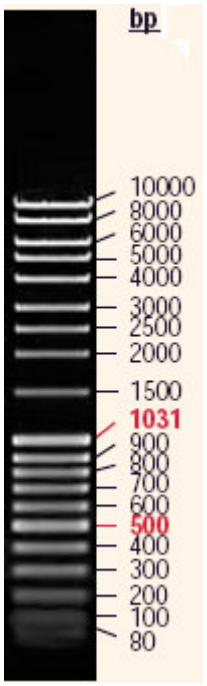
Mass Ruler
DNA Ladder
Low Range



Mass Ruler
DNA Ladder
High Range

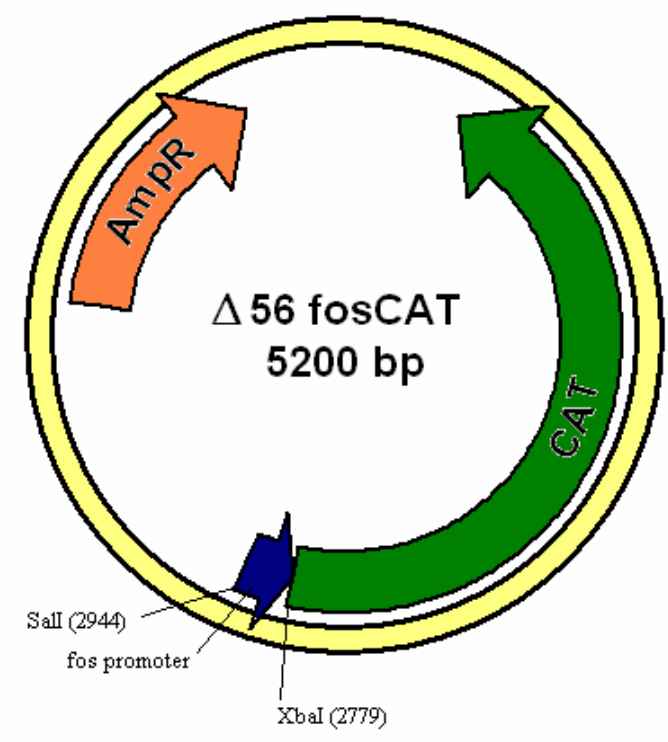


Mass Ruler
DNA Ladder
Mix

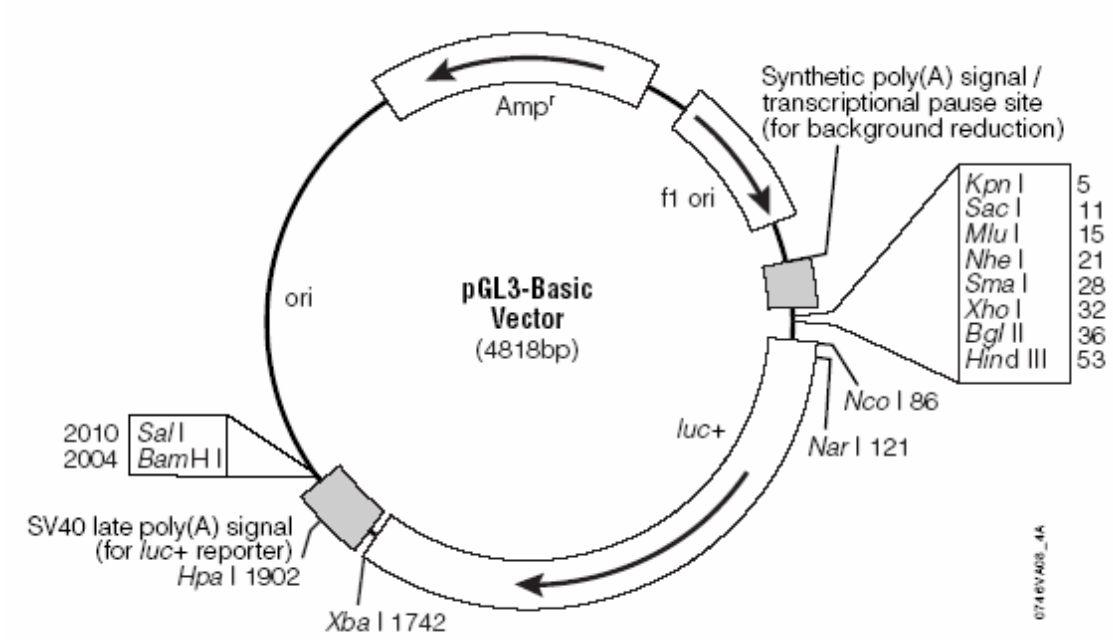


APPENDIX F

Schematic map of Δ56fosCAT vector



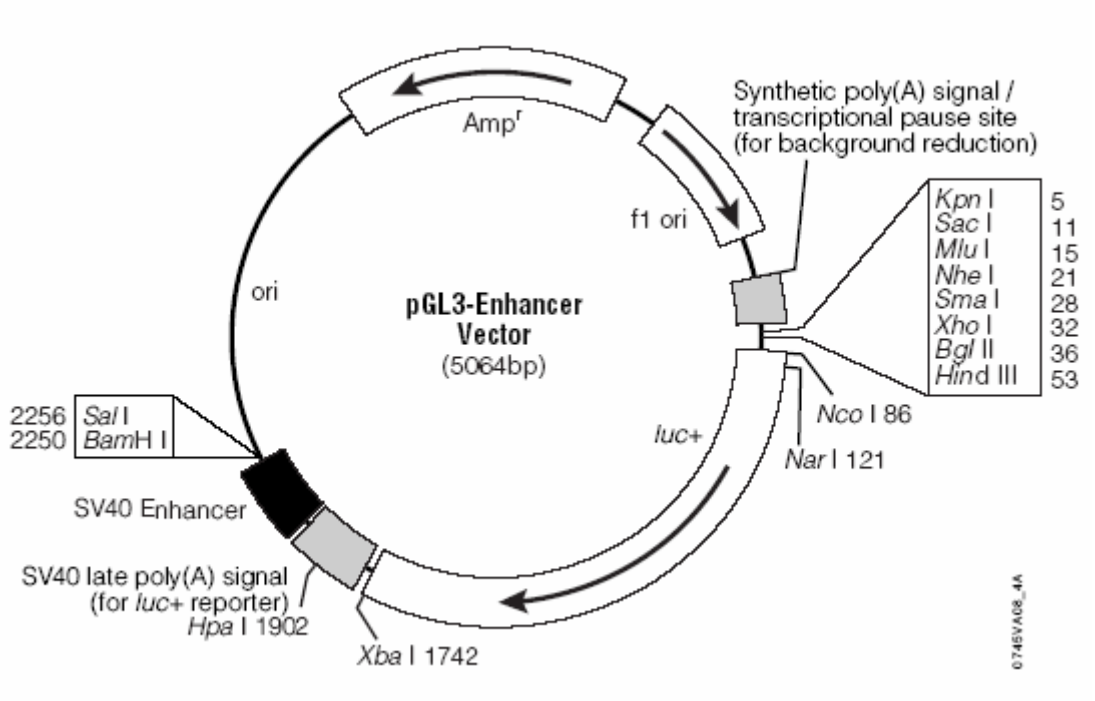
Vector map of pGL3 Basic



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 (Amp^r)	3080–3940
f1 origin	4072–4527
Synthetic poly(A) signal	4658–4811
RVprimer3 binding site	4760–4779

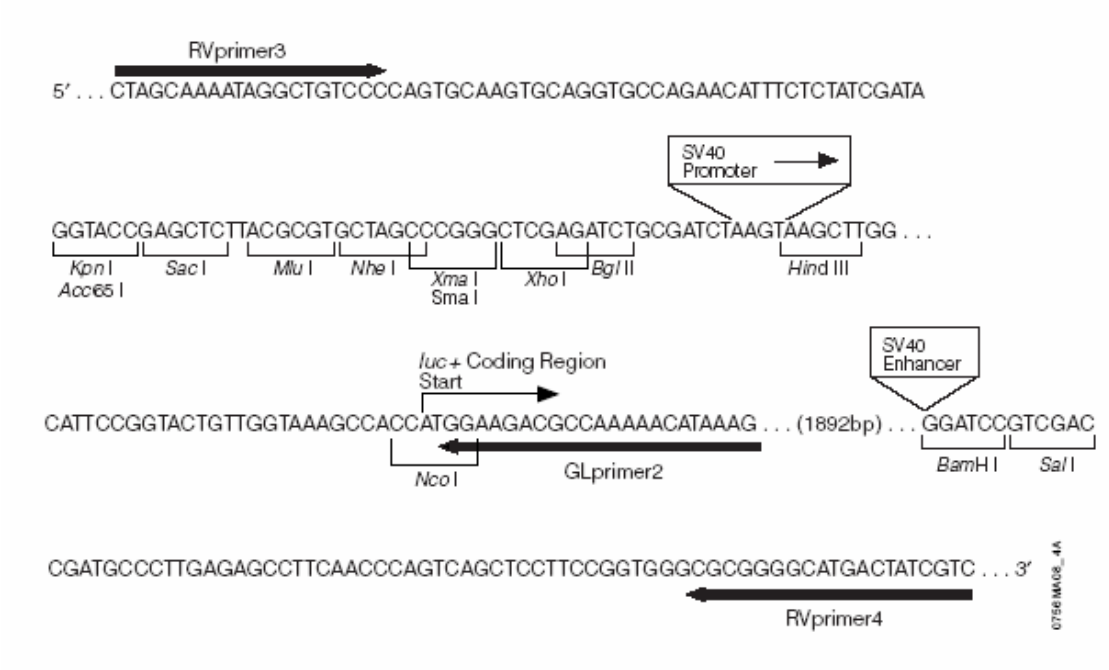
Vector map of pGL3 Enhancer



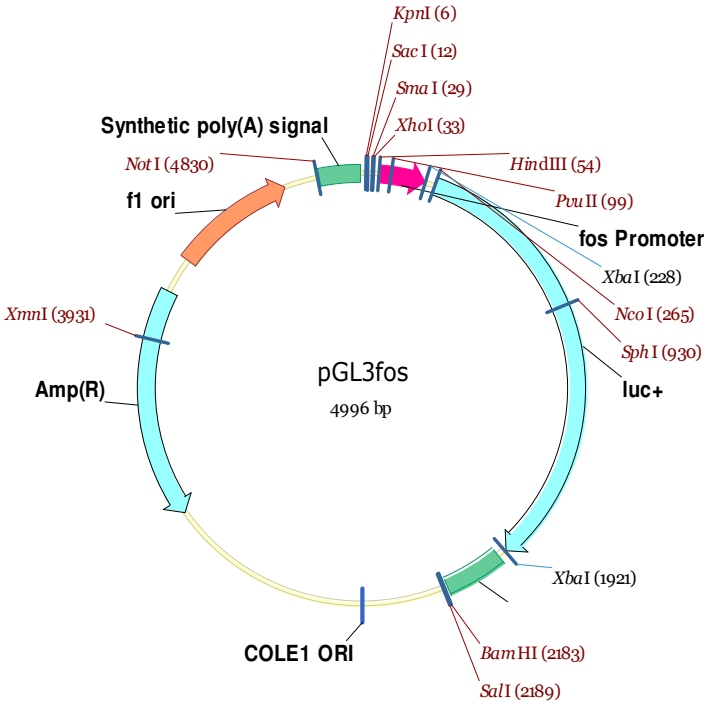
pGL3-Enhancer Vector Sequence Reference Points:

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

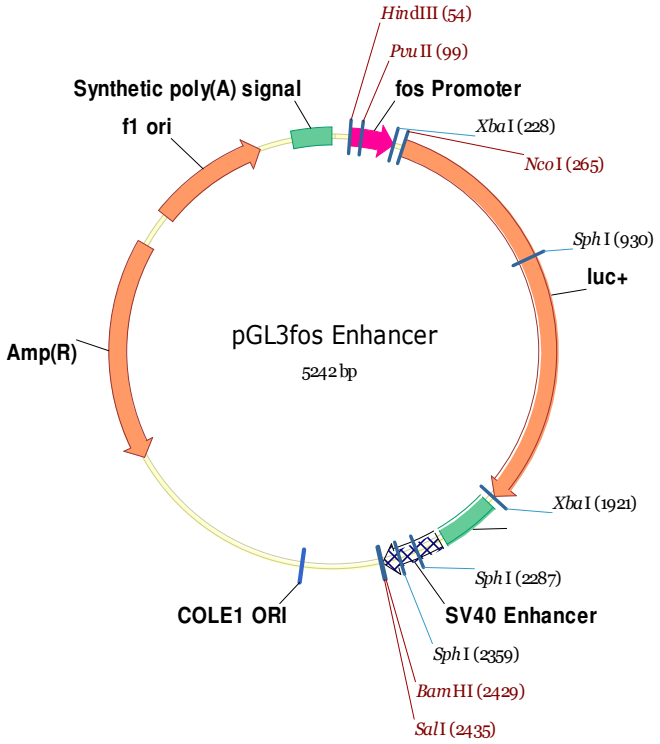
pGL3 Vectors multiple cloning regions:



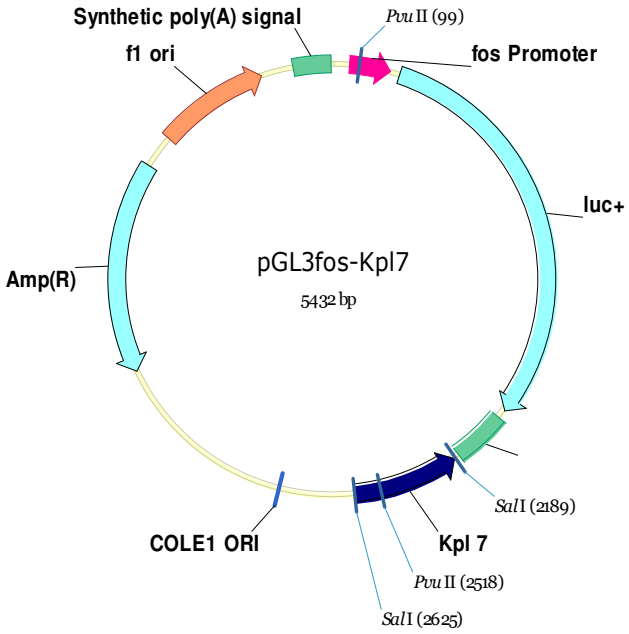
Vector map of pGL3fos



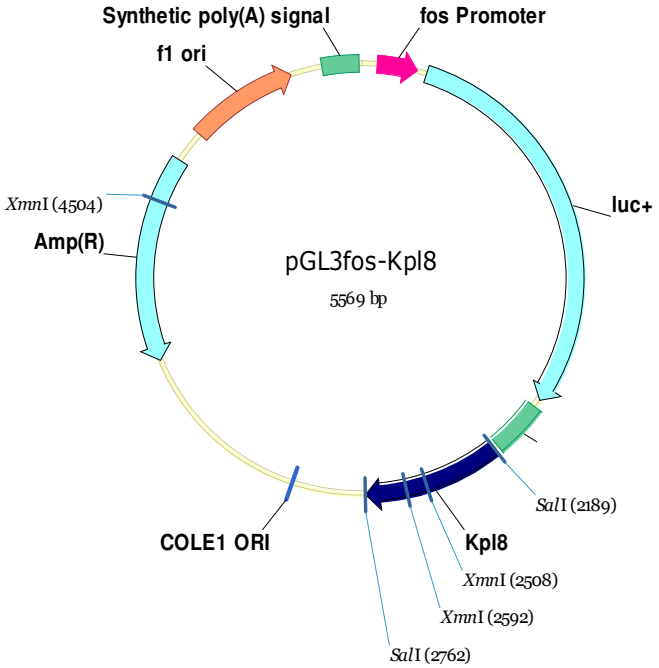
Vector map of pGL3fos Enhancer



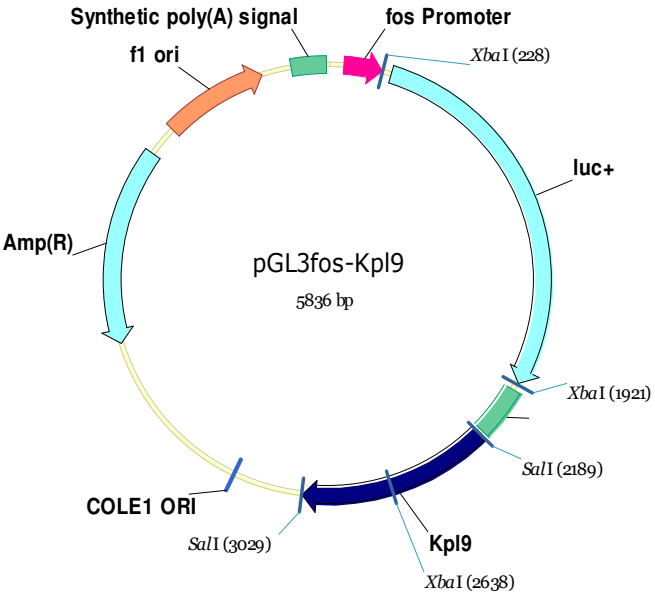
Vector map of pGL3fos Kpl 7



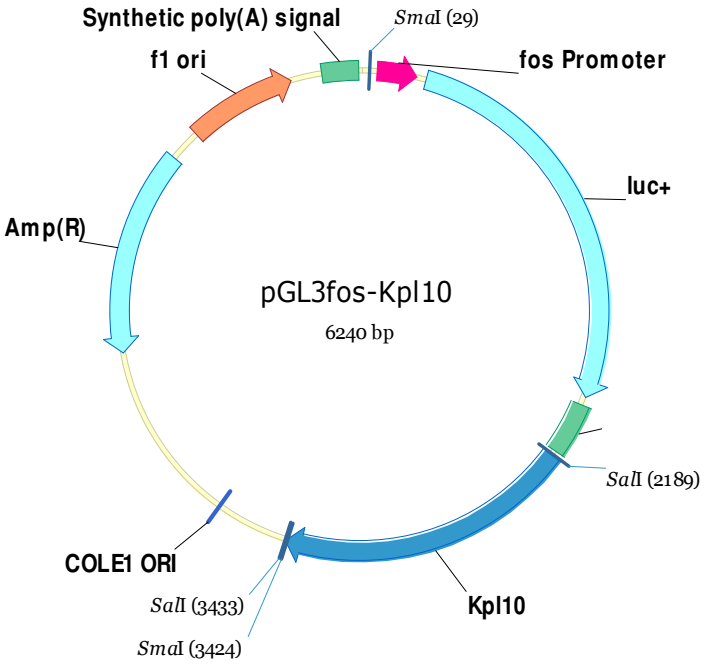
Vector map of pGL3fos Kpl 8



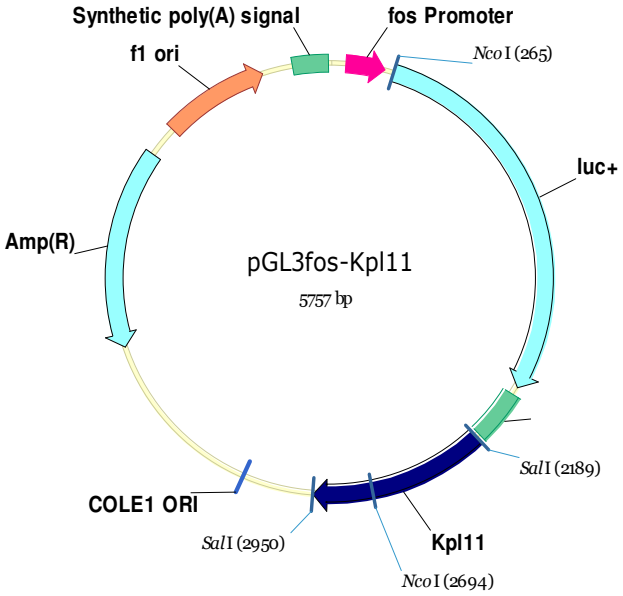
Vector map of pGL3fos Kpl 9



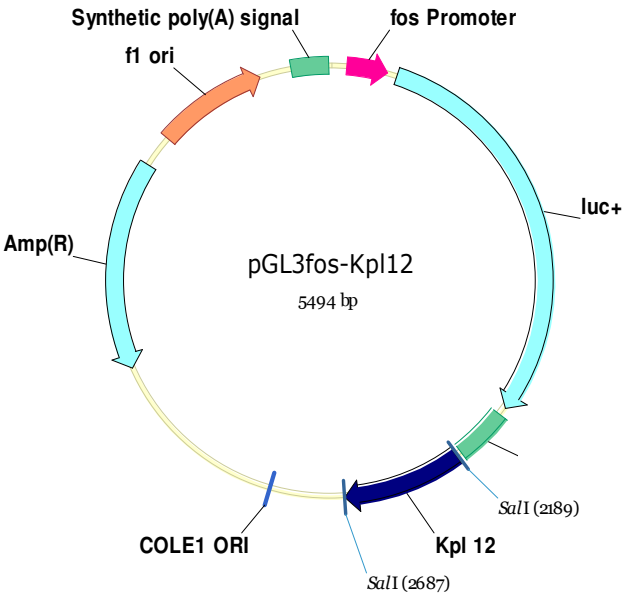
Vector map of pGL3fos Kpl 10



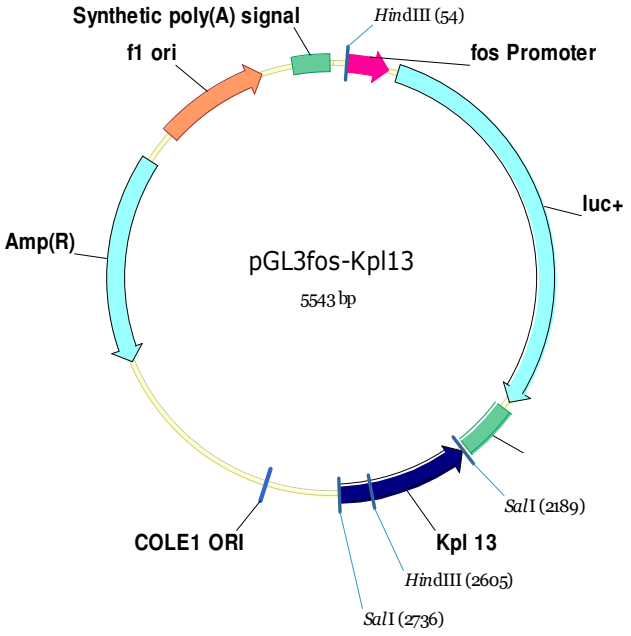
Vector map of pGL3fos Kpl 11



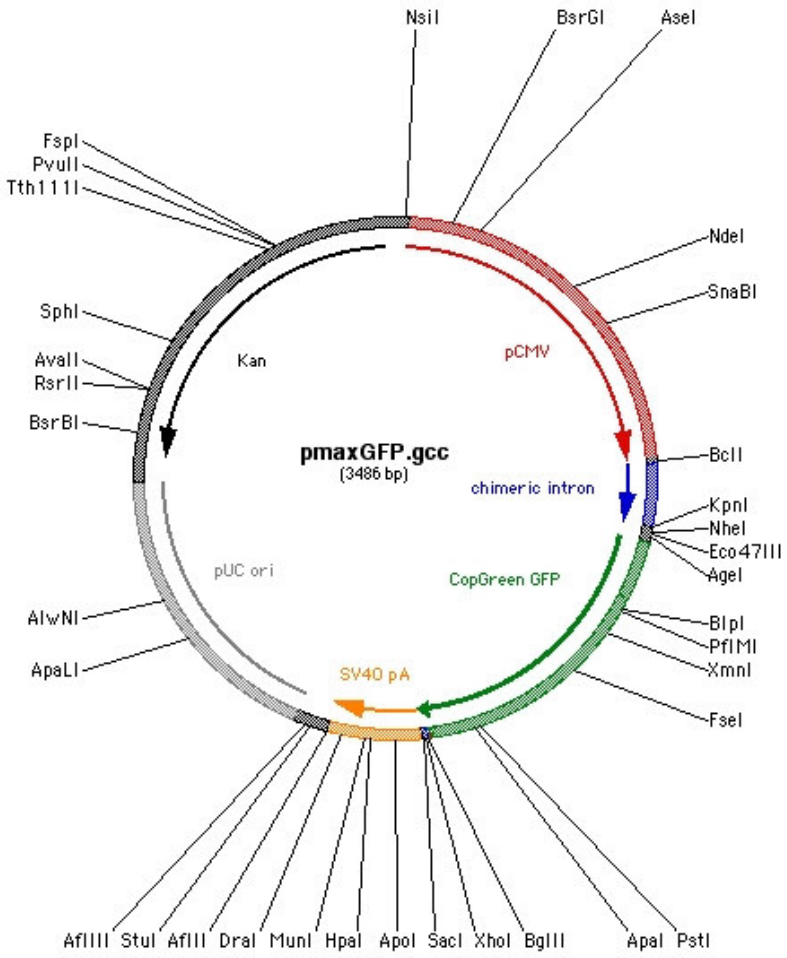
Vector map of pGL3fos Kpl 12



Vector map of pGL3fos Kpl 13



Vector map of pmaxGFP



APPENDIX G

Sequences Amplified and/or cloned DNA fragments

(in FASTA format)

> fos promoter
TCGAGGTCCATCCATTCACAGCGCTTCTATAAAGGCGCCAGCTGAGGCGCCT
ACTACTCCAACCGCGACTGCAGCGAGCAACTGAGAAGACTGGATAGAGCCG
GCGGTTCCGCGAACGAGCAGTGACCGCGCTCCCACCCAGCTCTGCTCTGCA
GCTCCCACCAGTGTCTCTAG

>Kpl 7
GGCTCCAAAGCTAACCAAAATGTCAGAGACGTTTATTCACGTCATCAAACC
TCACCCTCTCAACAAATTTAACTTTATATCCTATCTTCAAGGCATCTCAAAC
CAGCTGGTCAGCTCCCTGCACCTAAGTCAACTTAGTAGCTTTTACTTTTCCA
GAAGGCCCACTACTGCACATTTAACAGTGGATTTATGGTCATAATTCAAACC
ATGGGACTGCGTAGAAGGGTTTGAAAGTTTCCAATGGCACTGACTCACTGT
CTGCCTTACCAAGTGTGCCCTGTCAAATAAACCCTCATTGATGTCACTGCAT
TCTCATAACCCCAAAAACTAAGATTCAGAGCTGGGAAATGGTTCCACTTGC
ACCACATCTCTTAGACTGGTGCACAATGGTGTAATGGCATTGGTAATAATGT
GTGAGAGGACCACGTTC

>Kpl 8
TCGTAAACCTATCGTCTGTTGGTAGAACCTTCTTCAGTGCCTCTTCTCACCTT
TCCTCCCCCACCCATTTCGGAAGAAACACACATCCCTAGCACTACACATCTG
CCTCACACTGTGGGGCAGAAGGTGGGACAGATTACCAAAGTACATGAGATG
CATACATAATGTGAAAGTTTTATCTTCAGCCCTCAGAGTTAAGAAGTATTAT
TTCTTCCTCCTTACTGCTACATACACAGTTGAAAGGGCTTTTGTTCCAATCCT
CAAAGTTAAGAAGCATTATTTCTTCCTCCTCACTACTTACCCACATCTGAAC
ACATTCAGAGAGACACCTAAAATACACATATATCAGATCAACTAAGAAAAA
AAAATATGGCCACACTTGCATTTTGCTTTGAAGATTTTCTGATAATATCAAA
GATCATTTCCATAACCACAGTCCTAGTTTCACAGTCCAGGGAAAGTTTTTTA
ACCACTTCAGGAATATAACTAAATACAATTTTCTTGAGCTTTCTCTGTGCTA
CATTAGGCACAGACTTTAGCTAGGTTTCAGTGCAAACTATTGGGTCT

>Kpl 9
ACATTCCTCACAGGGAAGCAATACACAGACTGCACTGCCTTCTGTCCTGTGT
GTTCTTACCCACCAGGAACTGCTCCATTCTCTCTGACTAGGATTCTTTTACTC
TACACGCAACACTTCCACATTTTTTCCTTTGAAAGAAATTGCTCATGATTCC

AGCAATGGTGGTTCTGGCTACTGTTGACATGCATGAGTAATTTGTATTGTAA
TCGCTTCTTCAGTGGCTTTATAAACAAACATTTCTTGTGATCACAAAAGTAT
CTACCTAGAAAGTTGAGGGGAAAGGGTAATTGGTTTCTGTTTTCAATGTGAA
TCAGAGAAGGACTGAAAGTGGTGTTTTGGGACTGCTTGAAGAAGTGAGAGA
GGCTCTTCAAGCTGATGGGGCGCCTGTGGCAAGCATGAAATATAGATTCCA
AATTCCTTTTCAGTTGCTTCAACTCACTTCTAGAAATTCACTTTTCCAAATGA
TGAAATTTTAATTCCCAATAACTGTCACTTTAAACATGGTGGAAAATGCACT
TGGATTGCATATTTCAGCTCATTTGCAAATGTGACAAACAGGAGTGAATTAA
TATGTTGATGACCAAAAAGGAGAATAAGATTCTGAGCAACAGTGAAACAAA
AGCAATAAGAACTCAAGGGGAAAAATAAAAACAATACTAAAGTTCCAAGA
AATTGGCAATAAAAACATTTAAAACTCTCATTTTGCTGAAAGAGAGACAAA
TAAAAACTGATATAAAAAGAAAAGCTATATGTAAGAAATATTCATCTTACC
CCCCTACAAAGCACAGATGTAAATGCAGATACAGAGAATTCATTTTGCATT
CGATCAGC

>Kpl 10
CCTTCCCCCTCCAGATATAAACAGTGATGACTACCTTTATGCTTCATGACCT
ATATACTCACATCACTACTACATGTATGGTTCTTTGCTACATGTGTAACACA
TGCAAAAGGTGTTTGCTTCCCCATCAACCTGCCCATCATCTTTTCTCGTGTTC
TTCCTTTTGCTAATACATCTCTAGCTTTAGTTGGGTTCACATTTCTCTTCTTCA
TTTGAAACTAAAGTAACCATAACAACTATTTTTTCTGACCAATATGTTTCAA
GAAAAAAAATTAATCTCCAGAGAACACCTGGGTCGCAATATCCCTGCGAGA
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GAGGGATGAAGGCAGAACACAGGCTCATCAGGCCTGTGTCAGAGCACAGA
CAGAAGCAGTCTCTTAGCTGGCCTTTTCCTCAAACTACTCTGGGTGCTTTTCC
TGACTAGGGCATCACCACTGTAGTGGAAGGGATATTCATGCCTGCTTCTGAT
TCCTACCAGGGCATTATGAGCTTCCTTTCCTGAAAGCCTCTTGCTTTCTCTAA
GAACTCTTCCTCTTGTTAGCAGTTTAATCAAAATTTGAATTGAACCTGACGC
TAAAATACTCACTTGCTTATAGAAATGTCTTTAACAAGGAGTAAACCACCA
GAACTCACAGACTCTGTCTGGCATACCCAAAGATACAGTCAGAAATCTTAT
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GACTGAGTCAGCACAACTCATAAAGTGCTAAGACAATGCTGTTCAGTTGGA
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AGTTTTCACCTCACTTACCACCTGCTCTGCTAAGCAGATGTAATTATCTTCCC
AGAAGAAAGCAGGTGCCCAGGCTTCTTTCATATCTTAACTACAGAATCTACT
GACTAAGGCAGGTAGAGGGGTGCAGTTAGTACAGACCTGATGGAATGCATT
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>Kpl 11
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CCATGTCTGTCATGTGGAGGTCAGGGAGGACAGATAAGAAAAGGGAAGTTC
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CTTCCCGCACTCTATTTAGATTTCTCTCTGATGTCCTTTCAGCATCTCCTGCC
CCACCCAAAAGGGGTAAGCACACCAGTGGAAATCCCCTGAGCAAACTAGCA
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AGAATTTAAAGAGACATGATACAATTGTTCACCATGGAGTGATAGCCCATG
AAAGGCTGATGAGGTTGGGGTTAGTAGATATAATACATATGAATTTCTCCTT
AAACATTTTCACATAGCTTTGAGAATTTATCATTGTAGATTTCTAGAAAGCA
CATTCAACTTAGTGTGCAAAACAGGCTGATTTTGCGGTATGTTTTTCTGATG
AAGCTTTCAAAGCAAGATTACAAGCCAGAGTGTAAATAATTACCTGATTCC
CCAAATCATTTCTTTTGTTGCTTGGGAGA

>Kpl 12
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AGAGCAGAGAACTGGGCGTGACCATTGAGGGTTCAGCCCTAAGTGGGACAA
CTCTATCCTAGCATCATTATTCCTAAAACAAGAGCATCCCCAACCAACTGAG
GAGCATCGTAAGTGTGGATTTTGGCCTTGTGATCCTGTTACATTGGACCCTG
AGATCATCTGAAGCATCACTTGAAGTCTACCACCTTGCTCAAAAGTAAGCA
GTTAAGTTCAGGAGCTTCAGGGAATACCAAGATGCAGGAAAAAAAAAAGT
CACACTCTACCTCTCCGTTTTCTCTACCTCTGCTTACAGCAGCAATCCTTTTG
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ACTTGCACATACAAGCGTGCTTCT

>Kpl 13
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ACTCAGGGAGTTGTATCTTCTTCCTCCCCCACCAGCCATCGCTTGTTGCCAA
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CCATATAAGGAAAGTATGTCGAGGTCTGCAGTAGTAAAAGAGGACTTCTGA
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GTTTTCCTGAAGTCACTGGAGGAACAACGTCTTGGAGTTGAATGTCACAGA
ATACCCTCAGTCTGTCTTTGTTATTG