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THE STRUCTURE AND EXPRESSION OF A HUMAN Ly-6-RELATED GENE HOMOLOGOUS TO MOUSE TSA-1

by

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ABSTRACT

Mouse Ly-6 antigens belong to a family of structurally related GPI-anchored cell surface proteins encoded by tightly-linked, but independently regulated genes on chromosome 15. Functional data so far suggest roles in intercellular communication and trafficking of cells during hematopoiesis and immune responses. High sequence divergence between species has hampered homology cloning of human Ly-6 genes. The major part of this thesis reports the characterization of the first of two human Ly-6 homologues which have been discovered recently. Also reported is work on the generation of recombinant forms of Ly-6 proteins for use in Ly-6 ligand studies and in raising antibodies to the novel human Ly-6-related proteins. cDNA clone 9804, provided by collaborators at CytoMed (Cambridge Mass.), encoded a human Ly-6 sequence most like mouse TSA-1. It was used as a probe to isolate genomic fragments containing 9804 exons. Further cloning and characterization of overlapping fragments revealed that the 9804 gene consists of 4 exons, arranged like other Ly-6 genes, and spanning approximately 5 kb. Screening of human-hamster hybrid cell DNA by PCR associated the 9804 gene with chromosome 8. Fluorescence *in situ* hybridization, performed by collaborator D. Well, refined the mapping to 8q24.3, a region showing synteny with mouse chromosome 15 in the region containing Ly-6 genes. Northern blot analyses demonstrated a broad tissue distribution of 9804 expression, similar to that of TSA-1. While present in many different human tumor cell lines, 9804 mRNA was not detected in the B cell lines Raji and Daudi. 9804 mRNA levels were inducible *in vitro* by IFN-α and ATRA. The response to ATRA was restricted to myeloid leukemia cell lines and was a late event (36-48 hrs), whereas IFN-α produced a stronger and more rapid (8-12 hrs) enhancement in normal PBMC and a broad range of cell lines. The induction required protein synthesis, and was apparently at the level of transcription. Soluble recombinant 9804 and Ly-6E fusion proteins were expressed in a baculovirus system designed and used for secretion. Although not secreted, the Ly-6 fusion proteins were apparently folded correctly, and could be isolated from the insect cell extracts.

RÉSUMÉ

Les antigènes Ly-6 de la souris appartiennent à la famille des protéines de surface cellulaire liées par un groupe GPI. Celles-ci sont encodées par des gènes très rapprochés mais indépendamment régularisés sur le chromosome 15. A ce jour, les résultats confèrent un rôle aux antigènes Ly-6 dans la communication intercellulaire ainsi que dans le trafic des cellules pendant l hématopoïèse et les réponses immunitaires. Due à la haute divergence génétique entre les espèces, le clonage par homologie des gènes humains pour Ly-6 fut sans succès. La majeure partie de cette thèse rapporte la caractérisation du premier de deux homologues Ly-6 humains découverts récemment. La génération de formes recombinées des protéines Ly-6 pour fin d études portant sur Ly-6 comme ligand, ainsi que pour le développement d anticorps aux nouvelles protéines humaines reliées à Ly-6, est également présentée. Le clone 9804 d ADNc, donné par des collaborateurs à CytoMed (Cambridge Mass.), encode une séquence Ly-6 humaine semblable à TSA-1 de la souris. Cette séquence fut utilisée pour isoler des fragments genomiques contenant des exons 9804. Le clonage et la caractérisation des fragments se chevauchant ont révélé que le gène 9804 consiste de 4 exons. Ceux-ci sont organisés comme d autres gènes Ly-6 et s étalent sur une distance approximative de 5kb. L investigation de l ADN de cellules hybrides humaines-hamster par PCR a associé le gène 9804 au chromosome 8. L hybridization *in situ* par fluorescence, en collaboration avec D. Well, a défini cette région à 8q24.3, une région qui démontre un arrangement similaire au chromosome 15 de la souris contenant les gènes Ly-6. Des analyses par Northern blot démontrent l expression de 9804 dans plusieurs tissus, d une façon comparable à TSA-1. Bien qu il soit présent dans plusieurs lignées cellulaires cancéreuses humaines, l ARNm n est pas détecté dans les lignées cellulaires de type B Raji et Daudi. Les niveaux d ARNm pour 9804 sont induits *in vitro* par IFN-α et tous les *trans* retinoic acid (ATRA). La réponse à ATRA fut restreinte aux lignées cellulaires leucémiques myéloïdes et retardée (36-48). Par contre, IFN-α induit une augmentation plus rapide (8-12hrs) et plus élevée dans plusieurs lignées cellulaires incluant la PBMC normale. L induction requiert la synthèse de protéines et semble être au niveau de la transcription. Un recombinant 9804 soluble et des protéines de fusion Ly-6E ont été exprimées dans un système baculovirus désigné pour la sécrétion. Bien qu elles semblent avoir la bonne structure, les protéines de fusion Ly-6 ne furent pas sécrétées. Par contre, il a été possible de les isoler à partir d extraits cellulaires d insectes.

ACKNOWLEDGMENTS

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TABLE OF CONTENTS

ABBREVIATIONS

Ala	activated lymphocyte antigen
AML	acute myelogenous leukemia
APL	acute promyelocytic leukemia
ATP	adenosine triphosphate
ATRA	all-*trans* retinoic acid
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
°C	degrees centigrade
Ci, mCi	curie, millicurie
CNS	central nerve system
Con A	concanavalin A
CPM	counts per minute
CTL	cytotoxic T lymphocyte
3D	three dimensional
DAG	diacylglycerol
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetate
ELISA	enzyme-linked immunosorbent assay
ErBr	ethidium bromide
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
FCS	fetal calf serum
g	gram
mg	milligram
µg	microgram
ng	nanogram
GI	gastrointestinal
GPI	glycosyl phosphatidylinositol
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
HSC	hematopoietic stem cells
hrs	hours
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IL-1,2,3	interleukin 1,2,3
IEF	isoelectric focusing
IP_3	inositol 1,4,5-triphosphate
kb	kilobase
KCl	potassium chloride
kd	kilodalton
kb	kilobase
L, ml, µl	liter, milliliter, microliter
LPS	lipopolysacchride
LN	lymph node
mAb	monoclonal antibody
MALA	mouse activated lymphocyte antigen
$MgCl_2$	magnesium chloride
2-ME	2-mercaptoethanol

MHC	major histocompetibility complex
min	minutes
MLC	mixed lymphocyte culture
MLR	mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
MW	molecular weight
NaCit	sodium citrate
NaCl	sodium chloride
NaOH	sodium hydroxide
N-linked	asparagine-linked
O-linked	serine-linked
$OD_{260, 280}$	optical density at 260, 280 nanometers
PBL	peripheral blood leukocyte
PBMC	peripheral blood mononuclear leukocytes
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
pI	isoelectric point
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-bisphosphate
PI-PLC	phosphatidylinositol-specific phospholipase
PNA	peanut agglutinin
PMA	phorbol myristic acetate
RA	retinoic acid
RAR	retinoic acid receptor
RFLP	restriction fragment length polymorphism
RI	recombinant inbred
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	seconds
SSC	standard saline citrate
SSPE	standard saline phosphate
TAP	T cell activation protein
TAP_a	T cell activation protein associated
TCA	trichloroacetic acid
TFA	trifluoracetic acid
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
UV	ultraviolet
wt	wild type

LIST OF FIGURES AND TABLES

CHAPTER 1: INTRODUCTION

CHAPTER 2: STRUCTURE ANALYSIS AND MAPPING OF THE 9804 GENE

CLAIMS TO ORIGINAL RESEARCH

Because 9804 was discovered as a human Ly-6-related cDNA after the commencement of this thesis, all results, except the sequencing of the 9804 cDNA, presented in this thesis are contributions to original knowledge. In particular, I claim the following as my work:

1. Subcloned genomic fragments containing 9804 exons, introns and promoter. Performed nucleotide sequencing of the human Ly-6-related gene *9804* and described the structural organization of the gene. *

2. Mapped the *9804* gene to human chromosome 8. **

3. Analyzed the tissue distribution of the 9804 mRNA and compared with that of mouse TSA-1 mRNA.

4. First reported the second exon I for mouse TSA-1 and an irregular usage of the exon I in TSA-1 transcripts.

5. Performed and described the *in vitro* induction of the 9804 RNA expression by IFN-α and/or ATRA in normal PBMC and a range of cell lines of diverse origins. Studied the time course for these inductions and demonstrated a requirement for protein synthesis.

6. Analyzed the 5'-flanking region and promoter of the 9804 gene.

7. Expressed and analyzed the recombinant 9804 protein (as well as other Ly-6 protein) from a baculovirus expression system. Worked out the steps towards the purification of these recombinant proteins.

8. Performed cross-species hybridization of mouse Ly-6 in Southern blot analysis and found several rabbit genomic fragments hybridized with mouse Ly-6A cDNA and a guinea pig genomic fragment hybridized with mouse ThB cDNA. Screened rabbit and guinea pig genomic libraries and isolated several positive phage clones.

* Some of this work was assisted by honors project student Annie Bourdeau.

** During the course of this thesis, the chromosome mapping of RIG-E gene (identical to 9804) was reported [Mao *et al.*, 1996].

CHAPTER 1
INTRODUCTION

1.1 Serology And Tissue Distribution of Ly-6 Antigens

1.1.1 Detection of Ly-6 Antigens with Alloantisera

The murine *Ly-6* locus was discovered in late 1970s as a consequence of identifying new lymphocyte surface markers of functional subpopulations. The initial serological studies with alloantiserum, (BALB/c x A)F_1 anti-CXBD (a recombinant inbred (RI) strain formed between BALB/c and C57BL/6) lymphocytes, defined an Ly-6 specificity predominantly on peripheral T cells but absent from thymocytes and peripheral B cells of mouse strain C57BL/6 (McKenzie *et al*, 1977). The specificity was found also on allo-reactive cytotoxic T lymphocytes (CTLs) of mouse strain AKR (Woody *et al.*, 1977), but not on CTL precursors (Woody *et al.*, 1977; Horton *et al.*, 1979; Pilarski *et al.*, 1980). The Ly-6 was therefore considered to be a differentiation marker of peripheral T cells. However, antisera produced by alternate immunization protocols were also reactive with activated B cells (Horton *et al.*, 1979). Immunization of mice with mitogen (PHA, Con A, LPS)-stimulated lymphocytes recognized activated lymphocyte antigen-1 (Ala-1), which was expressed on activated lymphocytes of both T and B series, but absent from unstimulated cells (Feeney & Hämmerling, 1976). The studies led to the description of Ly-6 and Ala-1 to be lymphocyte differentiation antigens (Woody, 1977). Although initially thought to be distinct based on the different expression on peripheral B cells, Ly-6 and Ala-1 were shown by later work to be genetically inseparable (Feeney, 1978). Further serological and genetic testing (Horton *et al.*, 1978; Horton and Sachs, 1979; Potter *et al.*, 1980) also established a genetic linkage and antigenic relationships between the alloantigens Ala-1, Ly-6, DAG (Sachs *et al.*, 1973) and Ly-8 (Frelinger and Murphy, 1976).

Analysis of the strain distribution in many inbred and recombinant inbred mice with two reciprocal antisera (C3H anti-C58 and C58 anti-C3H) revealed two antithetical forms of Ala-1 (Feeney & Hämmerling, 1976). The observation implies that the antigen defined in the system has two alleles, Ly-6^a and Ly-6^b, and all strains carry one or the

other. The specificity, expressed on B6, AKR, C58, DBA and SJL-J strains, reactive with C3H anti-C58, was given the designation Ala-1.2, and that on C3H, BALB/c and A strains, detected by the reverse antiserum C58 anti-C3H, was designated Ala-1.1. Two alleles of Ly-6 locus, Ly-6^a and Ly-6^b, have been described, which control the Ala-1.1 (Ly-6.1) and Ala-1.2 (Ly-6.2) specificities, respectively.

1.1.2 Tissue Distribution Patterns of Ly-6 Molecules

With the production of monoclonal antibodies (mAbs) (Kimura *et al.*, 1980; Eckhardt *et al.*, 1980; Takei *et al.*, 1980; Auchincloss *et al.*, 1981; Kimura *et al.*, 1984; Hogarth *et al.*, 1984), it became apparent that the original alloantisera were composed of complex mixtures of antibodies. They recognized multiple Ly-6 specificities, each with a unique tissue distribution pattern in lymphoid tissues (reviewed by Kimura *et al.*, 1984; Hogarth *et al.*, 1984; Gumley, *et al*, 1995). Thus far, at least six Ly-6 specificities (Ly-6A/E, Ly-6B, Ly-6C, Ly-6G, ThB and TSA-1/Sca-1) have been defined. Genes controlling all but Ly-6B have been cloned. The seventh Ly-6 molecule, Ly-6F, has only been defined at mRNA level. Table 1.1 summarizes the tissue distribution of Ly-6 antigens and commonly used mAbs.

Ly-6A.2 and Ly-6D.2 have very similar tissue distribution profile on peripheral lymphocyte and activated T and B cells, but Ly-6D.2 is expressed on larger percentage of thymocytes (Kimura *et al.*, 1984; Hogarth *et al.*, 1984; Havran *et al.*, 1988). The reciprocal competition for binding their respective determinants by anti-Ly-6A.2 and anti-Ly-6D.2 mAbs (Kimura *et al.*, 1984; Houlden *et al.*, 1986) suggested that Ly-6A.2 and Ly-6D.2 determinants are identical. This was further confirmed by the observations that COS-7 transfectants of cloned Ly-6A.2 cDNA were shown to bind both anti-Ly-6A.2 and anti-Ly-6D.2 mAbs (Korty *et al.*, 1988). Ly-6A.2 was named T cell activating protein (TAP) as anti-TAP mAbs were able to activate T cells (Rock *et al.*, 1986; Yeh *et al.*, 1986). Ly-6A.2 is also expressed on hematopoietic stem cells and thus was named as stem cell antigen-1 (Sca-1) (Aihara *et al.*, 1986; Spangrude *et al.*, 1988c). The expression of Ly-6E antigen is associated with Ly-6^a strains and primarily detected on lymphoblasts. The basal level expression of Ly-6E.1 on resting lymphocytes is much lower (10-15%)

Table 1.1 Tissue Specificity of Ly-6 Antigens

Antigen	Synonym	Allo-mAb	Xeno-mAb	Tissue Distribution	Reference
Ly-6A.2	Ly-6.2 Sca-1 TAP TAPa Ala-1	S 8.106 5041-1.1 5041-24.2 5041-25.4	E13 161-7 (rat) YE3/19.1 (rat) D7 (rat) MTS23 (hamster)	50-70% peripheral lymphocytes all T & B blasts, HSC DN & a subset of SP endothelia, renal epithelia testicular Leydig & Sertoli cells	2, 144, 201, 204 275, 279
Ly-6B.2	Gm-2.2	SK 38.86 5119-4/7		50-70% BM cells; neutrophils; <5% peripheral lymphocytes	109, 145
Ly-6C.2		SK 140-112 SK 142.446 5075-3.6 5075-12.1 5075-1.11 5095-16.6	H9/25 (rat) HK1.4 (rat) 6C3 (rat) B4B2 (rat) 1G7.G10 (rat)	50% BM cells; 40% $CD8^+$ about 27% $CD8^-$	45,104 63,106 109, 161, 204, 276
Ly-6D.2		SK 142.476 5075-2.1 5075-19.1		like Ly-6A.2; plus 50-80% thymocytes	109, 145
Ly-6E.1	Ly-6.1 Ala-1	SK 70.94 SK 70.36	YE3/19-1 (rat) D7 (rat)	10-15% peripheral lymphocytes all T & B blasts	145, 202
ThB			53-9.2 (rat) 49-h4 (rat) 49-g7 (rat) 49-h5 (rat)	thymocytes; B cells	67, 159, 181
Sca-2	TSA-1		MTS 35 (rat) E3 81-2 (rat) PRST1(hamster)	75% BM leukocytes & mature B a subset of thymic medullary epithelial cells; thymic dendritic cells; immature thymocytes	2, 149, 290
Ly-6F				cDNA only. mRNA in testes	76
Ly-6G	Gr-1		RB6-8C5 (rat) 1A8 (rat)	granulocytic BM cells	76

than that of Ly-6A.2 (50-70%). Despite the apparent difference in the tissue distribution patterns, Ly-6E.1 and Ly-6A.2 have been determined to be allelic products by immunoprecipitation, Western blotting and competition studies (Takei, 1984; Palfree *et al.*, 1986a), and later confirmed by data from gene cloning (Palfree *et al.*, 1987; Reiser *et al.*, 1988). Ly-6B.2 (Gm2.2) is found almost exclusively on bone marrow cells and neutrophils (Takei *et al.*, 1980; Kimura *et al.*, 1984; Hibbs *et al.*, 1984). Although Ly-6C.2 and Ly-6B.2 have an overlapping profile of expression on bone marrow cells and neutrophils, the former is detected on 40% of $CD8^+$ and 27% $CD8^-$ T cells (Takei *et al.*, 1980; Auchincloss *et al.*, 1981; Kimura *et al.*, 1984) and the later is detected only on less than 5% of the peripheral lymphocytes. Ly-6C is also a marker of memory $CD8^-$ T cells (Walunas *et al.*, 1995). In a similar fashion to Ly-6A/E, the expression of Ly-6C on T cells is induced by mitogens. Ly-6F and Ly-6G are two recently identified members of the family (Fleming *et al.*, 1993a). Ly-6G antigen (Gr-1) was found on the granulocytic bone marrow cells (Fleming *et al.*, 1993b), but Ly-6F was only found in nonlymphoid tissues and has been studied so far at the level of mRNA. ThB is so named because it was originally detected on thymocytes and B cells (Yotogu *et al.*, 1974, 1976; Eckhardt and Hersenberg, 1980). Like its human homologue E48, ThB was also found recently on skin keratinocytes at a high level (Brakenhoff *et al.*, 1995). Sca-2 is another stem cell antigen, but it is expressed on a subset of lymphoid committed stem cells (Classon *et al.*, 1994). It is expressed on immature thymocytes and thymic medullary epithelial cells and thus was named as thymic shared antigen-1 (TSA-1) (MacNeil *et al.*, 1993; Kosugi *et al.*, 1994). In the peripheral lymphoid tissues, Sca-2/TSA-1 expression is basically found on B lymphocytes. Although freshly isolated T cells do not express detectable Sca-2/TSA-1 on their cell surface, Sca-2/TSA-1 expression, like that of Ly-6A/E, is markedly increased upon *in vitro* stimulation of the resting T cells with ConA (Kosugi *et al.*, 1994).

Although first described in lymphocytes, Ly-6 antigens have been detected in non-lymphoid cells and non-hematopoietic tissues as well. Ly-6A has been detected in many non-lymphoid tissues such as on kidney tubular epithelium and vascular endothelium (Blake *et al.*, 1993), osteoblasts (Horowitz *et al.*, 1994), testicular Leydig and Seritoli cells (Tokuda *et al.*, 1990). Ly-6C is expressed on the endothelium of small-

sized vessels of brain, heart, kidney, lung, spleen and lymph nodes (Jutila *et al.*, 1988; Hännien *et al.*, 1997). Sca-2/TSA-1 mRNA is detected in non-lymphoid tissues including testis, liver, heart and brain tissues. ThB, as described above, is expressed very abundantly on the keratinocytes (Brakenhoff, 1993). Moreover, Ly-6F has been detected exclusively in non-lymphoid tissues (Fleming *et al.*, 1993a). Most of the Ly-6 molecules are therefore not lymphoid restricted.

In addition to mitogen-induced expression of Ly-6 molecules, expression of several Ly-6 antigens on mature lymphocytes and in several other tissues is dramatically increased by interferons (IFNs). Ly-6A/E expression on T cells, bone marrow or thymic stromal cells, kidney tubular and endothelial cells, osteoblasts and cells in central nervous system is enhanced by IFN-α/β or IFN-γ (Dumont *et al.*, 1986a, Dumont *et al.*, 1986b; Dumont *et al.*, 1988; Khan *et al.*, 1993; Izon *et al.*, 1996; Blake *et al.*, 1993; Horowitz *et al.*, 1994; Cray *et al.*, 1990). Ly-6C expression on T cells is induced by IFN-α/β but not IFN-γ (Dumont *et al.*, 1986a, Dumont *et al.*, 1986b; LeClair *et al.*, 1989; Malek *et al.*, 1989; Jutila *et al.*, 1988).

1.1.3 Ly-6 Expression during Hematopoiesis

Hematopoiesis is a very complex process which involves the commitment of stem cells to precursors for different lineages and generation of mature blood cells and immune competent cells. Regulation of the process is largely controlled by the signals generated by local microenvironments and direct cell-cell interaction. These signals direct the proliferation, death and differentiation of hematopoietic cells. The decisive role of cytokines produced by stromal cells and the participation of direct cell-cell interactions in hematopoiesis can be inferred from numerous studies (reviewed by Lowry, 1995; Ritter and Boyd, 1993; Anderson *et al.*, 1996; Patel and Haynes, 1993; Paulson and Bernstein, 1995). The involvement of cytokines in hematopoiesis is most well studied, although a critical role of cell surface adhesion and costimulatory molecules in the later thymopoiesis is evident (Carlow *et al.*, 1992; Punt *et al.*, 1994; Foy *et al.*, 1995; Hardy and Megason, 1996).

Members of the Ly-6 family are individually regulated giving rise to a distinct pattern of expression during hematopoiesis (Fig. 1.1). Bone marrow and fetal liver hematopoietic stem cells (HSC) represent a small population (0.05-0.3%) with phenotype of Thy-1^{lo}CD8$^-$B220$^-$Mac-1$^-$Gr-1$^-$ (Thy-1^{lo}Lin$^-$) (Miller *et al.*, 1985; Berman and Basch, 1985; Muller-Sieburg *et al.*, 1986; Spangrude *et al.*, 1988a). Approximately 20-30% of the Thy-1^{lo}Lin$^-$ cells are Sca-1/Ly-6A$^+$. This subset contains highly enriched stem cell activity with the potential of self-renewing and populating all erythroid, myeloid and lymphoid lineages (Spangrude *et al.*, 1988b). The Thy-1^{lo}Ly-6A$^-$ bone marrow population, which expresses erythroid lineage markers, is enriched for erythroid precursors (Spangrude *et al.*, 1988b). Conversely, Ly-6A expression is maintained during the sequence of myeloid lineage commitment. However, as myeloid cells become mature, they gain Ly-6G, Ly-6B and Ly-6C expression and lose Ly-6A expression (Fleming *et al.*, 1993b; Julita *et al.*, 1988; McCormack *et al.*, 1993; Potter and McKenzie, 1984; Dumont *et al.*, 1985). Ly-6A expression is likely to persist during lymphoid commitment of the PSC, since lymphoid progenitors are Ly-6A$^-$. Another stem cell antigen, Sca-2/TSA-1, is expressed at a later stage of differentiation. It is expressed by Thy-1^{lo}Lin$^-$Ly-6A$^+$ cells but not by Thy-1^{lo}Lin$^-$Ly-6A$^+$ cells, and most Sca-2$^+$ cells also express the B cell marker B220 (Spangrude *et al.*, 1988a). The Sca-2$^+$ precursors lose the potential of populating erythroid lineage (Spangrude *et al.*, 1989; Wu *et al.*, 1991). Ly-6A, Sca-2/TSA-1 and ThB expression is maintained during B cell ontogeny. T cell differentiation in the thymus (Fig. 1.2) is a complex cascade of events that involves cellular proliferation, selection and deletion (Boyd and Hugo, 1991; Nikolic-Zugic, 1991). The stages are marked by changes in expression of thymocyte surface antigens, such as the most extensively studied CD3, CD4 and CD8. Ly-6 also undergoes a strictly controlled expression process. Expression of Ly-6A is down regulated at CD4$^-$CD8$^-$ double negative (DN) stage and eventually extinguished at transition of CD4$^+$CD8$^+$ double positive (DP) stage. It is re-expressed on most mature T cells in the thymus (Yeh *et al.*, 1986; Spangrude *et al.*, 1988c). Sca-2/TSA-1 is expressed on immature thymocytes (Godfrey *et al.*, 1992). Its expression on thymocytes is dramatically down regulated with the expression of CD3 molecule and disappears on post-transitional

Figure 1.1 Schematic Diagram of The Expression of Ly-6 Antigens During Hematopoiesis

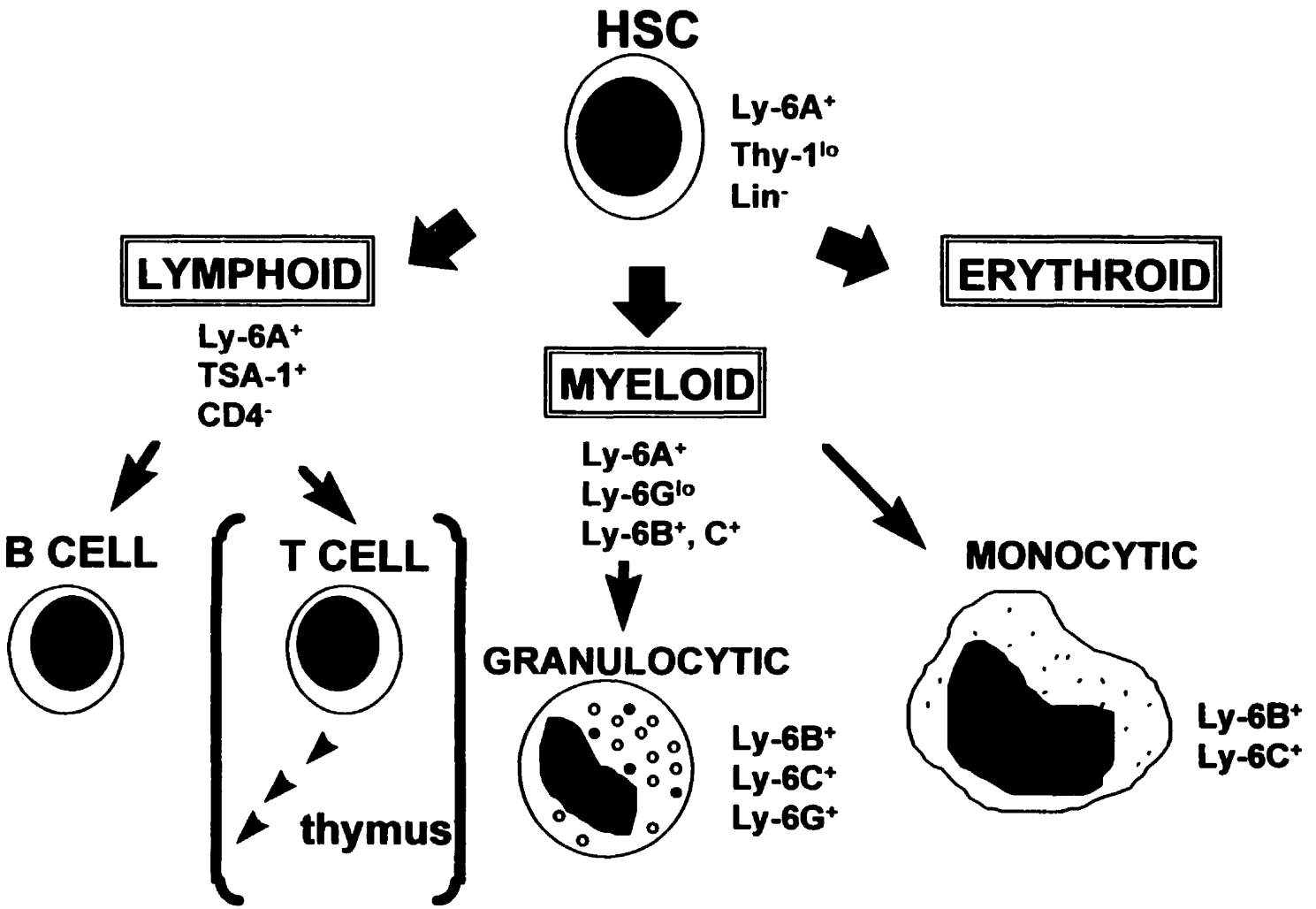
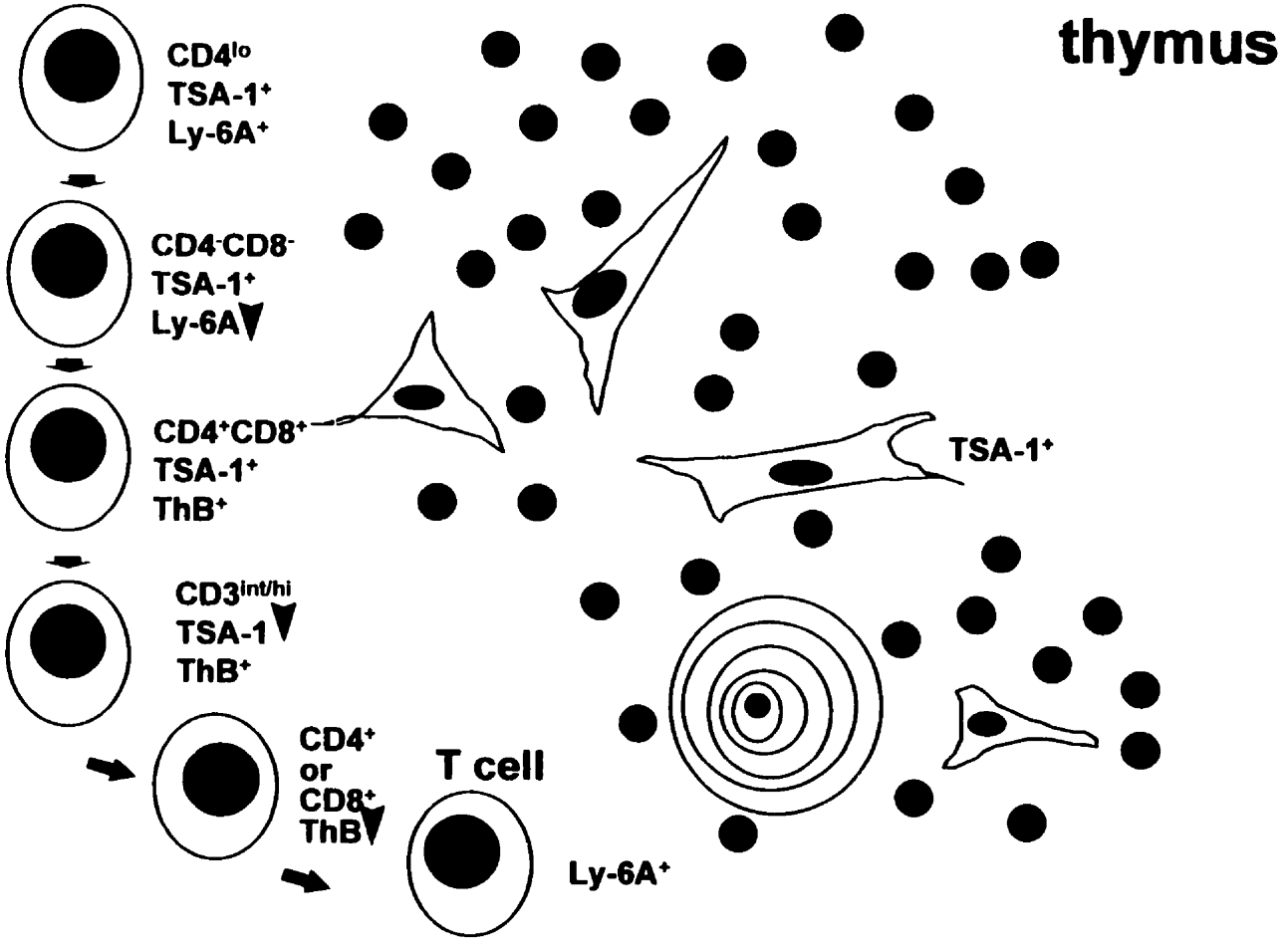

HSC
Ly-6A+
Thy-1lo
Lin-
LYMPHOID
Ly-6A+
TSA-1+
CD4-
MYELOID
Ly-6A+
Ly-6Glo
Ly-6B+, C+
ERYTHROID
B CELL
T CELL
thymus
GRANULOCYTIC
Ly-6B+
Ly-6C+
Ly-6G+
MONOCYTIC
Ly-6B+
Ly-6C+

Figure 1.2 Schematic Diagram of The Expression of Ly-6 Antigens during Thymic Development


thymus
CD4lo
TSA-1+
Ly-6A+
CD4-CD8-
TSA-1+
Ly-6A
CD4+CD8+
TSA-1+
ThB+
CD3int/hi
TSA-1
ThB+
CD4+
or
CD8+
ThB
T cell
Ly-6A+
TSA-1+

$CD3^{int/hi}$ $CD4^+CD8^+$ thymocytes (Randle *et al.*, 1993). ThB expression is turned on at DP stage and down regulated later as thymocytes become single positive (SP) (Stout *et al.*, 1975; Eckhardt *et al.*, 1980). Unlike that of Sca-2/TSA-1, the level of ThB expression is unrelated to the changes in CD3 expression.

The interesting tissue distribution pattern of each independently regulated Ly-6 antigen and the induction of Ly-6 surface expression by IFN or through cell activation suggest that Ly-6 molecules perform functions at specific times during hematopoiesis and immune responses. A requirement for strict regulation of Ly-6 expression during thymic development is implied by the results from an Ly-6A transgenic mouse recently reported by Bamezai *et al.* (1995). In the transgenic mice, Ly-6A.2 expression, which is normally turned off at the early stage of thymocyte development, was maintained at high level under the control of a CD2 enhancer. This overexpression of Ly-6A resulted in the arrest of thymocyte development at the $CD4^-CD8^-$ double negative (DN) stage. The requirement of proper surface expression of Sca-2/TSA-1 on thymocytes for the thymic development was also suggested by a study with fetal thymus organ culture (FTOC) (Randle *et al.*, 1993). Addition of anti-TSA-1 mAb MTS35 down-regulated TSA-1 expression, which dramatically reduced total thymocyte yield and blocked thymocyte differentiation beyond $CD3^-CD4^-CD8^-$ triple negative (TN) stage. The effects of anti-TSA-1 in FTOC were further studied by a reconstitution assay with immature T cells (McNeil *et al.*, 1993) at two defined differentiation stages, $CD45^+CD25^+$TN and $CD45^-CD25^+$TN, representing the last stage of T cell development before rearranging TCR β chain genes and direct precursors of $CD4^+CD8^+$, respectively (Godfrey *et al.*, 1993). No significant difference was observed in $CD45^+CD25^+$TN reconstitution FTOC between anti-TSA-1 or isotype control mAb. However, the presence of anti-TSA-1 mAb in the $CD45^+CD25^+$TN resulted in a skewed development toward the $\alpha\beta TCR^+CD4^-CD8^+$ phenotype. The data suggested that TSA-1 may play a role during positive selection and/or the decision of DP thymocytes to commit to CD4 or CD8 T cells. Since TSA-1 is expressed on both thymocytes and medullary epithelial cells, it is not known whether this inhibitory effect could be the result of reduced expression of TSA-1 on the thymocytes or

the antibody crosslinking between thymocytes and thymic stromal cells, delaying their migration to other microenvironments and subsequent differentiation.

As Ly-6A/E is expressed on DN thymocytes at a significantly high level, it is possible that Ly-6A/E expression plays critical role in the development of thymocytes from DN to mature T cells. Immature DN thymocyte have no surface TCR, they express low level of CD3 components (Zuniga *et al.*, 1993). As rearrangement of the TCR β chain occurs in the DN thymocyte, it could form pre-TCR with a partner chain, termed preTα (Groettrup *et al.*, 1993; Saint-Ruf *et al.*, 1994). The identification of these structures showed a possible signaling through TCR/CD3 components during early thymocyte development. ζ chain is necessary for targeting the TCR complex to the cell surface, nevertheless, in $\zeta^{-/-}$ mice a readily detectable number of the SP can be seen (Love *et al.*, 1993). A TCR-independent pathway is thus possible signaling through molecules such as Ly-6A which is expressed significantly on the DN cells (Shores and Love, 1996). However, Ly-6A knock-out mouse will be required to address the potential role of Ly-6A in the T cell development.

1.2 *Ly-6* Multigene Family And Gene Structure

Early recognition of the complexity of Ly-6 serological specificities raised the speculation as to whether the *Ly-6* locus contained multiple genes or whether the protein product of a single gene underwent variable post-transcriptional processing. Genetic evidence from backcross breeding experiments suggested a single *Ly-6* gene controlling all distinctive Ly-6 specificities (Feeney *et al*, 1978). But one recombination event was observed in the NZB mouse strains (Kimura *et al*, 1984). Cells from NZB mice were shown to be Ly-6A.2$^-$, Ly-6B.2$^+$, Ly-6C.2$^-$, Ly-6D.2$^-$, Ly-6E.1$^+$ suggesting that at least Ly-6B is encoded by a separate gene. In any case, the recombination frequency indicated that if many distinct *Ly-6* genes existed, they had to be extremely tightly linked. Southern blot analysis of mouse genomic DNA with Ly-6E.1 cDNA detected multiple fragments, suggesting that at least 10 distinct *Ly-6* genes or pseudogenes hybridize with Ly-6E.1 (LeClair *et al.*, 1986).

1.2.1 Cloning Studies of Ly-6 Genes

Definitive evidence for the existence of an *Ly-6* multigene family came from molecular biology studies and cloning of *Ly-6* genes. The Ly-6E.1 cDNA clone was first isolated from a MethA cDNA library using synthetic oligonucleotide probe designed from amino acid sequence data (LeClair *et al.*, 1986). A similar strategy was used to isolate Ly-6A.2 (Palfree *et al.*, 1987), Ly-6C.2 (Palfree *et al.*, 1988) and TAP/Ly-6A.2 (Reiser *et al.*, 1988) cDNA clones. Cos-7 expression systems were used to isolate cDNA clones encoding ThB and TSA-1 molecules from cDNA libraries (Gumley *et al.*, 1992; MacNeil *et al.*, 1993). Chromosomal genes of Ly-6C.1 (Bothwell *et al.*, 1988), TAP/Ly-6A.2 (McGrew and Rock, 1991), Ly-6E.1 (Stanford *et al.*, 1992; Sinclair and Dzierzak, 1993), Chromosomal genes of *ThB* (Gumley *et al.*, 1995) and *TSA-1* (Classon and Coverdale, 1996) have been cloned through use of the corresponding cDNA probes. Two other members of the family, Ly-6F and Ly-6G, have been cloned by cross-hybridizing with Ly-6A.2 cDNA probe (Fleming *et al.*, 1993a). The DNA sequencing showed distinct genes with high similarity. Among the genes, *Ly-6A/E, Ly-6C, Ly-6F* and *Ly-6G* are similar enough in nucleic acid sequence that they are certainly all among the multiple bands which were first detected in the Southern blot analysis of mouse genomic DNA with Ly-6E.1 cDNA probe (LeClair *et al.*, 1986).

1.2.2 Chromosome Localization of Ly-6 Genes

The *Ly-6* gene complex was initially assigned to chromosome 9 (Horton and Hetherington, 1980) and later to chromosome 2 (Meruelo *et al.*, 1982) by linkage relationships to known markers of the two chromosomes. The localiztion of *Ly-6* locus to chromosome 15 was definitively established by three studies: one base on antigen expression by the segregation of *Ly-6* alleles with *Xp-1-4, Gdc-1, Gpt-1* alleles in NXSM RI strains and Gdc-1 and Gpt-1 congeneic strains (Hogarth *et al.*, 1987), the others by *in situ* hybridization and RFLP analysis (Meruelo *et al.*, 1987; LeClair *et al.*, 1987). Using interspecies crosses, *Thb* (Gumley *et al.*, 1992) and *TSA-1* (MacNeil *et al*, 1993) genes have been mapped to chromosome 15E very close to *Ly-6* and linked to *Myc, Tgn, Ly-6*

and *Sis*. One *TSA-1*-related pseudogene (*Tsa1-rsl*) is located in the middle region of chromosome 12, which is not linked to *Ly-6* and *Tsa*-1 (MacNeil *et al*, 1993). Thus the chromosome mapping studies have placed all members of the *Ly-6* multigene family in an extremely tightly linked cluster on chromosome 15. Chromosome mapping of man-mouse linkage and synteny homologies (Nadeau and Reiner, 1988; Huppi *et al.*, 1988) revealed three major linkage groups mapped to mouse chromosome 15. The order of the three groups from most proximal to most distal in the mouse are *Gpt-1, Myc-1, Tgn* (human chromosome 8), *As-2, Dia-1, Sis* (human chromosome 22q), and *Gdc-1, Ela-1, Int-1* (human chromosome 12) which suggested the human homologue of *Ly-6* gene would lie on chromosome 22q or 8 depending on the site of the breakpoint.

1.2.3 Physical Map of Ly-6 Genes

To elucidated the general organization of the *Ly-6* multigene family, a detailed physical map of the *Ly-6* complex spanning about 1600 kb in C57BL/6 murine genome has been generated (Kamiura *et al.*, 1992). This segment of DNA contains at least 18 distinct *Ly-6*-related genes that hybridize with Ly-6-E.1 cDNA and are located on a 630-kb Sac II fragment. A total of 61 clones containing Ly-6-related sequences were isolated from a cosmid genomic library. The cosmids could be arranged into three clusters (A, B and C) and a solitary one c63, which contain 23 Ly-6-related *Hind* III fragments in C57BL/6 genomic DNA. Using specific oligonucleotide probes, *Ly-6A/E* and *Ly-6C* genes have been assigned to the clusters A and B, respectively. *Ly-6B* was not assigned to the map because of the absence of the cDNA sequence data. But it might be located on the cluster C as the fact that the *Hind* III fragment H21 in the cluster C exists in the RFLP on the NZB mouse DNA as it does on C57BL/6 (Ly-6^b) genome. *ThB* and *TSA-1* sequences appear not to be included in the cosmid clones, since they do not cross-hybridize with other known Ly-6 cDNAs (Matossian-Rogers *et al.*, 1982; MacNeil *et al.*, 1993). However, the mapping data of their being tightly linked to the *Ly-6* locus suggested that the *ThB* and *TSA-1* genes might reside elsewhere within the 1.600-kb DNA segment around the *Ly-6* complex (Erkhardt and Herzenberg, 1980; MacNeil *et al.*, 1993). By comparison of the restriction maps for Ly-6F.1 and Ly-6G.1 to the physic map

of the Ly-6 complex, it was suggested that Ly-6F.1 might correspond to fragments H22 and H12 in cluster C and Ly-6G.1 to fragments H18 and H19 in cluster B of the *Ly-6* locus (Fleming *et al.*, 1993a).

1.2.4 Gene Structure of Ly-6 Family

The *Ly-6* genes are organized into four exon structure with very similar usage of the splicing sites (Bothwell *et al.*, 1988). The first exon, together with a short 5' segment of exon 2, encodes 5' untranslated sequence (ut). The remainder 3' segment of exon 2 and first 8 nucleotides of exon 3 encode the leader sequence. The mature Ly-6 peptide is encoded by 3' segment of exon 3 and 5' part of exon 4. The rest of the exon 4 is responsible for C-terminal signal sequence for GPI attachment and 3'UT. While the sizes of the coding exons are highly conserved in *Ly-6A/E, Ly-6C, Ly-6F and Ly-6G*, the length of the introns varies. *Ly-6A.2* and *Ly-6E.1* are alternate alleles (Palfree *et al.*, 1986b) which differ only by three nucleotides in the open reading frame at positions 223, 362 and 324 and a deletion of nucleotide 595 in the 3' UT region of *Ly-6E.1* (LeClair *et al.*, 1986; Reiser *et al.*, 1988; Palfree *et al.*, 1987). *Ly-6C.1* and *Ly-6C.2* are also highly homologous alleles with only two nucleotide differences in position 271 and 393 in the open reading frame (Palfree *et al.*, 1988; Bothwell *et al.*, 1988). There is no allelic polymorphism found in *Ly-6G* and *Ly-6F*. The genes for Ly-6A/E, Ly-6C, Ly-6-F, and Ly-6G share high similarity. *Ly-6A/E* and *Ly-6C* have approximately 78% nucleotide sequence identity. Exon 2 has the highest (95%) and exon 3 the lowest (75%) sequence similarity between the two genes (McGrew and Rock, 1991). The overall nucleotide sequence homology of Ly-6F.1 and Ly-6G.1 to Ly-6E.1 are 84% and 82%, respectively, with >75% homology for non-gapped 5' exon I and for portions of the intron sequences (Fleming *et al.*, 1993a). The genomic organization of *ThB* and *TSA-1* is strikingly similar to that of other *Ly-6* genes, but the nucleotide sequence similarity with Ly-6A/E, Ly-6C, Ly-6F and Ly-6G is lower (Gumley *et al.*, 1995; Classon *et al.*, 1996). This explains the lack of cross-hybridization between *ThB* or *TSA-1* and other *Ly-6* genes (Gumley *et al.*, 1992; Classon *et al.*, 1994). The gene structure and sequence analyses indicated that these genes arose as a consequence of at least two periods of gene duplication, with early

divergence of *ThB* and *TSA-1* from the progenitor of the other members of the family which diverged following more recent duplications.

1.3 Biochemistry and Protein Structure of Ly-6

1.3.1 Molecular Weight of Ly-6 Antigens

In initial studies, it was found to be extremely difficult to radio-label or immunoprecipitate Ly-6 antigens. Thus a great disparity in molecular weight has been reported. A 78 kDa surface protein was precipitated by using Ly-6.2 (Ly-6A.2 using the nomenclature of Kimura *et al.*, 1984) alloantiserum in MLC blasts (Andersson *et al.*, 1981). Subsequent studies using mAb SK8.106 have challenged the report. The Ly-6.2 antigen was immunoprecipitated from murine tumor cell lines as a 34 kDa protein on the sodium dodecyl sulfate polyacrylamide electrophoresis (Matossian-Rogers *et al.*, 1982). The results were corroborated by using another Ly-6A.2 mAb, 5041-24.2 (Sutton *et al.*, 1985; Houlden *et al.*, 1986). However, a 56 kDa polypeptide was also reported to be detected in some immune precipitates and in an *in vitro* translation system containing poly(A)$^+$ RNA from the Ly-6.2$^+$ T cell lymphoma EL-4 (Sutton *et al.*, 1985).

From an early report using mAb H9/25 (Takei and Horton, 1981), Ly-6C.2 antigen was characterized biochemically as a 12 kDa protein in non-denaturing gel filtration. The partially purified radio-labeled precipitate of the antigen showed a molecular weight (M_r) of 15 kDa on denaturing and reducing SDS-PAGE (Takei 1982). Under nonreducing conditions, the precipitated material by mAb H9/25 migrated as a doublet of 15-16 kDa, but also showed a band with *Mr* of 34-36 kDa (Reiser *et al.*, 1986). The *Mr* of Ly-6C.2 was further confirmed by using another Ly-6C.2 mAb, 5075-12.1, as 14 kDa immunoprecipitate on reducing SDS-PAGE (Houlden *et al.*, 1986).

ThB antigen was immunoprecipitated with mAb 53-9.2 as a unique acidic protein of approximately 16 kDa from surface or metabolically labeled LPS blasts or a B cell lymphoma (Matossian-Roger *et al.*, 1982). The TSA-1 glycoprotein also has an *Mr* of 12-13 kDa reduced and 17 kDa nonreduced (Godfrey *et al.*, 1992). Ly-6G was immunoprecipitated by Ly-6G-specific mAb 1A8 and RB6-8C5 mAb, previously described to detect a myeloid-restricted Antigen (Gr-1), as a *Mr* 21 to 25 kDa protein

from metabolically labeled Ly-6G- and Ly-6C-transfected El4J cells (Fleming *et al.*, 1993b).

The detection of the 78 kDa antigen by Ly-6.2 antiserum has been attributed to possible contaminating antibodies in the polyclonal antiserum, while the discrepancy in the molecular weight of Ly-6 antigens using mAbs might be accounted for by the different sources of antigen or the different gel systems used in the analyses. In the absence of reliable immunoprecipitation procedures applicable to all Ly-6-related antigens, Palfree *et al.* initiated a comparison of the electrophoretic mobilities of a number of Ly-6 antigens in the presence of the non-denaturing, non-ionic detergent, octylglucoside, or by Western blot analysis under non-denaturing conditions (Palfree *et al.*, 1986a; 1986b). Octylglucoside has relatively high critical micellar concentration and provides efficient solubilization of the membrane proteins without affecting their charge, thus the electrophoretic motility of a membrane protein in polyacrylamide gels containing this detergent is dependent upon its size and its charge. These two contributions can be dissected by analysis of the mobility as a function of gel concentration. It was shown that Ly-6A.2, Ly-6B.2, Ly-6D.2 and Ly-6E.1 antigens had a similar size and charge and that Ly-6C.2 was similar in size, but differ in charge and in intrachain disulfide constrains (Palfree *et al.*, 1986a; 1986b). Two Ly-6-linked TAP and TAPa, described by Rock *et al.* (1986) and later proven to be identical to Ly-6A.2, were also found to have an *Mr* 10-20 kDa (Reiser *et al.*, 1986a).

1.3.2 Biosynthesis of Ly-6 Antigens

Ly-6A.2 was resolved on the gel as doublet or triplet in immunoprecipitation of the surface or metabolically labeled cells of appropriate sources. Ly-6E.1 appeared as a doublet on the gel of metabolically labeled immunoprecipitate. Pulse-chase experiments showed the middle band of the Ly-6A.2 triplet to be first synthesized and converted into upper and lower bands consequently (Reiser *et al.*, 1987). However, no precursor-product relationship was found with the Ly-6E.1 doublet (Palfree *et al.*, 1986a). Previous studies have shown that Ly-6 antigens are glycoproteins (Reiser *et al.*, 1986b). The biosynthetic labeling in the presence of tunicamycin, which inhibits the N-glycosylation,

and *in vitro* treatment by peptide N-glycanase F of the antigens do not alter the band patterns. In contrast, following treatment of Ly-6A.2 with trifluoromethanesulfonic acid (TFMS), a drug which deglycosylates all O-linked sugars, the upper band of the Ly-6A.2 triplet disappeared completely and resolved into middle and lower bands (Reiser *et al.*, 1987). The results indicated the absence of N-glycosylation. While there was no evidence found for phosphorylation of the Ly-6A.2 protein, the apparent size and charge heterogeneity of Ly-6A/E molecules might be the results of differences in O-linked carbohydrate or variation in the glycan now known to be present in the form of a GPI anchor at the C-terminus of the Ly-6 proteins.

1.3.3 GPI Attachment of Ly-6 Proteins

As was first evident in Ly-6A.2 (Reiser 1986b), Ly-6 molecules can be released from the plasma membrane in an intact and soluble form upon digestion with a phosphatidylinositol specific phospholipase C (PI-PLC) (Hammelburger *et al.*, 1987; Steiberg *et al.*, 1987; Godfrey *et al.*, 1992). These results indicated that the Ly-6 proteins are anchored to the cell surface via a phosphatidylinositol linkage (GPI). Furthermore, these proteins are not expressed on the plasma membrane of cells that have mutations in GPI anchor biosynthesis (Horton *et al*, 1982; Yeh *et al* 1988, Fatemi & Tartakoff 1988). This also indicates that an alternate transmembrane form of Ly-6 antigen does not exist and that the unprocessed protein must be degraded within the cell.

1.3.4 Protein Structure of Ly-6 and Structure Model

The genes of Ly-6 family encode peptides which consist of three distinct regions: (1) A hydrophobic leader sequence, characteristic of a secretory protein, (2) a mature peptide domain and (3) a C-terminal hydrophobic signal sequence required for the attachment of the mature protein to the cell surface through glycophosphatidylinositol (GPI). Immediately after translation, the N-terminal leader peptide is removed and the C-terminal signal peptide is replaced by a GPI anchor. The mature Ly-6 molecule is about 80 amino acids, 10 of which are cysteine residues. Comparison of the predicted amino acid sequences of the Ly-6 family showed a high conservation of the placing of the 10

cysteine residues and octapeptide (CCXXDLCN) at or near the proposed attachment site for GPI anchor. These conserved structural features are the hallmarks of members of the Ly-6 family. Structural identification of the human CD59, which inhibits the formation of complement membrane attack complex (MAC) (Davies *et al*, 1989; Sugita *et al.*, 1989; Sawada *et al.*, 1989), urokinase plasminogen activator receptor (uPAR) (Nielsen *et al*, 1988; Roldan *et al*, 1990) and squid brain glycoprotein Sgp-2 (Williams *et al.*, 1988) showed strong similarities with Ly-6 proteins, and extended the murine Ly-6 into a superfamily of GPI-anchored molecules (LY6SF) (Williams, 1991). Database homology search with either whole Ly-6 or the above octapeptide sequence as query showed top matches within the LY6SF and cytotoxin/neurotoxin families (Fleming *et al.*, 1993a). The sequence homology is striking for the cysteine residues and amino acids at the N- and C-termini, which are presumably involved in the overall folding and processing of Ly-6 domain. The crystal or solution structures of the five neurotoxins (neurotoxin a, erabutoxin b neuotoxic protein, α-bungarotoxin, "long" neurotoxin and neurotoxin I) have been solved (Tsernoglou & Petsko, 1977; Low *et al.*, 1976; Agard *et al.*, 1982; Walkinshaw *et al.*, 1980; Mikhailov *et al.*, 1990). As was seen in the snake venom neurotoxins, β-sheets were the predominant secondary structures predicted and resolved in Ly-6, CD59 and uPAR proteins (Fleming *et al.*, 1993a; Kieffer *et al.*, 1994; Ploug and Ellis, 1994). Based on the similarity in the structurally important amino acids and secondary structure predictions among the proteins of the LY6SF, a structural model was proposed for LY6SF members: Each protein of the LY6SF is folded into globular 3D structure similar to that of neurotoxins. There is a central core, from which three finger-like 'loops' extend in a nearly planar array (Agard *et al.*, 1982). Four disulfide bounds formed from eight of the ten cysteine residues are the major force holding the core structure, which is further stabilized by hydrogen bounding, electrostatic and hydrophobic interactions. The C-terminal GPI anchor holds the core proximal to the membrane, while the surfaces of the three loops are available to interact with the ligands. This LY6SF structure model provides a basis for further analysis of structure-function relationship of proteins in the LY6SF.

1.4 Functional Properties of Ly-6

Although serology and structure of the Ly-6 molecules have been well studied, their physiological functions are still not well understood. Ly-6 molecules were discovered as differentiation antigens on functional subsets of lymphocytes and hematopoietic cells at different developmental stages. Their expression on lymphocytes is highly regulated by cell activation and enhanced by certain cytokines. These characteristics may imply a functional relevance of Ly-6 molecules in the process of lymphocyte development and activation. Ly-6 antigens are also within a group of T cell surface molecules, such as Qa-2, sgp60, Thy-1, human CD59 and decay accelerating factor (DAF), which all have GPI anchors. GPI-anchored proteins can induce T cell activation after crosslinking (Hahn and Soloski 1989; Reiser 1990; Gunter *et al.*, 1984; Korty *et al.*, 1991; Davis *et al.*, 1988). They are considered to possess T cell accessory function and be capable of transducing signals through the GPI anchor. Studies of Ly-6 molecules have provided evidence for their functional properties in the regulation of T cell activation, signal transduction and inter-cellular adhesion.

1.4.1 Crosslinking Ly-6 mAbs Induces T Cell Activation

Malek *et al.* showed that a rat mAb D7, which recognizes a non-polymorphic framework determinant of Ly-6A.2/E.1 (Ortega *et al.* 1986), could induce T cell activation after crosslinking with anti-Ig reagents or by Fc receptor-bearing accessory cells (Malek *et al.*, 1986). The stimulation of resting murine T cells to proliferate required the addition of a costimulator, PMA (a PKC agonist), while D7 could induce IL-2 production from T cell hybridomas in the absence of a costimulator. It augmented antigen- or alloantigen-, but not mitogen-induced T cell proliferation. A rapid rise in intracellular calcium concentration $[Ca^{++}]_i$ was observed after crosslinking D7 mAb. A similar stimulating effect of crosslinking Ly-6A.2 molecule was also evident in T cell hybridomas and normal T cells by Ly-6A.2-specific mAbs 1A8 and 3A7 (Rock *et al.*, 1986; Yeh *et al.*, 1986). The activation seemed to be through an IL-2-dependent pathway, and occurs in magnitude to maximal antigen or lectin stimulation. It was not

surprising that no costimulator was required in the activation of the T cell hybridomas by Ly-6 mAb as it was noted that activation of most T cell hybrids appears to require only receptor triggering without any second signal (Rock and Benacerraf, 1983; Kappler *et al.*, 1983). Conversely, the presence of an additional signal, provided by accessory cells, was required for stimulation of normal T cells by these mAbs. Using a panel of mAbs to Ly-6A.2 antigen, Yeh *et al.* showed that soluble anti-Ly-6A.2 mAb was sufficient for T cell activation (Yeh *et al.*, 1987). However, this requires crosslinking mAbs because monovalent antibody Fab fragments are not stimulatory. Immediately after crosslinking there is a rapid rise in $[Ca^{++}]_i$, followed by interleukin-2 (IL-2) secretion and IL-2 receptor (IL-2R) expression. This indicates a pathway similar to that of TCR-mediated T cell activation. The potency of the mAbs in the T cell proliferation varied in the studies. This could be explained by the difference in the epitopes defined by the mAbs or in antibody affinities.

Cross-linking Ly-6C mAbs (nonpolymorphic rat 6C3, H9/25 and HK1.4, and Ly-6C.2 specific mouse 143-4-2) also induced T cell activation in the presence of nonmitogenic doses of PMA or IL-2 (Dumont *et al.*, 1986; Havran *et al.*, 1988; Leo *et al.*, 1987). 143-4-2 can promote the lysis of non-antigen-bearing target cells by alloreactive CTL clones (Leo *et al.*, 1987). Unlike other Ly-6 mAbs, HK1.4 can induce the cloned $CD8^+$ CTLs to proliferate and release cytokines in the absence of accessory cell, crosslinking of TCR, mitogen or PMA. Furthermore, proliferation of cloned CTL cells by stimulated HK1.4 was not blocked by the addition of anti-IL-2R mAb. Thus the activation induced by HK1.4 appears to proceed through a pathway of activation different from that by Ly-6A specific mAbs.

Anti-TSA-1 mAbs (hamster PRSY1 and rat MTS35) have no effect on $[Ca^{++}]_i$ in thymocytes (Kosugi *et al.*, 1994; Godfrey *et al.*, 1992). Similarly, there is no significant increase in the $[Ca^{++}]_i$ following cross-linking ThB on thymocytes with 53-9.2 mAb (Ledbetter *et al.*, 1987). It is possible that the epitopes for PRSY1, MTS35 and 53-9.2 are not associated with activation, as in the case of anti-Ly-6A/E and anti-Ly-6C for which only some of mAbs have the capability of stimulating T cell activation.

Alternatively, crosslinking GPI-anchored proteins on thymocytes activates pathways which differ from those activated in mature T cells.

A correlation of T cell activation and increased surface expression of Ly-6A and Ly-6C molecules has been observed. Crosslinking mAbs to Ly-6A and Ly-6C or treatment with IFNs induced surface expression of the antigens. On the other hand, the induced expression of Ly-6A and Ly-6C antigens dramatically augmented crosslinking Ly-6 mAb-induced T cell proliferation (Malek *et al.*, 1986; Dumond *et al.*, 1986). In the Ly-6A transcription and GPI linker mutants generated by chemical mutagenesis (Yeh *et al.*, 1988), the decrease or loss of Ly-6 expression impaired T cell activation through the TCR as measured by IL-2 production. However, the majority of the mutants responded to calcium ionophores and phorbol esters which are thought to bypass surface receptor stimulation of T cells and to activate these cells directly by mimicking receptor-generated intracellular second signals (Weiss *et al.*, 1986). Furthermore treatment of the normal T cells with phospholipid-specific enzyme PI-PLC to remove GPI linkage resulted in a similar defect in TCR stimulation as was observed with the mutants. The results point a role of GPI-anchored protein and particularly Ly-6A in the TCR pathway of activation in these cells.

1.4.2 Requirement of Intact TCR for T Cell Activation by Crosslinking Ly-6 mAbs

There is evidence that T cell activation mediated by crosslinking Ly-6 requires the intact TCR. A TCR loss mutant 2B4 of a mouse T cell hybridoma, which lacks mRNA for TCR α and β chains and thus the surface expression of TCR, did not secrete IL-2 after CD3 or Ly-6 cross-linking (Sussman *et al.*, 1988). A series of TCR negative variants of CTL clone KB5.C20 could no longer be activated either for killing or producing IFN-γ with lectins, anti-Thy-1 or anti-Ly-6 mAbs (Langlet *et al.*, 1988). Thy-1-mediated T cell activation also requires coexpression of CD3/TCR complex. In a TCR negative variant of the human Jurkat T cell tumor (Gunter *et al.*, 1987), TCR/CD3$^-$Thy-1$^-$ Jurkat cells failed to produce IL-2 in response to anti-Thy-1 mAb. Replacement of the defective TCR α or β chain genes reconstituted both surface expression of TCR/CD3 and responsiveness to Thy-1 in the IL-2 production. More recent experiments have indicated that expression

of the ζ chain of the TCR is the key component required for induction of IL-2 production by Thy-1 and Ly-6 mAbs (Wengener *et al.*, 1992; Codias *et al.*, 1992). The models to explain the requirement of TCR are that the GPI-anchored proteins directly interact with the TCR complex, that they interact indirectly through another transmembrane protein, and in the latter case the transmembrane protein may interact directly with TCR complex or indirectly through modulation of an intracellular signaling pathway that involves components of the TCR. However, there has been no evidence of coimmunoprecipitation of the GPI-anchored proteins with any component of TCR/CD3, so there is no evidence of a constitutive stable interaction.

1.4.3 Requirement of GPI-anchor for T Cell Activation by Crosslinking Ly-6 mAbs

Interestingly, the cell activation in response to crosslinking of GPI-anchored molecules required their attachment to the plasma membrane via GPI-anchor. Transmembrane forms of Ly-6A, DAF and Qa-2 do not transduce signals for T cell activation by mAbs to the GPI-anchored proteins (Su *et al.*, 1991; Shenoy-Scaria *et al.*, 1992; Robinson *et al.*, 1989). Thus the common feature for the activation through GPI-anchored proteins is the 'GPI-mediated signaling'. However, GPI-anchored proteins do not traverse the cell membrane, it is unclear how the intracellular signals are generated. The proposed mechanisms were that signals were derived either directly from GPI-anchored proteins, e.g., through cleavage of the GPI-anchor to yield second messengers or from interaction of a transmembrane protein with GPI-anchored protein (Malek *et al.*, 1994).

1.4.4 Ly-6-mediated Signal Transduction in T Cell Activation

Activation of T cells, initiated from the ligation of surface receptors with specific ligands, triggers a sequence of biochemical signals culminating in cellular proliferation and the induction of effector function. The central role of the multicomponent TCR in mediating the cellular events is well established (Cantrell, 1996). Among the early biochemical events, activation of tyrosine phosphorylation, elevation of intracellular calcium, activation of lipid-dependent kinases, and activation of Ras and its downstream

kinase cascade are well known (Weiss and Littman, 1994). Tyrosine phosphorylation of various cellular proteins is a crucial early event of T cell activation (reviewed in Salmoson *et al.*, 1992). Two non-receptor protein tyrosine kinases of the src family, $p56^{lck}$ and $p59^{fyn}$, have been implicated in the initial phosphorylation of tyrosine residues present in the cytoplasmic tail of the TCR/CD-ζ and ε chains, suggesting two separate T cell activation pathways (Irving *et al.*, 1993; Isakov *et al.*, 1995).

Antibody-mediated crosslinking of GPI-anchored proteins can induce rapid phosphorylation of multiple cellular substrates at tyrosine residues (Hsi *et al.*, 1989). Mutant cell lines with deficiencies in GPI biosythesis fail to induce tyrosine phosphorylation of TCR ζ-chain and ZAP-70, a non-receptor PTK critical for T cell activation (Romagnoli and Bron, 1997). In antisense Ly-6A transfectants, TCR expression and tyrosine phosphorylation of multiple cellular substrates by fyn kinase were significantly impaired (Lee *et al.*, 1994). The impairment of the kinase activity in the transfectants was not overcome by the rescue of TCR expression by transfection. Only complete reconstitution of TCR and Ly-6 molecules on the surface of antisense Ly-6 transfectants restored tyrosine phosphorylation of proteins following TCR stimulation by anti-TCR mAb. The data indicated that Ly-6A antigen was essential for the induction of tyrosine phosphorylation through TCR.

With a particularly gentle detergent lysis, the association between Ly-6A/E and intracellular $P56^{lck}$ and $P59^{fyn}$ kinase activities has also been observed by coimmunoprecipitation studies (Stefanova *et al.*, 1991; Shenoy-Scaria *et al.*, 1993; Bohuslav, *et al.*, 1993; Lee *et al.*, 1994). More detailed characterization of the coimmunopricipitates has provided surprising results (Cinek and Horejsi, 1992). Using column chromatography, it was found that all the kinases-associated with GPI-anchored proteins in T cell lysates are present in large lipid-rich complexes. The structure is similar to the membrane microdomains of lipid clusters, called caveolae, isolated from the cultured epithelial cells (Brown, *et al.*, 1992). The kinases bound to the transmembrane proteins in the lipid domain or directly attached to the lipid complex could be brought to close vicinity of the GPI-anchored proteins.

The complexes are resistant to solubilization by a number of non-ionic detergents (Brij-58, NB40, Chaps) at 0 °C and stable under the conditions which should disrupt protein-protein interactions, suggesting that the lipid might be important in maintaining their integrity. Some lipids were identified as particularly enriched in the lipid complexes, including cholesterol and glycosphingolipids (Brown *et al.*, 1992). While cholesterol could be a critical molecule which holds the lipid complexes (Rothberg *et al.*, 1990), glycosphigolipids and their catabolites may mediate transmembrane signal transduction (Hakomori *et al.*, 1990). There would be no requirement of transmembrane proteins in the lipid complex by this mechanism.

1.4.5 Physiological Function of Ly-6 Molecules in The T cell Activation

GPI-anchored proteins are a very diverse group of molecules sharing only a common means of surface attachment. They have different physiological functions *in vivo*. For example, CD59, also called 'protectin', serves to protect cells from homologous complement lysis by interfering with the assembly of the membrane attack complex (MAC) in the final stages of pore formation (Rosse *et al.*, 1989; Walsh *et al.*, *1992*; Davis *et al.*, 1993). DAF also inhibits the action of homologous complement but at the level of C3 convertase (Nicholson-Weller *et al.*, 1982; Medof *et al.*, 1994). uPAR has multiple physiological functions by interacting with uPA and pro-uPA whereby localizing uPA-mediated extracellular proteolysis to the vicinity of cell surface (Appella *et al.*, 1987; Tkachuk *et al.*, 1996; Pepper *et al.*, 1996; Mohanam *et al.*, 1995). The different physiological functions of these GPI-anchored proteins *in vivo* suggested a specificity contributed by the protein moieties.

1.4.5.1 Speficity of Ly-6 Function in Modulation of T Cell Activation

Although 'GPI-mediated signaling' has been demonstrated by mAb crosslinking Ly-6 molecules in T cell activation, data from the inhibition of crosslinking TCR/CD3-induced IL-2 production by mAbs to Ly-6A/E showed that the inhibitory effect was not simply due to mAb binding to a GPI-anchored protein (Codias *et al.*, 1992). mAbs to Ly-6C and Thy-1 did not inhibit IL-2 production in the presence of anti-CD3. Unlike in the

case of T cell activation, the inhibitory activity of anti-Ly-6A/E, somehow, does not require extensive crosslinking of the Ly-6 mAbs. Furthermore, Codias *et al.* also showed that the ability of anti-Ly-6A/E to inhibit IL-2 production induced by anti-CD3 is independent of expression of the ζ chain (Codias *et al.*, 1992). In the Ly-6A/E-negative EL-4J thymoma cells transfected with a chimera construct in which the GPI anchor of Ly-6E was replaced by a transmembrane domain and a portion of intracytoplasmic tail of H-2D^b, anti-ly-6A/E also blocked anti-CD3-induced IL-2 production (Fleming and Malek, 1994). The observations indicated that inhibitory events of anti-Ly-6 were operating through a pathway distinct from the 'GPI-mediating signaling'.

In the experiment using antisense oligonucleotides to Ly-6A mRNA, Flood *et al.* demonstrated an inhibition of Ly-6A antigen expression by 60-80% in antigen-primed lymph node T cells as well as in the D10 T cell clone. The inhibition of Ly-6A expression resulted in the inability to restimulate, *in vitro,* antigen primed T cells, and blocked activation of normal T cells and T cell clones by Con A, or mAbs to CD3 or Ly-6 (Flood., 1990), indicating the importance of the Ly-6 protein. Similar results were obtained by using Ly-6A.2 antisense stable transfectants of D10 clones which had reduced surface expression of Ly-6A (Lee *et al.*, 1994). The reduced Ly-6A expression on D10 cells correlated with functional impairment which appeared to be related to a reduced TCR expression, specifically a reduction in the TCR β chain expression. Reconstitution of the TCR by transfecting the TCR β chain cDNA did not restore the defect in TCR signaling. Rather, the expression of Ly-6E, reconstituted by transfecting Ly-6E cDNA, rescued TCR expression and partially restored the responsiveness to antigen, ConA+rIL-1 or TCR cross-linking. The results suggested that the signal through TCR is impaired due to the absence of the Ly-6 antigen, perhaps because signals through Ly-6A are necessary for maintenance of a functional TCR complex in the D10 clone.

1.4.5.2 Ly-6 Antigen in Cell-cell Interaction And Ly-6 Ligands

In addition to signaling, receptor-ligand interaction also increases the strength of adhesion between effector and target cells. An adhesion effect of Ly-6 molecules have been observed in several studies. Overexpressed Ly-6A on the transgenic thymocytes (Bamezai

et al., 1995a) was found to mediate *in vitro* homotypic adhesion and to aggregate normal thymocytes as well as peripheral T and B cells. This adhesion could be blocked by pretreatment cells with PI-PLC or anti-Ly-6A mAbs, indicating the presence of natural protein ligands for Ly-6A on normal thymocytes as well as T and B cells (Bamezai *et al.*, 1995b). It was recently found that mouse ThB was, in fact, expressed at a much higher level on skin keratinocytes than on thymocytes and B cells as was initially defined. It is thus very likely that ThB is involved in keratinocyte cell-cell adhesion just like its recently reported human counterpart E48 (Brakenhoff *et al.*, 1995).

Other lines of functional evidence for adhesion of Ly-6A/E and Ly-6C came from several experimental observations. Ly-6A/E was found to be expressed on bone marrow or thymic stromal cells or cell lines. A highly up-regulated expression of Ly-6A was observed after crosslinking Ly-6A/E on the stromal cells with mAb MTS23 (hamster anti-Ly-6A/E) (Izon *et al.*, 1994), followed by secretion of the granulocyte macrophage colony stimulating factor (GM-CSF) from the stromal cells (Izon *et al.*, 1996). As antibody crosslinking of cell surface proteins may mimic an interaction with natural ligand, this stromal cell activation process may be mediated by interaction of the surface Ly-6A/E molecule and its natural ligand(s). The ligand(s) could be on the thymocytes as implicated in the observation of the transgenic thymocyte aggregation (Bamezai *et al.*, 1995b) or on other stromal cells. In a particular CTL clone ($CD4^-CD8^-$ TCR-γ/δ^+, specific for HSV gI), the activation of cytolysis of the HSV gI-transfected L cell targets and the production of IFN-γ by the CTL clone can be inhibited significantly by mAbs specific to Ly-6C (Johnson *et al.*, 1993). The inhibition of Ag-specific lysis by a Ly-6C-specific nonactivating mAb (7B10) was demonstrated only when 7B10 was bound to Ly-6C present on the surface of susceptible T cells. The CTL clone seemed to use Ly-6C as accessory molecule and interact with an undefined ligand on the target cell for antigen-mediated cytolysis and cytokine production. In a more recent study of endothelial adhesion and homing of $CD8^+$ T cell, Hänninen *et al.* (1997) showed that pretreatment of the T cells with mAb to Ly-6C (rat 1G7.G10) significantly reduced lymphocyte binding to lymph node high endothelial venules (HEV) *in vivo* or *in vitro*, and that further crosslinking Ly-6C mAb abrogated the inhibitory effect. The mAb crosslinking may have mimicked the effect of the

Ly-6C binding to its natural ligand. Results from *in vitro* T lymphocyte aggregation assay suggested that Ly-6C participated in the adhesion process by activating integrin-dependent pathway. Although not identified, Ly-6 ligands have been suggested from the functional studies and implied through analogy with CD59, uPAR and neurotoxins which interact with protein ligands. From the diversity of potential functions for Ly-6 molecules, one would expect distinct and possibly multiple ligands for each Ly-6 molecule.

1.5 Ly-6 Studies in The Species Other Than Mouse

The structure and biology of the mouse Ly-6 family have been reasonably well characterized and multiple genes been cloned since 1986. The existence of Ly-6 gene family in other species was also evident. Several Ly-6-related cDNA clones (RK6, RK10 and RK11) have been identified by crosshybridizing with mouse cDNA probe (Friedman *et al.*, 1990). The structure similarity between rat and mouse Ly-6 domains are striking and Southern blot analysis uncovered the presence of a multigene family in rat just as in mouse. Recently, an apparent TSA-1/Sca-2 homologue, ChSca-2, was discovered in chicken (GenBank accession number: L34554, CHKSCA2A).

Several molecules more distant to Ly-6 family have been found in various species. A glycoprotein, sgp-2, was isolated from the nervous tissue of squid (Willian *et al.*, 1988). Biochemically, sgp-2 is a small cell surface GPI-anchored glycoprotein of about 18 kDa. Amino acid sequence analysis revealed a protein structurally similar to mouse Ly-6. Eight of the ten cysteine residues were well aligned with mouse Ly-6A and Ly-6C. A sheep lymphocyte surface molecule B5 was found to be small GPI-anchored protein migrating on the SDS-PAGE as multiple bands within 11-17kb (Hein and Beya, 1989). The B5 molecule was also known to be involved in T cell activation by crosslinking with anti-B5 mAb, and thus was considered as a sheep homologue of mouse Ly-6 (Hein *et al.*, 1988). Human CD59 was discovered and hailed as a human homologue of mouse Ly-6 by the structural similarity and its T cell activation function after crosslinking (Okada *et al.*, 1989). It has a wider cellular range of expression and low overall sequence identity to mouse Ly-6. It is also not induced by IFNs. Crosslinking CD59 induced T cell activation might be attributed to the GPI-anchor of the

molecule. Most importantly, it is encoded by a single gene located on the chromosome 11 (Forsberg *et al.*, 1989) which is not syntenic to the chromosome region of *Ly-6* locus. Along with another GPI-anchored human protein uPAR, which has three Ly-6 domains, CD59 belongs to LY6SF which also include snake venom neurotoxins, snake plasma PLA-2 inhibitor (Ohkura *et al.*, 1994) and sperm acrosomal protein SP-10 (Palfree, 1996).

As Ly-6-related genes have been discovered from a wide range of the species other than mouse. There is reason, therefore, to believe that Ly-6 antigen is conserved through evolution in the species including human. However, the considerable divergence of *Ly-6* genes between species prevented direct cloning of human homologues through cross-hybridization. As a strategy to step through species and finally clone human *Ly-6* genes, I used mouse Ly-6 cDNA probe and low stringency conditions to detect cross-hybridizing sequences in genomic DNAs from various species. From the cross-species Southern blots, multiple bands were detected in rabbit with an Ly-6A cDNA probes, and a single band in guinea pig with a mouse ThB cDNA probe. There was no crosshybridization found in other species which I tested outside rodentia. The work on cloning and characterization of these Ly-6-related species was interrupted when a human Ly-6-related cDNA became available. Since this was a major breakthrough in the quest for human Ly-6, studies of this novel gene turned into the major project for this thesis.

The human Ly-6-related cDNA, termed 9804, which was made available to us in February 1994, was initially cloned by Cohen *et al.* through subtractive screening a U937 cDNA library for genes uniquely or much more highly expressed in the parental U937 than in a costimulation-deficient U937 subclone (clone 6). The U937 clone 6 failed to provide costimulation in an *in vitro* intercellular communication system in terms of IL-2 production by T cells (Fig. 1.3), and was found to express basal level of 9804 gene relative to the parental U937 cells (Fig. 1.4). Database similarity search revealed that 9804-encoded polypeptide sequence was homologous to the murine *Ly-6* gene family, most like TSA-1. The mature 9804 polypeptide shared about 50% identity with the mature mouse TSA-1. In particular, the number and spacing of cysteines, which are the characteristic of Ly-6 proteins, are highly conserved being nearly identical in the alignment with mouse TSA-1.

Figure 1.3 Costimulatory Activity of U937 Clones Compared with The parental Cell Line

Normal human T cells and U937 cells were cocultured, each at 10^5 per well, along with immobilized anti-CD3 in 96 well plates. For the null control, U937 cells were omitted. After 24h, the supernatants were assayed for IL-2 on the CTLL-2 cell line. One unit produces half maximal CTLL-2 proliferation (B. Landgraf and E. Cohen).

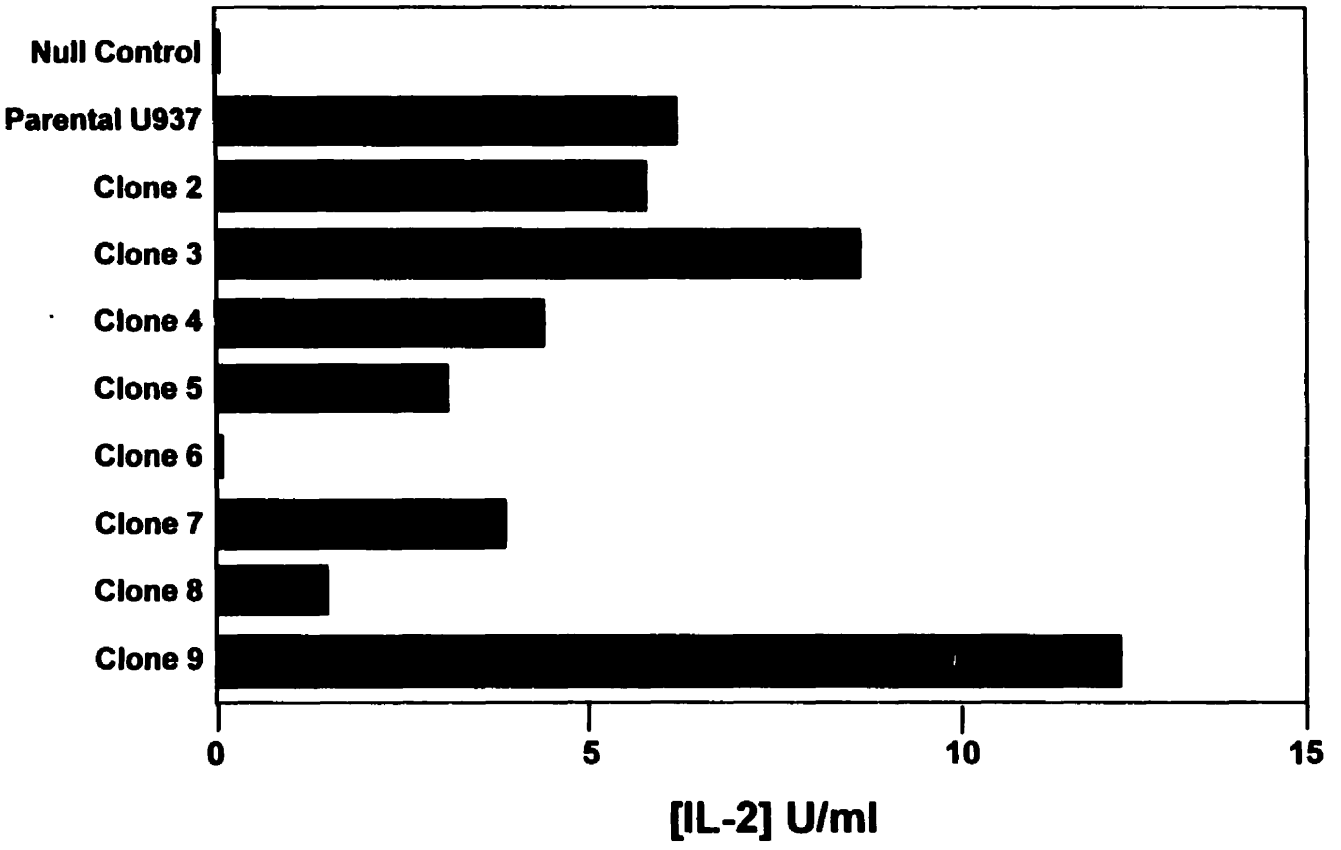

Null Control
Parental U937
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
0
5
10
15
[IL-2] U/ml

Figure 1.4 Northern Blot Analysis of 9804 mRNA Levels in Clone 6 and Parental U937 cells

5μg of polyA$^+$ RNA from clone 6 and the parental U937 cell line were electrophoresed in an agarose gel, transferred to nitrocellulose, and probed with 9804 cDNA probe. Relative to the size markers, 9804 mRNA was approximately 1.3kb (B. Landgraf and E. Cohen).

Clone 6
Parental
kb
9.49
7.46
4.40
2.37
1.35
0.24

Through the application of bioinformatics analysis to the dbEST of GenBank, Capon *et al.* (1996) identified a cDNA encoding human TSA-1/Sca-2, which turned out to be identical to the 9804. Interestingly, the same cDNA was isolated independently by another group as a retinoic acid-inducible gene, RIG-E (Mao *et al.*, 1996). Another human gene, which encodes the keratinocyte adhesion molecule E48, has been recently reported and is apparently the human homologue of mouse ThB (Brakenhoff *et al.*, 1995). Interestingly, the chromosomal mapping of 9804 (as it will be detailed in the coming chapters)/RIG-E and E48 placed the genes at the same region 8q24-qter on chromosome 8, syntenic to mouse *Ly-6* locus on chromosome 15. During the preparation of this thesis, another human gene was reported as GPI-anchored molecule-like protein (GML) (Kimura *et al.*, 1997). The gene is induced by tumor suppresser p53 and encodes a GPI-anchored protein of about 17 kDa, similar in structure to Ly-6 proteins. The genomic structure of GML is typical four exon organization of *Ly-6* genes. And the structural gene of GML is also located in the same region of chromosome 8 as that of E48 and RIG-E/9804. The discovery of human counterparts of mouse Ly-6 molecules has moved the Ly-6 study into the human arena with the emergence of a human Ly-6 multigene family.

1.6 Summary

Ly-6 molecules are a set of structurally related, GPI-anchored cell surface proteins that belong to a superfamily including CD59, uPAR, SP-10, and more distantly the snake venom neurotoxin family. These molecules were initially identified in mouse as lymphocyte differentiation antigens. Studies with antisera and monoclonal antibodies have identified six distinct mouse Ly-6 antigens: Ly-6A/E, B, C, G, TSA-1/Sca-2 and ThB, and a seventh, Ly-6F, has been identified at the molecular genetic level. The genes coding all but Ly-6B have been characterized, and their genomic sequences have been reported. Although the biological role for these molecules are not well understood, there is mounting evidence that they participate in intercellular adhesion and signaling. Crosslinking Ly-6 molecules can affect intracellular signaling pathways, cytokine production, and cell differentiation. Together with the observation that each has a

distinctive range of expression in lymphoid tissues, the results have inspirited the view that Ly-6 molecules may participate in the control of leukocyte development and activation. Immunogenetic and RFLP analyses have demonstrated that Ly-6 molecules are encoded by genes that are tightly linked on mouse chromosome 15 and distal to *Myc-1* and proximal to *sis*. On the basis of synteny, it has be proposed that the human homologues of *Ly-6* genes would reside on chromosome 8, distal to *Myc-1* or on chromosome 22 near *sis*.

Direct cloning of human homologues through cross-hybridization was unsuccessful because of the considerable divergence of the *Ly-6* genes between species. However, recent discovery of two human counterparts, E48 and 9804, the latter being the main focus of this thesis, has moved Ly-6 studies into the human arena. Subsequent chapters of this thesis describe (1) the characterization and mapping of 9804 gene, (2) analysis of 9804 RNA expression, (3) transcriptional regulation of the gene and (4) 9804 recombinant protein expression. The earlier preliminary work on the cross-species hybridization of murine Ly-6 probes will be appended to this thesis.

CHAPTER 2

STRUCTURE ANALYSIS AND MAPPING OF THE *9804* GENE

2.1 Introduction

Intercellular communication and cell-cell adhesion are pivotal for eliciting a variety of cellular responses (Singer, 1992). These interactions, known to be essential determinants in developmental biology, also regulate the normal physiological responses observed in several tissues. The cellular interactions responsible for the development of a functional immune response are just one example. In this regard, the two signal hypothesis serves as a paradigm for a form of intercellular communication involved in immune activation (Bretscher and Cohen, 1970; Bretscher, 1992). This hypothesis provides the conceptual basis for the requirement of an antigen-specific and a non-specific contact-mediated signal as the decisive interactions for promoting the appropriate biochemical responses of T and B lymphocytes to foreign and self antigens.

The second signal, which is antigen-independent and imparted by the presence and pairing of unique cell-surface molecules, is known as costimulation (Jenkins, 1992). One of the most extensively studied costimulatory pairs to regulate T cell activation is CD28-B7 (June *et al.*, 1994). This form of intercellular communication appears to be influenced, if not regulated, through additional cell-surface proteins (Linsley *et al.*, 1991). Other well known cell-surface molecules were also described as initiating responses similar to the CD28-B7 interaction (Damle *et al.*, 1992; Larsen *et al.*, 1996). Given this apparent redundancy, it is not surprising, therefore, that CD28 gene knockout did not cause any profound immune dysfunction (Sharpe, 1995; Shahinian *et al.*, 1993). In support of this, it is well known that different soluble effectors can elicit duplicate or redundant physiologic responses (Ihle *et al.*, 1994). It is highly probable that novel costimulatory interactions remain to be discovered. Indeed, Johnson and Jenkins recently reported that a human monocytic cell line, U937, possesses a B7-independent form of T cell costimulation (Johnson and Jenkins, 1994). Since the phenomenon could not be attributed to any known cell-surface antigens, the possibility exists that novel mechanisms for promoting T cell activation remain to be elucidated.

A costimulation-deficient U937 clone (clone 6) was isolated as a useful tool in a search for novel proteins that mediate such interactions by Cohen *et al.*. A subtracted probe was prepared to screen a parental U937 cDNA library for unique or more highly expressed genes in the parental line relative to clone 6. One such gene, designated 9804, was found to encode a polypeptide sequence homologous to the murine Ly-6 protein superfamily (Cohen *et al.*, 1996; Shan *et al.*, 1998). We obtained the 9804 cDNA through collaboration and started further studies on the novel human gene. As an essential step towards further understanding Ly-6 functions and identification of additional human Ly-6 genes, this chapter describes the structural characterization and chromosomal mapping of the *9804* gene. The *9804* gene structure is completely analogous to that of other genes in the Ly-6 family. It is located at chromosome 8q24.3, which exhibits synteny with the region of mouse chromosome 15 containing the Ly-6 locus.

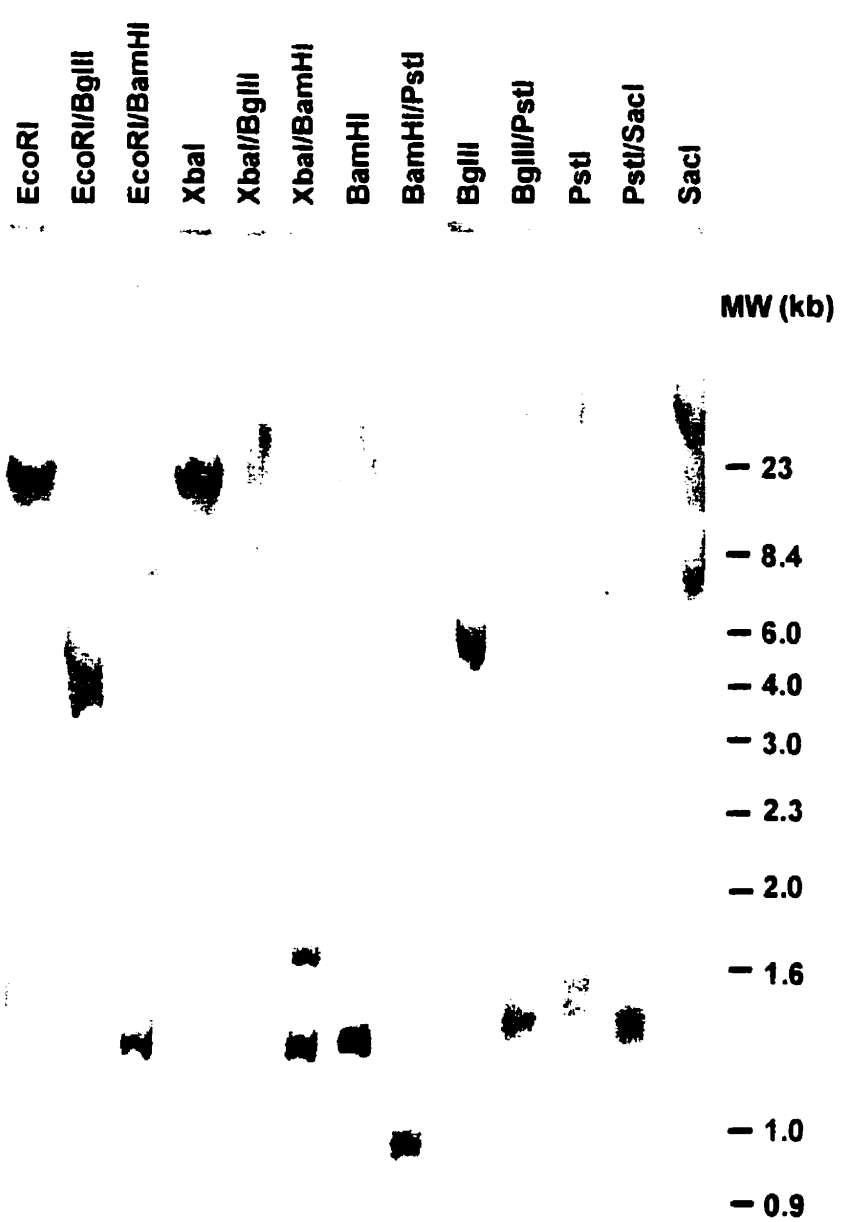
2.2 Southern Analysis Of Human Genomic DNA

Human high molecular weight genomic DNA was digested with restriction enzymes *Eco*R I, *Xba* I, *Bam*H I, *Bgl* II, *Pst* I and *Sac* I, singly or in combination, and processed for Southern blotting. After hybridization with a (^{32}P)-dCTP labelled probe generated from 9804 cDNA coding sequence by use of a random primer labelling kit, the blot was washed and autoradiographed. *Eco*R I, *Xba* I or *Sac* I digestion each revealed a single hybridising band of size 20 kb, 20 kb and 7 kb, respectively. The single bands can be broken down further by double digestion with *Bgl* II, *Bam* H I or *Pst* I. Single digestion with *Pst* I or *Bgl* II resolved two bands of about 1.2 and 1.4 kb or 5 and 1.2 kb, respectively (Fig. 2.1). The hybridising banding pattern indicated that, like mouse TSA-1, 9804 cDNA does not cross-hybridise with other members of a multigene family as was seen in the case of genes for mouse Ly-6A/E, Ly-6C, Ly-6F, and Ly-6G. The *9804* gene or at least the coding region and 3' untranslated (ut) region of the gene is likely contained within the 20 kb *Eco*R I, the 20 kb *Xba* I or the 7 kb *Sac* I fragments. Two *Pst* I and two *Bgl* II fragments might be contiguous within the gene and useful for subcloning and sequencing.

A cosmid clone CMC9 containing 9804 coding sequence was obtained from CytoMed, Cambridge, Massachusetts, and was analysed by restriction digestion and

Figure 2.1 Southern Analysis of Human Genomic DNA with 9804 cDNA

Human fetal lung high molecular DNA (20 μg/lane) was digested with restriction enzymes indicated on top of each lane, blotted and hybridized with ^{32}P random priming labeled 9804 cDNA probe. The sizes of hybridizing genomic fragments were estimated by comparison with molecular standards (1 kb ladders and λ Hind III fragments from GIBCO, BRL).


EcoRI
EcoRI/BglII
EcoRI/BamHI
XbaI
XbaI/BglII
XbaI/BamHI
BamHI
BamHI/PstI
BglII
BglII/PstI
PstI
PstI/SacI
SacI
MW (kb)
23
8.4
6.0
4.0
3.0
2.3
2.0
1.6
1.0
0.9

Southern blotting with 9804 cDNA probe (Fig. 2.2). The sizes of hybridising bands from Figure 2.1 and Figure 2.2 are summarised in Table 2.1. *Bgl* II, *Pst* I, *Eco*R I/*Bgl* II, and *Bgl* II/*Pst* I digestions of the cosmid confirmed a similar hybridization banding pattern to that of high molecular genomic DNA, indicating that the cosmid may contain the complete *9804* gene.

2.3 Construction of Human Genomic Fragments of 9804

Two hybridising *Pst* I fragments of about 1.3 kb were isolated by gel electrophoresis and ligated into the pBluescript II KS$^-$ vector. The subclones of the *Pst* I fragments were sequenced with flanking T7 and KS primers and internal primers as listed in Table 2.2. The sequencing data demonstrated that they contained all three coding exons. Analysis of a PCR fragment (Fig. 2.3) generated from the CMC9 template between primers 9804LEAD and 9804X4R1 (Table 2.2) within the first and last of these exons proved that the two *Pst* I fragments were indeed juxtaposed. As a typical Ly-6 gene contains 4 highly conserved exons, we expected to find a short upstream exon that contained only 5'-untranslated sequence, but the entire sequence of our 9804 cDNA clone was accounted for in these three exons. During this work, routine database searches revealed several 5' extended cDNA sequences, corresponding to the *9804* gene, recently submitted to EMBL/Genbank by researchers involved in the Washington University/Merck EST Project. Comparison of these with our 9804 cDNA clone and genomic sequences confirmed the presence of another exon containing 5' untranslated sequence. In order to locate this exon I, two more neighbouring *Pst* I fragments were isolated by walking along the DNA through overlapping *Bgl* II fragments according to the strategy shown in Figure 2.3. The full sequence of the region spanned by these four *Pst* I fragments was determined, and accounted for 5,543 bp of genomic DNA.

2.4 Nucleotide Sequence of *9804* Gene

The 5,543 bp of genomic DNA included four exons, 1,166 bp of 5', and 483 bp of 3' flanking sequences (Fig. 2.4). The four exons are separated by three introns of different length. Intron 1 is large, 2,298 bp, whereas introns 2 and 3 are only 321 and 131 bp

Figure 2.2 CMC9 Cosmid Restriction Analysis

Cosmid CMC9, provided by CytoMcd, Cambridge, Massachusetts, (20 μg/lane) was digested with restriction enzymes indicated on top of each lane. The gel was blotted and probed with 9804 cDNA. The band sizes were estimated by molecular standards λ *Hind* III fragments (GIBCO, BRL).

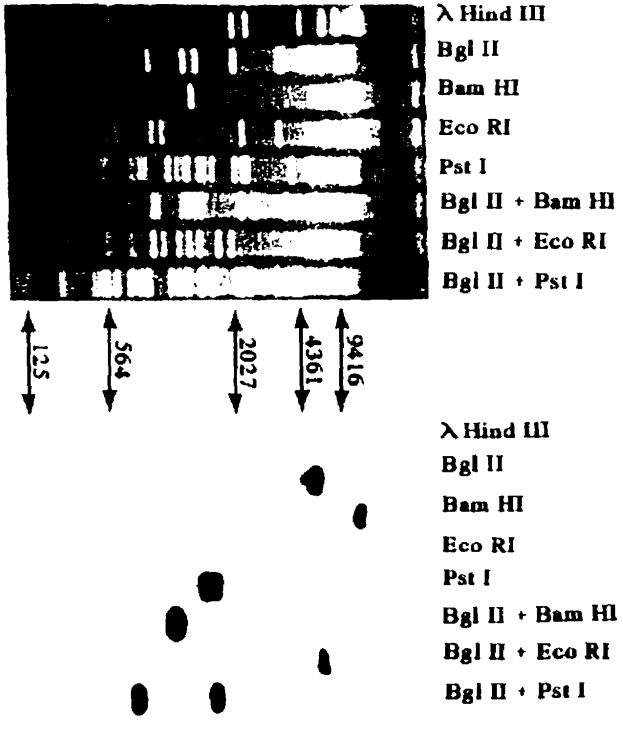

λ Hind III
Bgl II
Bam HI
Eco RI
Pst I
Bgl II + Bam HI
Bgl II + Eco RI
Bgl II + Pst I
125
564
2027
4361
9416
λ Hind III
Bgl II
Bam HI
Eco RI
Pst I
Bgl II + Bam HI
Bgl II + Eco RI
Bgl II + Pst I

Figure 2.3 Genomic Organization, Restriction Map And Cloning Fragments, and Sequencing Strategy of the 9804 Gene

The exons are boxed; translated sequences are represented by solid boxes. Arrowed lines indicate the subcloned fragments and arrowheads the internal primers used for sequencing or PCR. The restriction enzyme sites are designated as follows: *Pst* I (P), *Nco* I (N), *Msc* I (M), *Sac* I (S), *Bgl* II (Bg), *Bam* H I (Bm), *Sma* I (Sm).

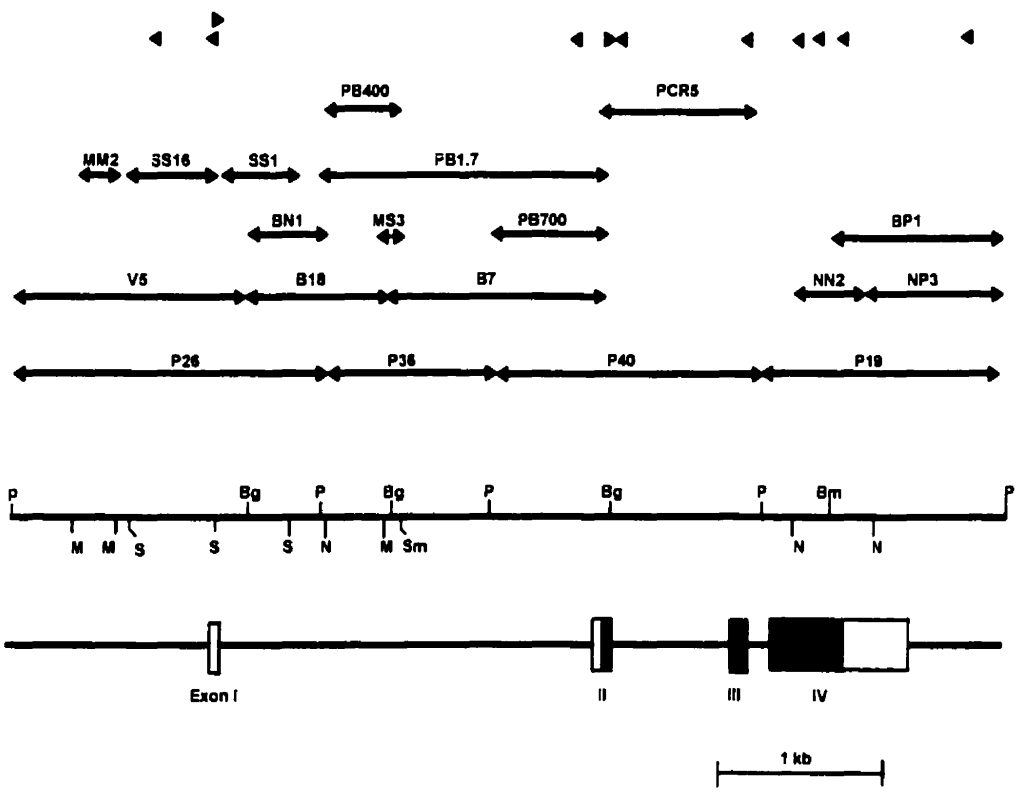

PB400
PCR5
MM2
3S16
SS1
PB1.7
BN1
MS3
PB700
BP1
V5
B18
B7
NN2
NP3
P26
P36
P40
P19
P
Bg
P
Bg
P
Bg
P
Sm
P
M
M
S
S
S
N
M
Sm
N
N
Exon I
II
III
IV
1 kb

Figure 2.4 Nucleotide Sequence of The 9804 Gene

Exons are denoted by uppercase letters and underlined. Donor and acceptor sites within the introns are denoted by italic boldface type. Predicted transcription initiation sites are indicated in single boldface capital letters. GC box and polyadenylation signal are boxed. Translation start codon and termination codon are indicated in boldface. Amino acid sequence is predicted and indicated with single letters. Potential ISRE and RARE in promoter region are indicated in boldface and labelled near the sites. An *Alu* repetitive sequence in intron 1 is underlined with a dashed line. The triangle indicates 5' end of the S1 nuclease protected sequence.

1 ctgcagagacagaacccccctgccctcaccgccaacattccagaggggaagatgccctgcctgtgccccac 70
71 gtaagacccagaggcagggactcccccgggccacagcctactggaggctcctgtccccagagacagctgggccatcgccccaaagctgtg 160
161 gaagccccacccacgatgccatcataccacggccaggacatctcgtgacacacacagccccagcctcagcctccatacacacacacagbc 250
251 accacacacactcactcatgcaaaacacacacacagccacattgactgcacccctagatttgagacaggggtgttacaggtgtcttgtca 340
341 gccccagcccccctnctgaaccacatggccacctgccccaccctcacatttccccacaccctcccccctactcccgtgtggggttcagaaga 430

ISRE

431 cccctccctggtgctcgccctgtcaccc***agaaaggaaac***gcactgtcaactggagaacaggcggtgcagagccccagcgcccacagccct 520
521 cggagagggctggcgggaggagaggcacccaccgatgcagagcctcagtgccctcgcagaagccagcacccacaacctcccacctcccaa 610
611 ccccttgccggtctccccacccccgcctgagggatggccaaggaacagcccagctggagcgggaagcaggcacaagatgcacctcaggaa 700
701 tcccaggctgagggggtgcctgcgtcctccgnncccgagctccactccgcaggcgagacccccaac**A**ctgctccccgacactctccgagg 790
791 cacccctcctccgtgctcacacccaaacacgtctgctcacccgtgggcttcccaccccgaacacaacgaggactgtccaggtgtcagtga 880

RARE **RARE** ∇

881 ***cggtca***ccccgacctggtnccctgcncggcctngcc***aggtca***gcacccgtccgtcnncacagcagggtccgggtggaaggaggtccgggc 970
971 ctggtccgtggctcagctccacctcactgggaaactgggaggtggtctcagaacttctgggcctcagcttccccatctatcccaaggagc 1060
1061 cggggtagcctgggcggcgcgggg**A**ccccaaccgggctc**G**ccgactcc**G**cccgcgg**A**ggcccctcccgtc**C**gcccctgccccccggggc 1150
1151 gcgcggctgcctgggagGCTCCGGCCAGCCGCGGTCCAGAGCGCGCGAGGTTCGGGGAGCTCCGCCAGGCTGCTGGTACCTGCGTCCGCC 1240
1241 CGGCG***gtgagt***ccgcgcggccccggcgggacgcccccgccacctgcgcgcaccgtcagacccggcggcctcggctgcggtgcacgcggcc 1330
1331 cggctcagccacngcggcggaaggccctgcgggggcgggggagagggtgggagagagcaagaggggngnnnggggaggggcaggagaggg 1420
1421 gctggggctgccggtgggttggtccccagagagctgagcttctcctcccatcccccgcaggcctccccgaatgtttccaaagatctgggg 1510
1511 cgggacggggcggagactgccgcaggagcctcccggcnnccccaggctgcgcagccactggagcccatcacccaactccagacgctcctg 1600
1601 gctcctctacgtgggccgggagggacagccttgaggactaggggagggggcacgggaccttgcagagctcctggccggaaagggaggatt 1690
1691 gaccgcccccggcatatcaccccggagcactggaacccgcccccgcttcttgttttggcactggtggtgcttgcggtgaggtccaaggag 1780
1781 cccagcctccctgagtggaccgccgggcccctccccgttccgggacacaggagagctcccgccccttgctggctggcagcccctagata 1870
1871 cctggctcccaggggccagcttccctgagcctggggatgagccatgagcctgcagcctggccccagggcggcccccacggcctgccggc 1960
1961 tggctccctccggctccatggcccacccggccttcctaattgccttcgcgtcccaccggtgccttagctcagcctggtggcccagcggtt 2050
2051 gggtgccacccagtgagcaggtggcggcaccagctggacctgtttgtggccctgtatgctaggacttcctcagagacagctcagggaccc 2140
2141 cccccacctaccagaacctcacttaggggggtgggggaaaaggaattggagatccttcctcctcggctactccctggaggcggggatagcc 2230
2231 ccagccggacttggcactgttttggccacctggggctcccactcccaaccccaggatgtcagcccaggtctcactgtcctggcctgctg 2320
2321 ctctccccttaggcctctgcgggcccctctccagatctgttctctgagggcatcctccttacccccagtgcccagcactagctccccagg 2410
2411 cccgggatgtccctcccactcctctgcccacgcgtgtcccctgaagaaggcagcagctccccctccaacaccatgcactcacaaaacaga 2500
2501 gaatcacgaccccagctggtgtgttccagattctttctccagcagtgcagagggtcctgtgcagaggccgaggagcagtacagcgaccca 2590
2591 tctggccctttcctgctggtgggaaccagtggcaacgcagccttgtctctcggagcccatttcccagccacagaatggggagtagcagat 2680
2681 actgagtggggtgctttcctcggaactgagtaacatcaggaatggggagtgctttccccctcagccatgccccacctggccccaggtctc 2770
2771 catgtgaggggagctgcctgggctcaaaaccactgagccatcccaaggaaaattctagatacagattagctaatatacactgacagatac 2860
2861 acatatagatgtgtatatagatttctcttttttttgagacagggtctcactctgttgcccaggctgcaggaatgcatggcatgatcatag 2950
2951 tatacaacttcagtacttgaacatccctagagcatcaagagatccatcccacctcagacctcccaagatagcatgaccacaggcacgtac 3040
3041 cacgcccggctaattttgtatttttgtagaaaacaggaggtctccactgtgttgcccagggtggtctcaaacccctggcctcatatga 3130

Table 2.1 The Band Size of Human Restriction Genomic Fragments Hybridized with 9804 Probes

Restriction enzyme	hybridizing bands (cDNA probe)	Hybridizing bands (9804X1F)
EcoRI	~ 20 kb	~20 kb
XbaI	~ 20 kb	N/A
HindIII	N/A	~20 kb
BglII	5, 1.2 kb	~6 kb
BamHI	1.2 kb	~10 kb
SacI	7 kb	N/A
PstI	1.2, 1.4 kb	2 kb
EcoRI/BglII	4.2 kb	>12 kb
EcoRI/BamHI	8.5, 1.5 kb	>12 kb
EcoRI/HindII	N/A	~10 kb
XbaI/BglII	3.4, 0.9 kb	N/A
XbaI/BamHI	1.7, 1.2 kb	N/A
BamHI/PstI	1.1, 0.4, 1.2 kb	N/A
BglII/PstI	1.2, 1.9 kb	1.5 kb
PstI/SacI	1.1, 1.2 kb	N/A

Table 2.2 Oligonucleotides Used in 9804 Study

Oligonucleotide	Position[a]	Direction	Nucleotide sequence[b]
9804SR1	807-826	reverse	GCAGACGTGTTTGGGTG
9804X1F	1169-1193	forward	CTCCGGGCCAGCCGCGGTCCA
9804X1R	1218-1243	reverse	CCGGGCGGACGCAGGTACCAGCAGC
9804ABF1	3364-3384	forward	CCTAGAGCATGTCTGCACTG
9804X2R	3419-3436	reverse	CTCGCTCCACACCCAGA
9804LEAD	3575-3596	forward	GGCAGGACGGCCATCCTCTCC
9804X3F1	3972-3987	forward	CAGCCAGCTCGCTGATGTGCTTCTCC
9804J12R	4333-4348	reverse	ATTGCACAGAAAGCT
9804ABR1	4505-4535	reverse	CAACAGATCAGGCACGTGAGGAGTC
9804X4R1	4435-4452	reverse	GGGCGGTCAGGGGCCAAA
9804ABR2	4535-4562	reverse	GACCAAGGGCACAGATCAGGCACGTC
9804X3R	4672-4091	reverse	CACCAATGCCGGCACTAGC
AR431F	NA	forward	ATCAGCTTGCAAGAAAGCCC
AR431R	NA	forward	GATGTGTAGCAAGTTGGCTAGG

[a] Positions correspond to numbering in Figure 2.4.

[b] Sequences are written in 5' to 3' direction.

respectively. A comparison of the genomic sequence with the 9804 cDNA sequence permitted location of the intron/exon boundaries, and these were found at positions which corresponded well with those of other Ly-6 genes (Fig. 2.5). The splicing sites are compared with the consensus splicing site sequences in Table 2.3. From the longest reported 5' ut sequence of a cDNA clone, we knew that the first exon is more than 80 bp in length. Exon II is 107 bp long and contains 55 bp of untranslated region and the first 52 bp of coding region. This, together with the first 8 bp of the exon III, encodes the 20 amino acid leader sequence. The mature protein is encoded by the last 112 bp of the exon III and the first 125 bp of the exon IV. The remainder of the exon IV, 716 bp, is 3'-untranslated sequence.

The only other known human Ly-6 homologue is E48, which is the homologue of mouse ThB (Brakenhoff *et al.*, 1995). Although the E48 genomic structure is not available at this time, we have some data relating to it which came from our involvement in chromosomal mapping of *9804* and *E48*. Based upon the ThB genomic structure, we hypothesised that the E48 gene, like those of ThB and 9804, had a short intron between the last two exons. We used primers in these exons to amplify an E48-specific fragment. Characterization of the fragment confirmed a short intron, almost the same size (132 bp), as the corresponding 9804 intron (131 bp), yet the sequences showed little similarity when aligned by the Clustal w program (Fig. 2.6) (Thompson *et al.*, 1994).

2.5 Characterisation of the 9804 promoter

In an attempt to identify potential transcription initiation sites, we used the programs ProScan and EPPNN (Reese and Eeckman, 1995). Although the former failed to predict any promoter for the gene, the later found twelve potential transcription initiation sites when a reduced cut-off value of 0.5 was provided. Some of the potential initiation sites are presented in Table 2.4, and indicated by bold capital letters in Figure 2.4. None of the predicted initiation sites have upstream TATA or CAAT consensus sequences, but the 100 bp region 5' to the longest reported exon I is GC-rich (78%). Several GC boxes were found in the region in either forward or reverse orientation and one of them is presented in the Figure 2.4 .

Figure 2.5 Sequence Alignment of E48, ThB, 9804 and TSA-1 Intron 3

Nucleotide identities between neighbouring sequences are indicated by vertical lines. Pairwise alignments between these sequences gave between 46% and 54% identity, while identities of between 38% and 53% were observed in several alignments in which one of the sequences was randomized (not shown). Intron sizes are indicated in base pairs. The E48 intron is between the first and second base of the Valine codon in the sequence corresponding to the amino acid sequence...CKTTNTVEPLRGNL... in the predicted E48 polypeptide. Alignments were generated with Clustal w program.

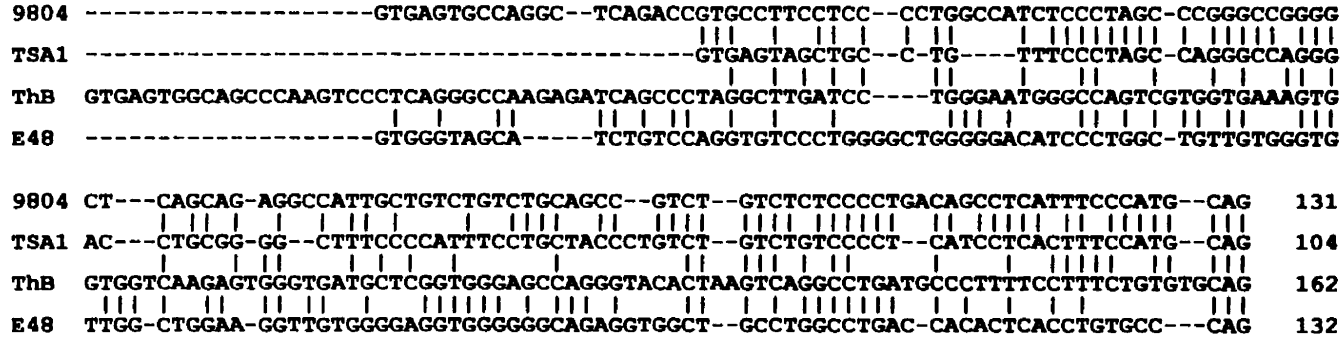
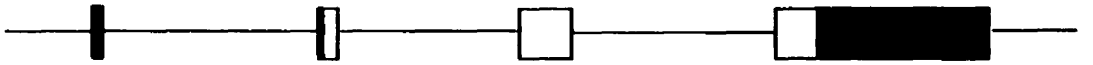

9804 ----------GTGAGTGCCAGGC--TCAGACCGTGCCTTCCTCC--CCTGGCCATCTCCCTAGC-CCGGGCCGGGG
TSA1 ----------GTGAGTAGCTGC--C-TG----TTTCCCTAGC-CAGGGCCAGGG
ThB GTGAGTGGCAGCCCAAGTCCCTCAGGGCCAAGAGATCAGCCCTAGGCTTGATCC----TGGGAATGGGCCAGTCGTGGTGAAAGTG
E48 ----------GTGGGTAGCA-----TCTGTCCAGGTGTCCCTGGGGCTGGGGGACATCCCTGGC-TGTTGTGGGTG
9804 CT---CAGCAG-AGGCCATTGCTGTCTGTCTGCAGCC--GTCT--GTCTCTCCCCTGACAGCCTCATTTCCCATG--CAG 131
TSA1 AC---CTGCGG-GG--CTTTCCCCATTTCCTGCTACCCTGTCT--GTCTGTCCCCT--CATCCTCACTTTCCATG--CAG 104
ThB GTGGTCAAGAGTGGGTGATGCTCGGTGGGAGCCAGGGTACACTAAGTCAGGCCTGATGCCCTTTTCCTTTCTGTGTGCAG 162
E48 TTGG-CTGGAA-GGTTGTGGGGAGGTGGGGGGCAGAGGTGGCT--GCCTGGCCTGAC-CACACTCACCTGTGCC---CAG 132

Figure 2.6 Comparison of The Intron/exon Organization of Ly-6 Genes

The typical structure of Ly-6 genes consists of four exons drawn in boxes. The 5'- and 3'-untranslated regions (ut's) are filled. The numbers indicate the corresponding intron or exon sizes of each gene in base pairs (bp).



Ly-6A/E	**53**	215	**98**	961	**117**	999	**601**
Ly-6C.1	**53**	203	**98**	628	**117**	1674	**581**
Ly-6F.1	**60**		**98**		**117**		**491**
Ly-6G.1	**60**		**98**		**117**		**603**
ThB	**?**		**86**	672	**99**	162	**533**
TSA-1	**68**	2059	**101**	391	**120**	104	**839**
9804	**>80**	2298	**109**	321	**120**	131	**838**

Table 2.3 Splicing Site Comparison Between Ly-6-related Genes and Eukaryote Consensus

Intron (Gene)	Splicing Sites[a]																		
Consensus[b]	A_{64}	G_{73}	g_{100}	t_{100}	a_{62}	a_{68}	g_{84}	t_{63}	.....	6	Py_{74-87}[c]						c_{65}	a_{100}	g_{100}	N
1 (Ly-6A)	A	A	g	t	a	a	g	g	.....	t	c	t	g	t	g		c	a	g	G
2 (Ly-6A)	A	G	g	t	g	a	g	g	.....	g	t	c	t	c	t		c	a	g	C
3 (Ly-6A)	G	G	g	t	a	a	g	t	.....	t	t	c	t	t	g		c	a	g	A
1 (Ly-6C.1)	A	G	g	t	g	a	c	a	.....	a	c	t	t	c	c		a	a	g	G
2 (Ly-6C.1)	A	G	g	t	g	a	g	g	.....	g	t	c	t	c	t		c	a	g	C
3 (Ly-6C.1)	G	G	g	t	a	a	g	t	.....	t	t	c	t	t	g		c	a	g	A
2 (ThB)	A	G	g	t	g	a	g	c	.....	a	t	c	c	t	t		c	a	g	C
3 (ThB)	A	G	g	t	g	a	g	t	.....	t	g	t	g	t	g		c	a	g	T
3 (E48)	A	G	g	t	g	g	g	t	.....	t	g	t	g	c	c		c	a	g	T
1 (9804)	C	G	g	t	g	a	g	t	.....	t	c	t	c	t	c		c	a	g	A
2 (9804)	A	G	g	t	g	a	g	g	.....	c	c	t	t	c	g		c	a	g	C
3 (9804)	T	G	g	t	g	a	g	t	.....	c	c	c	a	t	g		c	a	g	G

[a] Donor and acceptor sites are shown with lowercase for introns and uppercase for exons.

[b] The subscriptions indicate the percent occurrence of the specified bases at each consensus position.

[c] Py, pyrimidine.

Table 2.4 Promoter Prediction in the 5`-flanking Region of 9805 Gene[a]

Start[b]	End	Score	Promoter sequence[c]
727	770	0.64	ctccgnnccc**g**agctccactccgcaggcgagacccccaac**A**ctgctcccc
1045	1095	0.51	atctatcccaaggagccggggtagcctgggcggcgcgggg**A**ccccccaccg
1060	1110	0.60	ccggggtagcctgggcggcgcggggacccccaccgggctc**G**ccgactccg
1069	1119	0.65	cctgggcggcgcggggacccccaccgggctcgccgactcc**G**cccgcggag
1077	1127	0.71	gcgcggggacccccaccgggctcgccgactccgcccgcgg**A**ggcccctcc
1091	1141	0.74	accgggctcgccgactccgcccgcggaggcccctcccgtc**C**gccctgcc

[a] Predictions were produced in an EPPNN analysis (Reese and Eeckman, 1995).
[b] Positions correspond to numbering in Figure 2.4.
[c] The boldface uppercase letters indicate the potential transcription initiation sites.

To determine the transcription initiation site, a reverse primer, 9804X1R (Table 2.2), of 25 nucleotides complementary to 3' end of the predicted exon I was synthesised and applied in primer extension experiments. 20 μg total RNA from Jurkat, KG-1a, SK-N-MC or IFN-α-induced NB4, TF-1 and U937 cells were used in each reaction. Different experimental conditions including annealing temperature and reverse trancriptase were tried without success. The primer was not extended beyond 50 nucleotides (data not shown). It might reflect the difficulty for the polymerase to extend the primer through the extremely G/C-rich region (87%) of the exon I.

An alternative procedure, S1 nuclease protection assay was also performed to determine the transcriprion initiation site. As diagrammed in Figure 2.7, single stranded probes were synthesised either by vent DNA polymerase in an asymmetric PCR or by Klenow fragment of DNA polymerase I in an *in vitro* DNA synthesis (see section 7.2.3). The probes were uniformly labelled by incorporating (^{32}P)-dCTP during the PCR cycles or end-labelled by 5' end phosphoraylation of primer 9804X1R with T4 polynucleotide kinase, respectively (Fig. 2.7). The labelled probes were separated from the template plasmid P26 by size difference on alkaline denaturing gel (see section 7.4.2) and ethanol precipitated. The single stranded probes were hybridised with 20 μg of total RNA from human cell lines KG-1a (myeloid leukaemia) and A431 (epidermoid carcinoma). A single band of 294 nucleotides was detected only with A431 using either probe but not with KG-1a using either probe (Fig. 2.8). The most 5' end of the protected fragment in A431 is about 220 bp further upstream to the longest cDNA sequence from dbEST (Fig. 2.4). The reason for not detecting the S1 nuclease protected fragment in the KG-1a cells was unclear from the S1 nuclease assay. However, the detailed studies on transcription of the 9804 gene suggested an alternative usage of the exon I in A431 and KG-1a cell lines. The results will be presented and discussed in the Chapter 3.

Many potential transcription factor binding sites can be found in this region, but we have presented only potential ISRE and RARE in Figure 2.4. In addition, a sequence comparison of these potential response elements with ISRE and RARE known from several different genes is presented in Table 2.5 (Darnell *et al.*, 1994; Giguere, 1994; Bluyssen *et al.*, 1994).

Figure 2.7 Single Stranded Probe Preparation for S1 Nuclease Assay

Two 9804 exon I-containing genomic clones (P26 and SS16) in pBluescript KS^+ were used as templates in the probe preparations. Single stranded SS16 (SS16(ss)) was prepared from SS16 top strand packaged M13 helper phages. 9804X1R Primer (◂) was end-labeled (◂●, see section 7.2.1) and extended by Klenow fragment of DNA polymerase I to obtained probe 1 of 450 bp. Double stranded P26 (P26(ds)) was linearized by Bgl II (B) digestion and used in PCR with T7 (o) and 9804X1R (◂) primers in sequence. The reverse strand DNA was uniformly labeled with [^{32}P]-dCTP during the PCR to generate probe 2 of 1.2 kb. Both probes were isolated from alkaline denaturing gels by electrophoresis.

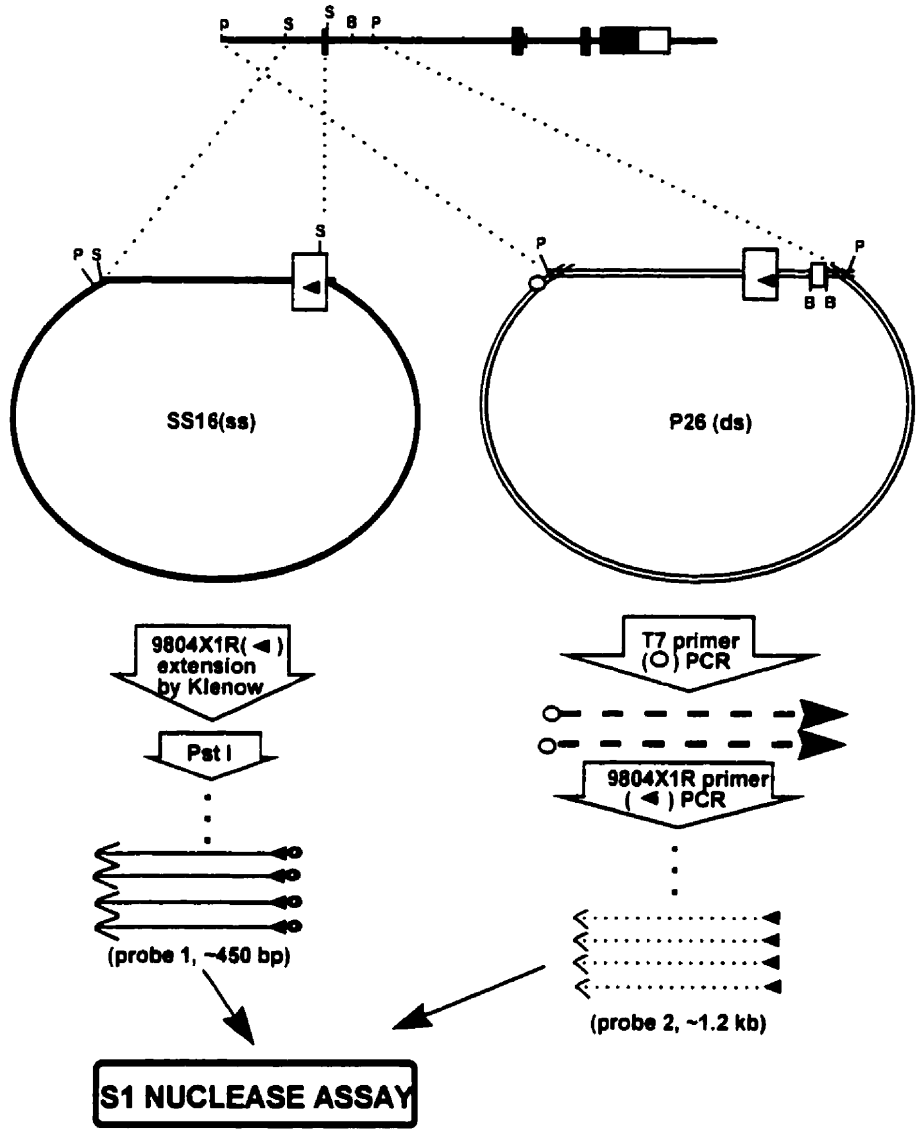

P
S
S
B
P
P S
S
SS16(ss)
P
P
B B
P26 (ds)
9804X1R(◄)
extension
by Klenow
Pst I
(probe 1, ~450 bp)
T7 primer
(○) PCR
9804X1R primer
(◄) PCR
(probe 2, ~1.2 kb)
S1 NUCLEASE ASSAY

Figure 2.8 S1 Nuclease Protection Assay

20 μg of total RNA from A431or KG-1a was hybridized to either probe 1or probe 2 (see Figure 2.7 and section 7.7.1). The S1 nuclease protected products were resolved on 7M urea polyacrylamide denaturing gel. Sequencing reaction using 9804X1R primer and P26 template plasmid DNA was loaded on the gel for comparison. The protected band size is about 245 bp from the primer as estimated by reading the known 9804 sequence.

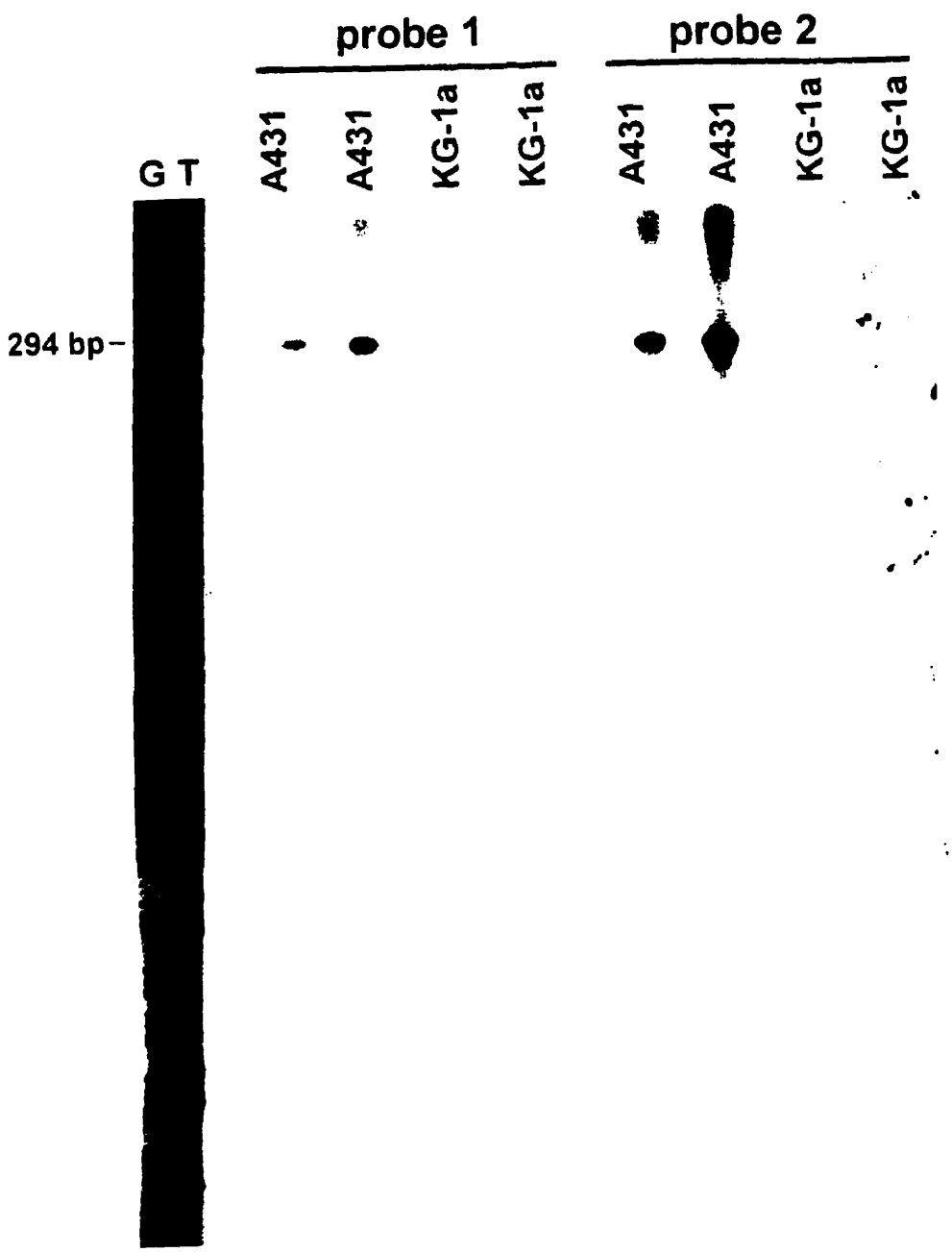

probe 1
probe 2
A431
A431
KG-1a
KG-1a
A431
A431
KG-1a
KG-1a
G T
294 bp

Table 2.5 Sequence Comparison of DNA Binding Sites

Gene	Nucleotide sequence	Reference
IFN-α-stimulated response element (ISRE)		
GBP[a]	ATGAAACTGAAAGT	
OAS[b]	AGGAAAC.GAAACC	
9-27[b]	AGGAAATAGAAACT	
2',5'[b]	AGGAAAC.GAAACC	
6-16[b]	GGGAAAATGAAACT	
ISG54[c]	GGGAAAGTGAAACT	
ISG15[c]	GGGAAAGGGAAACC	
9804	CAGAAAG.GAAACG	
consensus	RNGAAANNGAAACT	
Retinoic acid response element (RARE)		
rCRBP-II[d]	GGTCACA.....GGTCA	
rCRBP-I[d]	GGTCAAAA.....GGTCA	
hRAR 2[e]	GTTCACCGAAA.....GTTCA	
mγF-crystallin	TGACCCTTTTAACCA.GGTCA	
TREpal[f]	GGTCA.....TGACC	
hOXY[g]	GGTCA(N_{47})GGGTCAAGGTCA	
9804	GGTCA(N_{30}).GGTCA	
consensus	GKTCA	

[a] GBP, guanylate binding protein.
[b] OAS, 9-27, 2',5' and 6-16, oligoadenylate synthetases.
[c] ISG, interferon stimulated gene.
[d] CRBP, cellular retinol binding protein.
[e] RAR, retinoic acid receptor.
[f] TREpal, synthetic palindromic T3 response element.
[g] OXY, oxyticin.

2.6 Comparison of 9804 Genomic Structure with Ly-6 Family

Database sequence similarity searches have suggested that the predicted polypeptide encoded within the 9804 cDNA is highly homologous to the murine Ly-6 gene family encoding several differentiation and activation antigens. Within this family of molecules, the sequence for TSA-1/Sca-2 is clearly the most closely related to 9804. In this instance, the nucleic acid sequence identity with TSA-1/Sca-2 is 69%, while the unprocessed polypeptide sequence identity is approximately 60%. The similarity between 9804 and other Ly-6 modules was further studied. The presence of common motifs at the N- and the C-termini of the mature protein is a hallmark for Ly-6 and Ly-6-related proteins. These motifs are an N-terminal LXCXXC, that is present upon cleavage of the signal peptide, and CCXXXXCN at the C-terminus after removal of the hydrophobic sequence. Another feature indicative of Ly-6 family members is the spacing of the 10 cysteine residues that all of these related proteins possess. These three features, present in the predicted, mature 9804 protein (Fig. 2.9), further strengthen the similarity of 9804 with this gene family and Ly-6 protein superfamily (Fleming *et al.*, 1993).

The amino acid sequence of 9804 was further analyzed using the PredictProtein (Rost and Sander, 1993a; Rost and Sander, 1993b; Rost and Sander, 1994) for secondary structure. This analysis identified four regions of predicted β-sheet structure from sequences Met^{2} to Leu^{7}, Tyr^{27} to Ala^{33}, Asp^{39} to Leu^{47} and Val^{61} to Cys^{78} as predominant secondary structure for the 9804 protein. When these predictions were compared with the known areas of β-sheets for human CD59 (Kieffer *et al.*, 1994), two crystallized neurotoxins (Fuse *et al.*, 1990; Agard *et al.*, 1982) and predicted Ly-6 secondary structures (Fleming *et al.*, 1993), similarities in β-sheet spacing were evident (Fig. 2.10). Three regions of overlap were especially noted by Fleming *et al.* and indicated by boxes in Figure 2.10 (Fig. 2.10A). The amino acid sequence of mouse TSA-1 was also subjected to the same analysis and the resultant secondary structure was not surprisingly similar to that of 9804 (Fig. 2.10B). The result is consistent with the notion that like mouse Ly-6, human 9804 protein shares regions of similar secondary structure with other members of Ly-6/neurotoxin superfamily and is likely to share the same basic fold with them.

Figure 2.9 Amino Acid Sequence Alignment of Ly-6 Modules

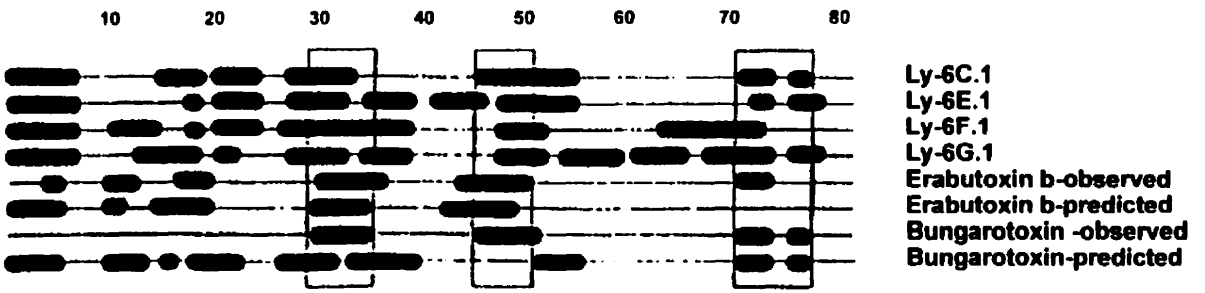
Alignment of the predicted 9804 polypeptide Ly-6 module sequence with several members of the Ly-6 family and superfamily. Only the Ly-6 modules of each are aligned (motif starts XXCXXC, usually immediately after the signal peptide, and ends CCXXXXCN). In the case of uPAR, which contains three Ly-6 modules, they are labelled 1 to 3 starting from the outermost, N-terminal domain. Alignments were generated with the Clustal w program and slightly adjusted to align Cys 7 in CD59 and uPAR 3, which were out by 2 places. The cysteine residues are in boldface type.

```
         1               15  16              30  31              45  46              60  61              75  76              90
9804     LMCFSCLNQKSNLYC LK----PTICSDQD- NYCVTVSASAGIGNL --VTFGHSLSKTC-- SPACPIP----EGVN VGVASMGISCCQS-FLCN  79
SCA2     LMCFSCTDQKNNINC LW----PVSCQEKD- NYCITLSAAAGFGN- --VNLGYTLNKGC-- SPICPSE--N-VNLN LGVASVNSYCCQS-FSCN  79
CHSCA2   LICFSCSDASSNWAC LK----PVKCAENE- EHCVTTYVGVGIGG- ---KSGQSISKGC-- SPVCPS-----AGIN LGIAAASVYCCDS-FLCN  76

E48      LRCHVCTS---SSNC KH----SVVCPASS- RFCKTTNTVEPLRG- ---NL---VKKDC-- AESCTPSYT--LQGQ VSSGTSSTQCCQE-DLCN  73
ThB      LRCHVCTN---SANC KN----PQVCPSNF- YFCKTVTSVEPLNG- ---NL---VRKEC-- ANSCTSDYS--QQGH VSSGSEVTQCCQT-DLCN  73

CD59     LQCYNCPN--PTADC KT----AVNCSSDF- DACLITKAGLQVYN- ----------KCWK FEHC--NFND-VTTR LRENELTYYCCKK-DLCN  70

Ly6A     LECYQCYGVPFETSC P-----SITCPYPD- GVCVTQEAAVIVDS- --QTRK-VKNNLC-- LPICPPNIES-MEIL GTKVNVKTSCCQE-DLCN  79
Ly6F     LECYNCLGVSLGIAC K-----SITCPYDP- AVCISQQVELIVDS- --QRRK-VKNKLC-- FPFCPANLEN-MEIL GTTVNVNTSCCKE-DLCN  79
Ly6G     LECYNCLGVPPETSC N-----TTTCPFSD- GFCVALEDRVVVDS- --HRSK-VKSNLC-- LPICPTTLDN-TEIT SNAVNVKTYCCKE-DLCN  79
Ly6C     LQCYECYGVPIETSC P-----AVTCRASD- GFCIAQNIELIEDS- --QRRK-LKTRQC-- LSFCPAG----VPIK DPNIRERTSCCSE-DLCN  76
RK10     LNCYNCTMIPFGNTC SS----TATCPYPD- GVCTIQVAEVVVSS- --VRLK-VKSNLC-- LPGCPKSPQT-PEVL GTVVHVNTDCCNT-DLCN  80
RK11     LKCYSCIEVPLNANC S-----TATCPYSD- GVCVSQVLEAVEGS- --VRRT-AKSNLC-- LPICPKFPQR-TEIL GTVVYTKVSCCNT-DLCN  79

UPAR3    RQCYSCKG-NSTHGC SSEETFLIDCRGPM- NQCLVATGTHEPKN- ---QS--YMVRGCAT ASMC--QHAH-LGDA FSMNHIDVSCCTK-SGCN  81
UPAR2    LECISCGS--SDMSC ERGRHQSLQCRSPE- EQCLDVVTHWIQEGE EGRPKDDRHLRGCGY LPGCPGS----NGFH NNDTFHFLKCCNT-TKCN  85
UPAR1    LRCMQCKT---NGDC R-----VEECALGQ- DLCRTTIVRLWEEG- ---EELELVEKSC-- THSEKTNRTLSYRTG LKITSLTEVVCGL-DLCN  77
```

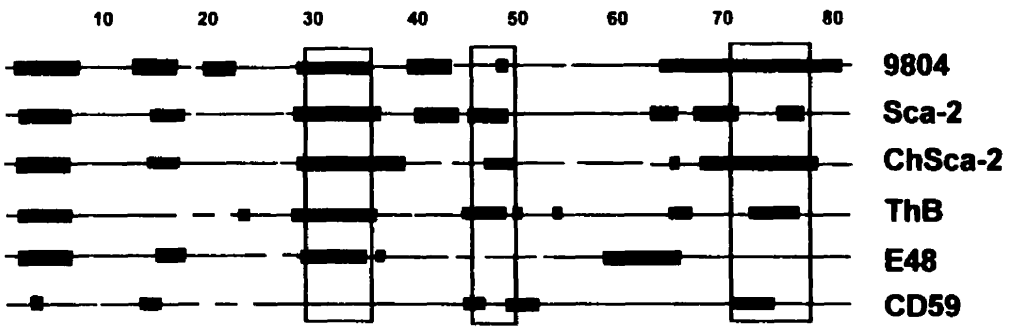
Figure 2.10 Secondary Structure Prediction of β-sheets in Ly-6 Molecules

The secondary structure of 9804, Sca-2, ChSca-2, ThB, E48 and CD59 (panel B) was predicted using the PredictionProtein program through Web site: http://www.embl-heidelberg.de/prediction/predictprotein.html. The spacing of residues is as found in Figure 2.8. Boxed areas indicate common areas of predicted β-sheets by comparison with observed areas of β-sheets in Ly-6C, Ly-6E, Ly-6F, Ly-6G and neurotoxins (panel A, adapted from Fleming *et al.*, 1993).

A


10
20
30
40
50
60
70
80
Ly-6C.1
Ly-6E.1
Ly-6F.1
Ly-6G.1
Erabutoxin b-observed
Erabutoxin b-predicted
Bungarotoxin -observed
Bungarotoxin-predicted

B


10
20
30
40
50
60
70
80
9804
Sca-2
ChSca-2
ThB
E48
CD59

Although the primary and secondary structure analyses place the most similar sequences next to each other, a better way to visualize the degrees of relatedness between them is through a diagrammatic representation of the results of a distance analysis. Such an analysis was performed by comparison of alignments of several members of this protein superfamily, including three other human polypeptides (CD59, E48 and uPAR), using the PHYLIP software package from Joe Felsenstein and PROTDIST as described in the Experimental Procedures. The result is presented in Figure 2.11 in a phenogram style. This analysis provides a strong indication of the divergence of the subsets. Furthermore, this analysis substantiates the relationship of 9804 to the Ly-6 family and most especially to the murine TSA-1/Sca-2 antigen.

2.7 Physical Mapping of 9804 Gene

Primer pairs were designed for specific amplification of 9804 gene fragments by PCR from human genomic DNA. In preliminary screening of DNA from BIOS-MAP human-hamster hybrid cell panel 1, amplification of specific fragments correlated with the presence of human chromosome 8 (data not shown). A more refined study was performed by our collaborator D. Wells (University of Houston) on human-hamster cell hybrids CL-17, 3;8/4-1, MC2F, 21q+ and TL/UC, which contain chromosome 8 deletions or translocations (Parrish *et al.*, 1994; Wagner *et al.*, 1991). The portion of chromosome 8 present in each of these chromosomes is shown in Figure 2.12 along with the PCR results. Amplification of the 9804 gene fragment was seen for template DNA from CL-17, 21q+ and TL/UC in addition to the control total human DNA. No amplification was seen when the template DNA was derived from 3;8/4-1, MC2F, total hamster DNA or when no DNA template was included. The results sublocalize the primer to the telomeric end of the q arm in interval I-9 of chromosome 8 (Spurr *et al.*, 1995).

The primer pair 9804ABF1/9804X3R was then applied in screening YACs in the distal portion of a large YAC contig (Chen *et al.*, 1996). This contig extends into interval I-9 and about 2 megabases distal to the *cMyc* gene located in 8q24.2. All YACs within this contig were negative indicating that the 9804 primers were distal to *cMyc*. The primers were then used to screen CEPH mega YAC A and B as well as a BAC libraries (Research

Figure 2.11 Degree of Similarity Between the Ly-6 Domains

Phenogram showing diagramatically the degrees of similarity between the Ly-6 modules aligned in Figure 2.8. Programs from the PHYLIP software package were used for this analysis as described in Experimental Procedures.

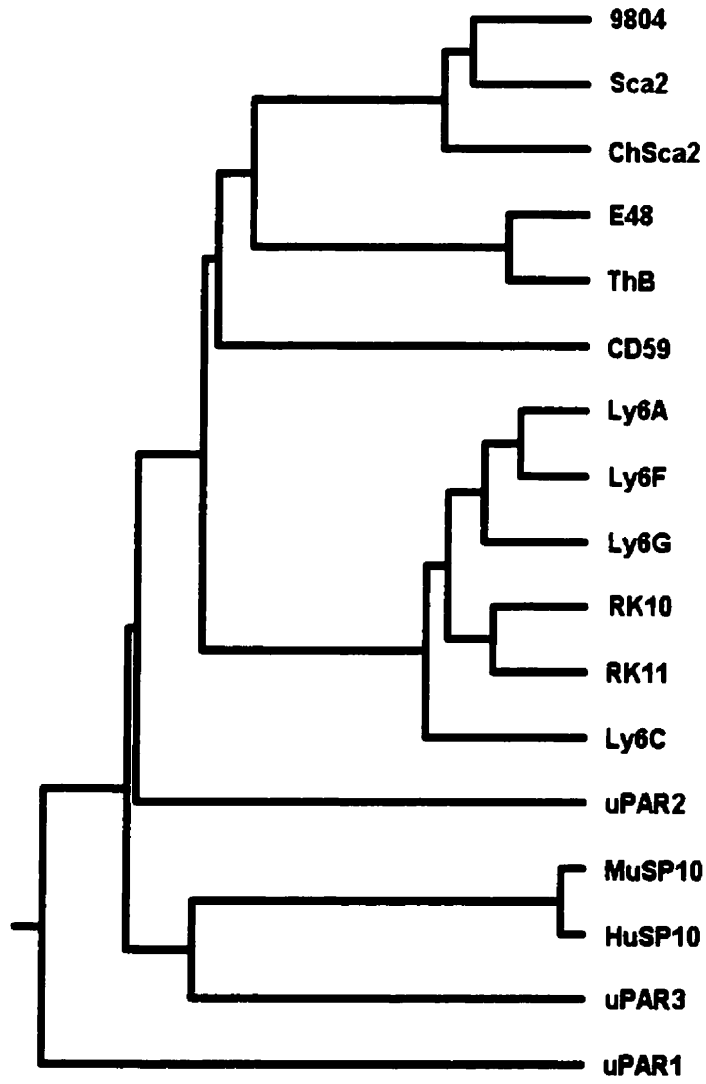
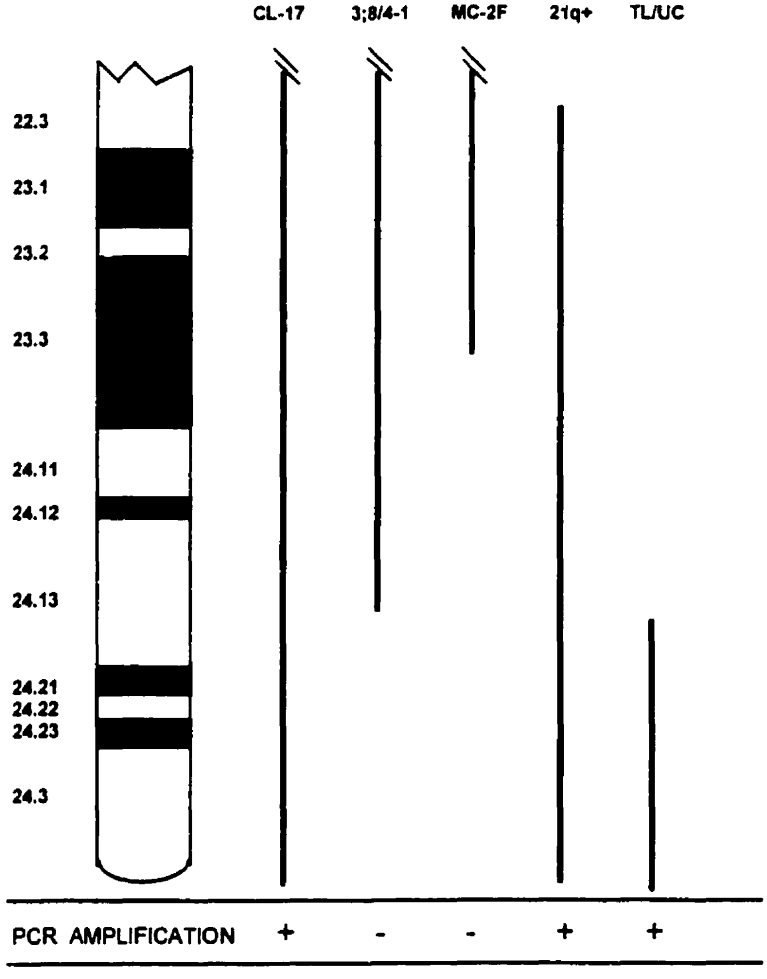

9804
Sca2
ChSca2
E48
ThB
CD59
Ly6A
Ly6F
Ly6G
RK10
RK11
Ly6C
uPAR2
MuSP10
HuSP10
uPAR3
uPAR1

Figure 2.12 Mapping of 9804 in the Distal Portion of Chromosome 8

The vertical lines represent the portions of chromosome 8 present in each of the human-hamster hybrid cell lines. Positive/negative PCR signals are indicated under each line with +/- (This Figure was generated by our collaborator D. Wells).


CL-17
3;8/4-1
MC-2F
21q+
TL/UC
22.3
23.1
23.2
23.3
24.11
24.12
24.13
24.21
24.22
24.23
24.3
PCR AMPLIFICATION
+
-
-
+
+

Genetics) and a chromosome 8 cosmid library, LA08NC01 (Wood *et al.*, 1992). Although no positives were seen for the YAC or BAC libraries, 2 positive cosmids, c101F1 and c157F6, were isolated from the cosmid library. These cosmids were subsequently used to rescreen the library and develop an overlapping cosmid contig (Fig. 2.13). Overlaps between cosmids were confirmed by comparison of *Eco*R I restriction enzyme digestion patterns and by direct hybridisation of cosmids to each other. The 4.3 kb *Eco*R I restriction enzyme digestion patterns and direct hybridisation of cosmids to each other. PCR using the STS primer pair AR431, developed from sequence near one end of cosmid c101F1, amplified fragments from c101F1, c93F9, c16E6, and c182H9 but not c157F6 and c189A10 (Fig. 2.13).

Cosmid c101F1 was fluorescently labelled and hybridised to metaphase spreads from CL-17, which contains intact human chromosome 8 as its only human material. Figure 2.14 shows a typical metaphase spread of chromosome 8, distinguished by hybridisation with a chromosome 8 specific centromere probe, and showing positive fluorescence in 8q24.3 close to the telomere (Fig. 2.14).

2.8 Discussion

Ly-6 molecules have been studied most intensively in mice. These molecules are encoded within a tightly-linked, mostly cross-hybridizing multigene family of about 20 members located on mouse chromosome 15. The chromosomal genes for Ly-6A/E, Ly-6C, Ly-G, Ly-6F and ThB have been cloned and characterised. Except for ThB, which appears to have just three exons, the Ly-6 genes typically possess four exons, the first of which contains part of the 5' untranslated sequence. The boundaries of the other three exons correspond to particular sites within the encoded polypeptide. Exon II terminates approximately three codons before the end of the signal peptide, while the boundary between exons III and IV corresponds to a position in the loop between the fifth and sixth of the 10 cysteine residues in the mature Ly-6 domain (Fleming *et al.*, 1993). We have found this same organization in the structure of 9804 gene, and in the recently reported mouse TSA-1 genomic sequence (Classon and Coverdale, 1996). There is considerable variation in the size of corresponding introns in Ly-6-related genes. Intron 3 varies from

Figure 2.13 Overlapping Cosmid Contig in the Distal Portion of Chromosome 8

The horizontal lines represent cosmid clones. The numbers above the lines indicate the sizes of *Eco* RI digestion products. 9804 and AR431 are the two primer pairs used in PCR screening (This Figure was generated by our collaborator D. Wells).

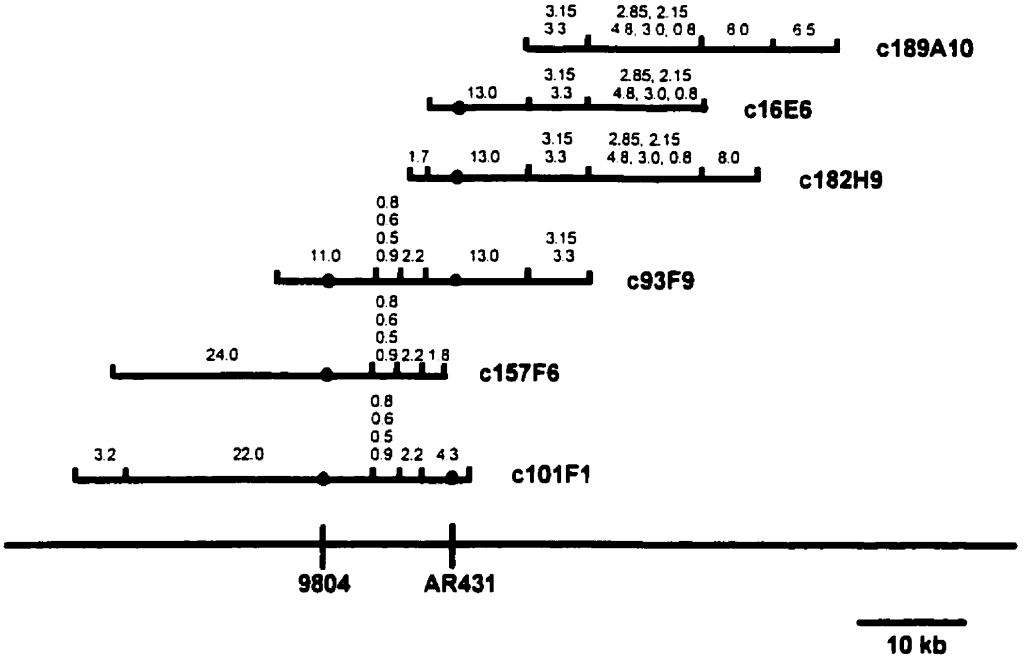
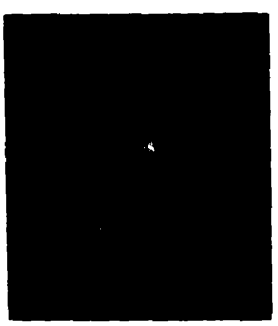

3.15
3.3
2.85, 2.15
4.8, 3.0, 0.8
8.0
6.5
c189A10
3.15
13.0
3.3
2.85, 2.15
4.8, 3.0, 0.8
c16E6
3.15
1.7
13.0
3.3
2.85, 2.15
4.8, 3.0, 0.8
8.0
c182H9
0.8
0.6
0.5
3.15
11.0
0.9
2.2
13.0
3.3
c93F9
0.8
0.6
0.5
24.0
0.9
2.2
1.8
c157F6
0.8
0.6
0.5
3.2
22.0
0.9
2.2
4.3
c101F1
9804
AR431
10 kb

Figure 2.14 Fluorescent *in situ* Hybridization of 9804

Metaphase chromosomes were prepared from CL-17 hybrid cell line. The cosmid c101F1, positive for the 9804 primers, was used as the 9804 region specific probe. The fluorescence is located very close to the telomere. The identity of chromosome 8 was confirmed by means of a chromosome specific centromere probe (This Figure was generated by our collaborator D. Wells).



approximately 7 kb in the CD59 gene down to 162 bp in the ThB gene, 104 bp in the TSA-1 gene, and now 131 bp in the 9804 gene. We have been able to compare these with the corresponding intron from the ThB-like E48 gene (Fig. 2.5), since preliminary work in a study of the two new human Ly-6 genes produced a PCR fragment of the E48 gene which included this intron. Interestingly, the sizes of the E48 intron (132) and 9804 intron 3 almost identical, and are much smaller than intron 3 in mouse Ly-6A (approx. 1kb) or Ly-6C (approx. 1.7 kb) genes. There is, however, little sequence similarity between these small 9804, E48, ThB and TSA-1 introns, so no evolutionary relationship can be inferred.

The full sequence of our 9804 cDNA clone is contained in the three exons, but the most common structure for Ly-6-related genes includes an additional exon, exon I, which contains the beginning of the 5' untranlated sequence. Shortly after data to accumulate from the Washington University-Merck Expressed Sequence Tag (EST) project, routine searches of GenBank showed an accumulation of sequences in the database which overlapped with the 9804 sequence. Several were found to begin upstream of the original 9804 cDNA sequence, and these defined the 3' end of exon I.

As is the case for mouse Ly-6 genes, there is no TATA box in the promoter of the 9804 gene. But the 5' flanking region is GC-rich (78%) and contains several GC boxes. The S1 nuclease protection study predicted an exon I of about 294 bp which is 220 bp further upstream to the longest known cDNA sequence of the 9804 gene. This seems very large comparing with other Ly-6 exons I. However, if it is an artefact relevant to the single strand probes, we should have detected a similar band also in KG-1a line. One possibility could be the presence of additional exon further upstream of the exon I, since the most apparent variations in Ly-6 gene structure occur around the region of the exons I. Overall, the introns 1 of human Ly-6-related genes (e.g. 5.4kb in CD59, 5.5 kb in GML) are much larger than that of most mouse Ly-6 genes (Petranka *et al.*, 1992; Kimura *et al.*, 1997), and there is no evidence for the absence of additional exons. To confirm the 9804 exon I, we need to employ primers, both forward and reverse, for RT-PCR in the 5' sequence of the S1 nuclease protected fragment.

As will be presented in next two chapters, 9804 has much broader tissue distribution compared with E48, which is expressed exclusively in the skin. The mRNA levels are

enhanced strongly by IFN-α and slightly by IFN-γ in normal human peripheral blood mononuclear cells and most malignant cell lines tested so far, although not in the B cell lines Daudi and Raji. These distinctive expression patterns will be under the control of response elements for tissue specific and inducible transcription factors. A large number of potential transcription factor binding sites were revealed by computer analysis of the 5' flanking region of the 9804 gene, most of which will have no evident physiological relevance. But we also looked especially for retinoic acid response elements (RARE), IFN-γ activation sites (GAS), and sequences similar to known IFN-α–stimulated response elements (ISRE) because of the known inducibility of 9804 gene expression by IFNs (see chapter 4) and retinoic acid (Mao *et al.*, 1996) and because ISRE and GAS are known to be involved in the induction of mouse Ly-6A expression by IFN-α/β and IFN-γ (Bothwell *et al.*, 1988; Sinclair *et al.*, 1993; Khan *et al.*, 1993). No typical GAS site was found in the 9804 promoter region, and consistent with there being only a slight effect, which may be an indirect one, of IFN-γ on 9804 mRNA levels in PBMC. A potential ISRE and two possible RARE half sites were found in the promoter region of 9804 (Fig. 2.4). I have started preparing vectors containing fragments encompassing these potential regulatory sequences upstream of a luciferase reporter gene (Chapter 7), but no data have been acquired yet in this regard.

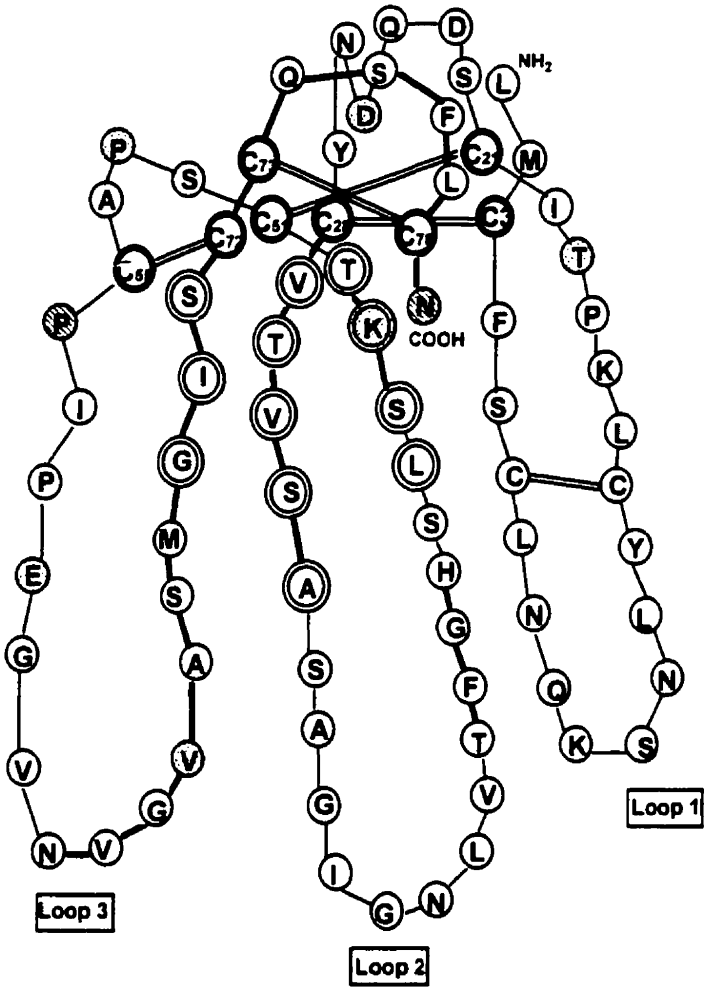
The similarities between 9804 and other members of the Ly-6 superfamily with respect to structurally important amino acids, particularly cysteines, and the predominant β-sheet secondary structures almost certainly imply their similar three dimensional structures. We, therefore propose that 9804 folds into the same general structure as that of α-bungarotoxin and CD59 (Fig. 2.15): A globular core at one end that is stabilised by four intrachain disulfide bounds. From this core, three loops protrude and run antiparallel to one another. From this model, the GPI-anchor would place the globular core toward the plasma membrane leaving the loops available for interaction with its potential ligand(s).

Mapping of the 9804 gene to human chromosome 8 in the q24.3 region telomeric to *c-myc* is significant, since this region is homologous to the mouse Ly-6 gene locus on chromosome 15. It is of further significance that the recently reported gene for E48, the human homologue of mouse ThB, has also been mapped to 8q24 (Mao *et al.*, 1996). Taken

together, these data strongly suggest the existence of a multigene family for human Ly-6 in the region q24 of human chromosome 8. We consider it likely, therefore, that additional human Ly-6 genes will be found in the vicinity of 9804 and E48.

Figure 2.15 Structural Model of 9804

Schematic model the mature peptide folding of 9804 was proposed from Figure 2.8 and Figure 2.9 based on the α-bungarotoxin crystal structure (Agard *et al.*, 1982) and the structural model of Ly-6 molecules (Fleming *et al.*, 1993). The conserved cysteine residues are indicated with thick-lined empty circles (O) and disulfide bounds with double lines. The Areas of β-sheets in 9804 are indicated with thick lines and the structurally conserved β-sheets are indicated with a double circle (◎). Loop numbers are boxed under the appropriate loop.


NH2
COOH
Loop 1
Loop 2
Loop 3

CHAPTER 3

9804 RNA EXPRESSION AND COMPARISON WITH TSA-1

3.1 Introduction

Ly-6 molecules, initially identified as lymphocyte differentiation antigens, are expressed mainly on hematopoietic cells. There is a limited expression of Ly-6A in the nonlymphoid tissues such as kidney (Blake *et al.*, 1993), skin (Brankenhoff *et al.*, 1995), testicular Leydig and Sertoli cells (Tokuda *et al.*, 1990). Ly-6A is transcriptionally active in many tissues such as brain, liver, muscle, heart and spleen (Reiser *et al.*, 1988). The high level of Ly-6A expression in some tissues rich in blood supply may be due, in part, to the expression of this molecule on the endothelial cells. TSA-1/Sca-2 has a most broad tissue distribution among the Ly-6 family. As assessed by immunofluorescence staining with monoclonal antibodies, it is expressed on 75% of bone marrow leukocytes, mature B cells, a subset of thymic medullary epithelial cells, thymic dendritic cells and immature thymocytes (Godfrey *et al.* 1992). It is the only marker which allows the distinction of low CD4 intrathymic precursor from bone marrow stem cells (Spangrude and Brooks, 1990). With a sensitive avidin-biotin system, a broader expression of TSA-1/Sca-2 was detected in subpopulations of peripheral $CD8^+$ lymphocyte and granulocytes (Antica *et al.*, 1997). Northern analysis, however, reveals that TSA-1/Sca-2 message is present in a wide range of non-lymphoid tissues (Tucek *et al.*, 1992). Levels in liver and lung are as high as seen in immature thymocytes and medullary epithelial cells, but low levels are found in kidney, brain, muscle, testes and heart.

As shown in Chapter 2, 9804 is a structural homologue of TSA-1. It is therefore anticipated that 9804 will be found to be a functional homologue of mouse TSA-1 as well. A preliminary survey of 9804 expression in a variety of human tissues and tumor cell lines was performed by Northern blot analysis. Circumstantial evidence for functional similarity came from a similar pattern of expression in developing and mature tissues. Northern blot analysis was also performed to detect TSA-1 gene expression in mouse tissues. At this time we also noticed occasional use of a different exon I in the mouse *TSA-1* gene, and included a survey of tissue alternate exon I usage. Since many

EST copies of the *9804* gene, like our original cDNA, do not extend into exon I, we also entertain the idea that an alternative promoter is used in 9804 transcription.

3.2 Tissue Distribution Pattern of 9804 RNA And Comparison with TSA-1

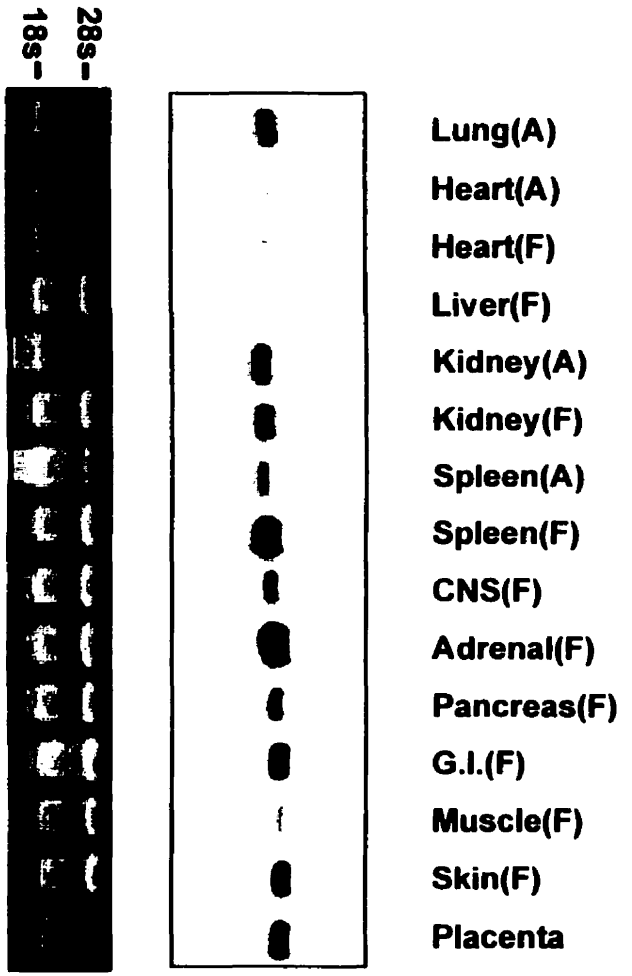
Human multiple tissue Northern (MTN) blots were purchased from Clonetech (Palo Alto) and processed for hybridization by Drs. E. Cohen and B. Landgraf at CytoMed Inc. RNA from various human fetal and adult tissues, kindly supplied by Dr. A. Bateman in the Endocrine Laboratory, McGill University, was processed for Northern analysis. Figure 3.1 shows the results of the Northern blot analysis of human tissues with 9804 cDNA probe. There is only slight 9804 expression in adult or fetal skeletal muscle and low messenger levels in pancreas and testis. The highest messenger levels in the adult are found in the liver, kidney, ovary and peripheral blood leukocytes (PBLs). In contrast to adult liver, fetal liver contains a very low amount of this messenger. Other tissues containing moderate levels include adult brain, placenta, lung and colon. Lower levels are present in the heart, spleen, thymus, prostate and small intestine. Fetal spleen has a relatively high amount of 9804 mRNA as compared with adult. This same high level is evident also in fetal adrenal. Moderate levels are found in fetal skin and similar to adult, it is also present in moderate amounts in fetal gastrointestinal (G.I.) tissue.

For comparison with TSA-1 mRNA expression, total RNA was prepared from various tissues of C57BL/6 and C3H mice and subjected to Northern blot analysis. With TSA-1 cDNA probe, a transcript was detected in all the tissues tested (Fig.3.2). The expression level is consistent with the previous observations by Godfrey *et al* (Godfrey *et al.*, 1992) and Tucek *et al* (Tucek *et al.*, 1992). It is high in thymus, bone marrow, lung, liver and adrenal, moderate in brain, heart, uterus, G.I, placenta, testis, thymocyte and C3H kidney; low in muscle. The high level of TSA-1 RNA expression in mouse adrenal is reminiscent of 9804 expression in human adrenal, and has not been reported before now.

In mouse, a polymorphism which controls antithetical structural epitopes or level of surface expression has been observed in Ly-6A/E, Ly-6B, Ly-6C and Ly-6F or ThB. Generally, mice of Ly-6.2 phenotype exhibit levels of expression in certain tissues which

Figure 3.1 Tissue Distribution of 9804 RNA Expression

Northern analyses were performed in a human multiple-tissue Northern blot (MTN, Clontech; A) and total RNA from adult and fetal (18 weeks) tissues (B). An attempt was made to load equal amounts (20 μg) of each. To show loading differences and check on the quality of the RNA preparations, the intensity of ethidium bromide staining was monitored and is presented below the blot. The blots were probed with 9804 cDNA probe. Relative to the size markers, 9804 mRNA was approximately 1.3kb.


28s
18s
Lung(A)
Heart(A)
Heart(F)
Liver(F)
Kidney(A)
Kidney(F)
Spleen(A)
Spleen(F)
CNS(F)
Adrenal(F)
Pancreas(F)
G.I.(F)
Muscle(F)
Skin(F)
Placenta

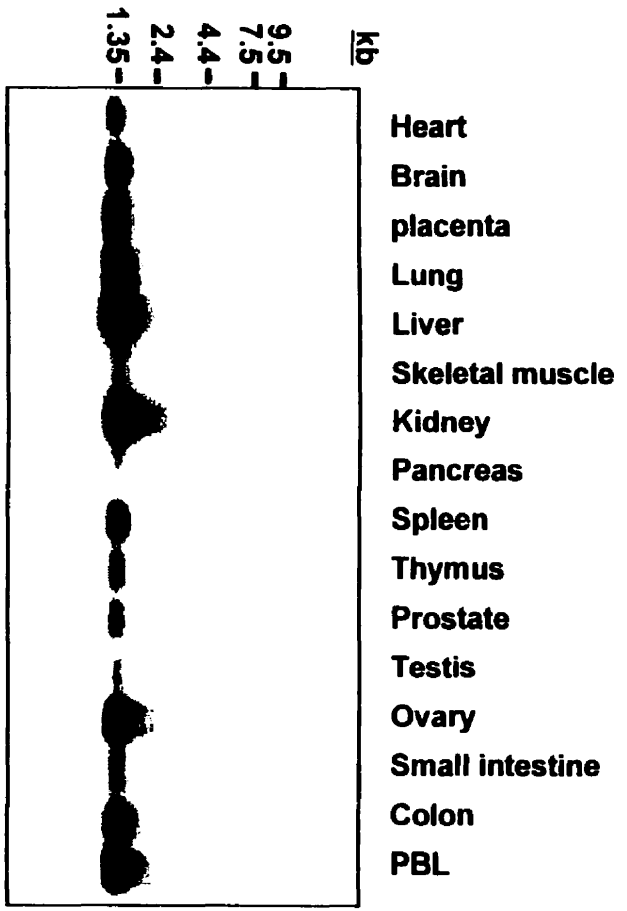
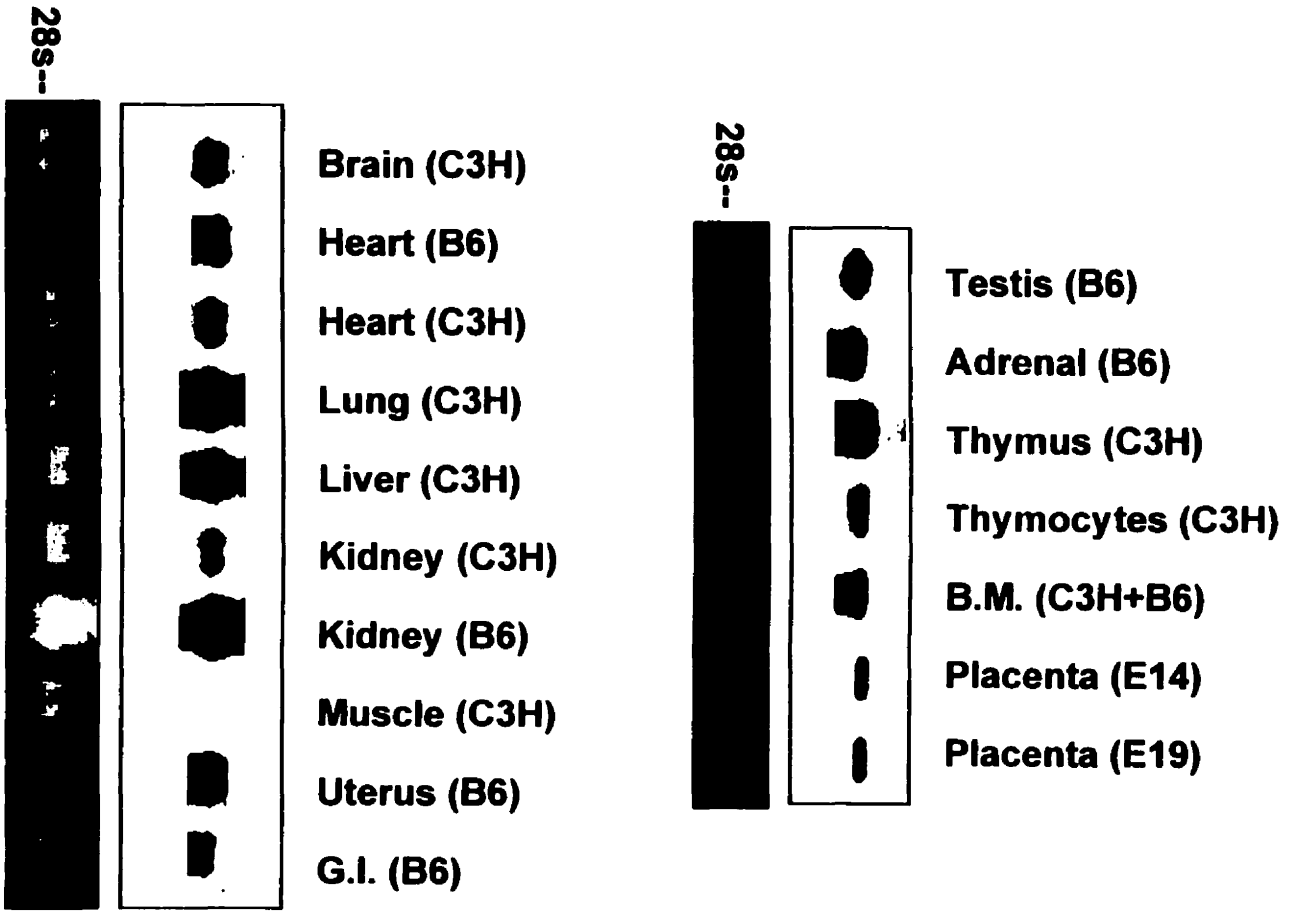

kb
9.5
7.5
4.4
2.4
1.35
Heart
Brain
placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small intestine
Colon
PBL

Figure 3.2 Tissue Distribution of Mouse TSA-1 RNA

Total RNA was extracted from C57BL/6 (B6) and C3H mouse tissues. 10-20 μg RNA was used in each lane. The loading difference is shown in the bottom panels of ethidium bromide staining. The blots were hybridized with mouse TSA-1 cDNA probe (Pan-TSA-1).


28s--
Brain (C3H)
Heart (B6)
Heart (C3H)
Lung (C3H)
Liver (C3H)
Kidney (C3H)
Kidney (B6)
Muscle (C3H)
Uterus (B6)
G.I. (B6)
28s--
Testis (B6)
Adrenal (B6)
Thymus (C3H)
Thymocytes (C3H)
B.M. (C3H+B6)
Placenta (E14)
Placenta (E19)

are higher than found in mice of the Ly-6.1 phenotype. For example, ThB on B lymphocytes is higher in Ly-6.2 mice. No such polymorphism has been reported with TSA-1. In our Northern analysis, although no such differential expression was observed in most tissues tested, the expression level of TSA-1 in C57BL/6 (Ly-6.2) kidney was much higher than that was observed in C3H (Ly-6.1) kidney. The observation was consistent in several experiments with either the full cDNA probe (Fig. 3.2) or the exon I probe (Fig. 3.7). Further studies on TSA-1 expression in additional mouse strains will be needed to demonstrate consistency and a link with Ly-6 phenotye.

3.3 Expression of 9804 RNA in Cell Lines

Northern analysis was performed on RNA preparations derived from a panel of human tumor cell lines (Table 3.1) covering lymphoid, myeloid, epithelial, mesodermal and neuroepidermal origins which had been grown under conditions recommended by ATCC (Fig. 3.3). 9804 is expressed moderately in T cell lines Jurkat and Hut78 but not B cell lines Daudi and Raji. Myeloid cell lines express different levels of 9804 transcript. It is interesting to note that within the myeloid cell lines, the most primitive cell lines, KG-1 and KG-1a, have the highest levels, while the most mature ones, HL60 and U937, have the lowest levels. The expression in NB4 and TF-1 falls in between. Although normal CNS has a low level of expression, the neuroblastoma cell line SK-N-MC expressed an impressively high level of 9804 mRNA. The glioblastoma U373MG, however, has a very low expression level. This suggests a major contribution by the parenchymal cells of the brain to 9804 expression in that tissue. In comparison with the normal counterpart, skin, the epidermoid carcinoma cell lines A431 and CaSki had an elevated level of 9804 RNA expression. A low level 9804 expression was detected in the leiomyeosarcoma line HTB88 and moderate expression in the renal adenocarcinoma ACHN.

3.4 Detection of 9804 Expression with Exon I Probe 9804X1R

With a reverse (9804X1R) primer derived from the noncoding exon I of the 9804 gene, we didn't succeed with primer extension in RNA from any cell lines. However, we did detect a protected fragment of 294 bp in A431, but not KG-1a, cell lines with an S1

Figure 3.3 Expression of 9804 RNA in Human Cell Lines

Northern analysis was performed with RNA (20μg/lane) prepared from the human cell lines. The blots were probed with [^{32}P]-random labeled 9804 cDNA. The intensity of ethidium bromide staining is presented in the bottom panel.

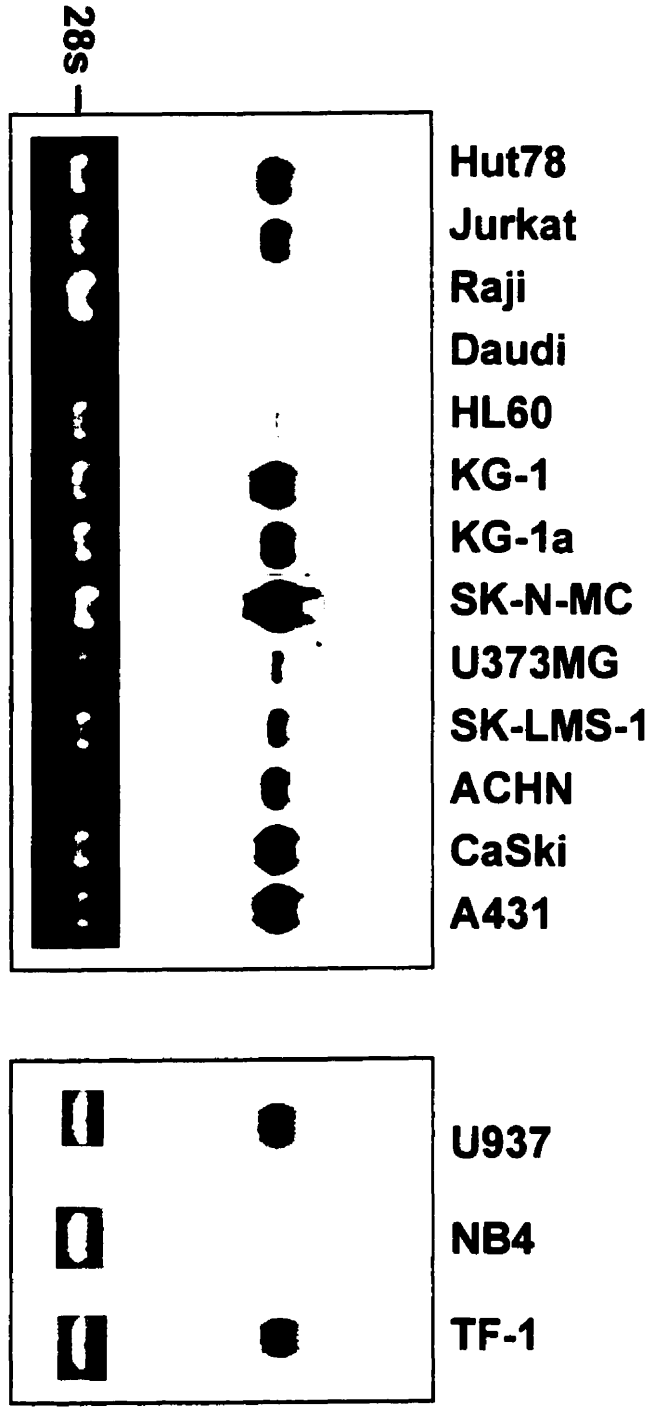

28s
Hut78
Jurkat
Raji
Daudi
HL60
KG-1
KG-1a
SK-N-MC
U373MG
SK-LMS-1
ACHN
CaSki
A431
U937
NB4
TF-1

Table 3.1 Human Cell Lines Used in 9804 Expression

Hut78	cutaneous T cell lymphoma	(ATCC TIB 161)
Jurkat	T cell lymphoma	(ATCC CRL 8163)
Raji	Burkitt lymphoma, B lymphoblast-like	(ATCC CCL 86)
Daudi	Burkitt lymphoma, B lymphoblast	(ATCC CCL 213)
HL60	peripheral, promyelocytic leukemia	(ATCC CCL 240)
KG-1	bone marrow, acute myeogenous leukemia	(ATCC CCL 246)
KG-1a	variant subline of KG-1	(ATCC CCL 246.1)
SK-N-MC	matestatic neuroblastoma	(ATCC HTB 10)
U373MG	glioblastoma, astrocytoma, grade III	(ATCC HTB 17)
SK-LMS-1	leiomyosarcoma	(ATCC HTB 88)
ACHN	renal adenocarcinoma	(ATCC CRL 1611)
CaSki	epidermoid carcinoma	(ATCC CRL 1550)
A431	epidermoid carcinoma	(ATCC CRL 1555)
U937	histiocytic lymphoma	(ATCC CRL 1593)
TF-1	erythromyeloid leukemia	(ATCC CRL 2003)
NB4	acute promyeloid leukemia	(DSM ACC 207)

nuclease assay (Fig. 2.8). These observations may indicate that exon I is not utilized in some particular tissues or cell lines. Although exon I exists in most Ly-6 genes, no such exon I has been found in ThB gene thus far. Moreover, as will be described later in this chapter, we recently found that a second exon I was occasionally utilized for transcription initiation in TSA-1. This alternative exon I is upstream of the usual TSA-1 exon I.

We used 9804X1R to probe our human RNA preparations, in order to investigate the possibility that this exon is not always present in 9804 transcript. The transcript was detected in liver, spleen, adrenal, CNS, skin, G.I., and kidney and barely detectable in heart, muscle, pancreas and (Fig. 3.4). The intensity of the band corresponds well to what was seen with 9804 cDNA probe. These RNA preparations, however, were from mixed cell types, so differences due to one cell type may not be noticeable. Relatively homogeneous cell lines were likely to be more informative.

Among the cell lines (Fig. 3.5), the expression of exon I was detected in U937, TF-1, Jurkat, U373MG, CRL1161, HL-60, NB4, KG-1, KG-1a, CaSki and A431. There was no detectable 9804 mRNA found in the cell lines Raji, Daudi and U373MG as was expected from the results with the full cDNA probe, which also showed little, or no expression of 9804. However KG-1a, which has a high level of 9804 mRNA detectable with the full cDNA probe, also showed little 9804 expression with the 9804X1 probe. This is consistent with the result of the S1 nuclease assay that no protected band was seen in KG-1a (Fig. 2.8). It is therefore likely that, in the KG-1a cell line, exon I is barely used. Transcription may start from the first coding exon, the exon II, of the *9804* gene or an alternative exon I which has yet to be discovered. Prediction of a potential promoter in the intron 1 of the *9804* gene (Table 3.2) defined several potential transcription initiation sites, one of which is located at 1,808 bp 5' to the exon II with a score of 0.97 (score range 0 to 1.0). Thirty-eight bp downstream there is a sequence typical of well conserved splicing donor site (Fig. 3.6). This possible promoter region is GC-rich (70%) and a few GC boxes are also found in this region (Fig. 3.6). So far no 9804 sequence includes this possible alternate exon I has been added to the EST database, so this is at present purely hypothetical.

Figure 3.4 9804 Exon I Expression in Human Tissues

Northern analysis was performed with RNA (20μg/lane) prepared from human tissues. The blots were probed with end-labeled oligonucleotide probe 9804X1R.

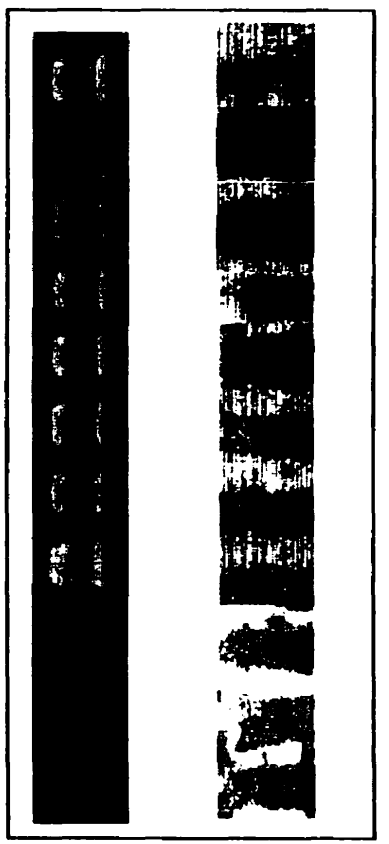

Heart (F)
Heart (A)
Liver (F)
Spleen (F)
Adrenal (F)
CNS (F)
Skin (F)
Muscle (F)
G.I. (F)
Pancreas (F)
Kidney (F)

Figure 3.5 9804 Exon I Expression in Cell Lines

Northern analysis was performed with RNA (20μg/lane) prepared from human cell lines. The blots were probed with end-labeled oligonucleotide probe 9804X1R.

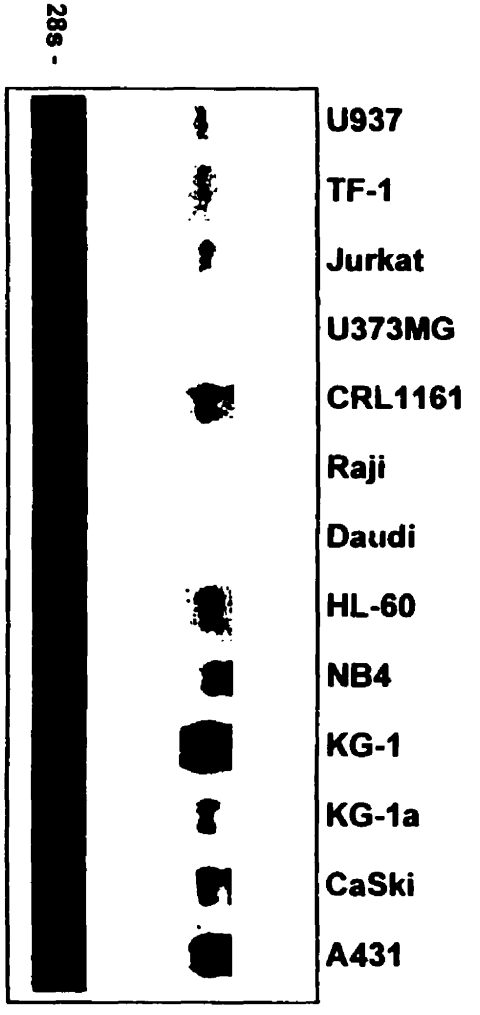

28s -
U937
TF-1
Jurkat
U373MG
CRL1161
Raji
Daudi
HL-60
NB4
KG-1
KG-1a
CaSki
A431

Figure 3.6 Potential Promoter and Alternate Exon I in the Intron 1 of the 9804 Gene

Five potential transcription initiation sites corresponding to those shown in Table 3.2 are presented in boldface uppercase letters. The predicted transcription initiation site with the hignest prediction score (0.97) is at the nucleotide position 543 (G). 31 nucleotides downstream from the G, a highly conserved splicing donor site is indicated with bold face italic letters.

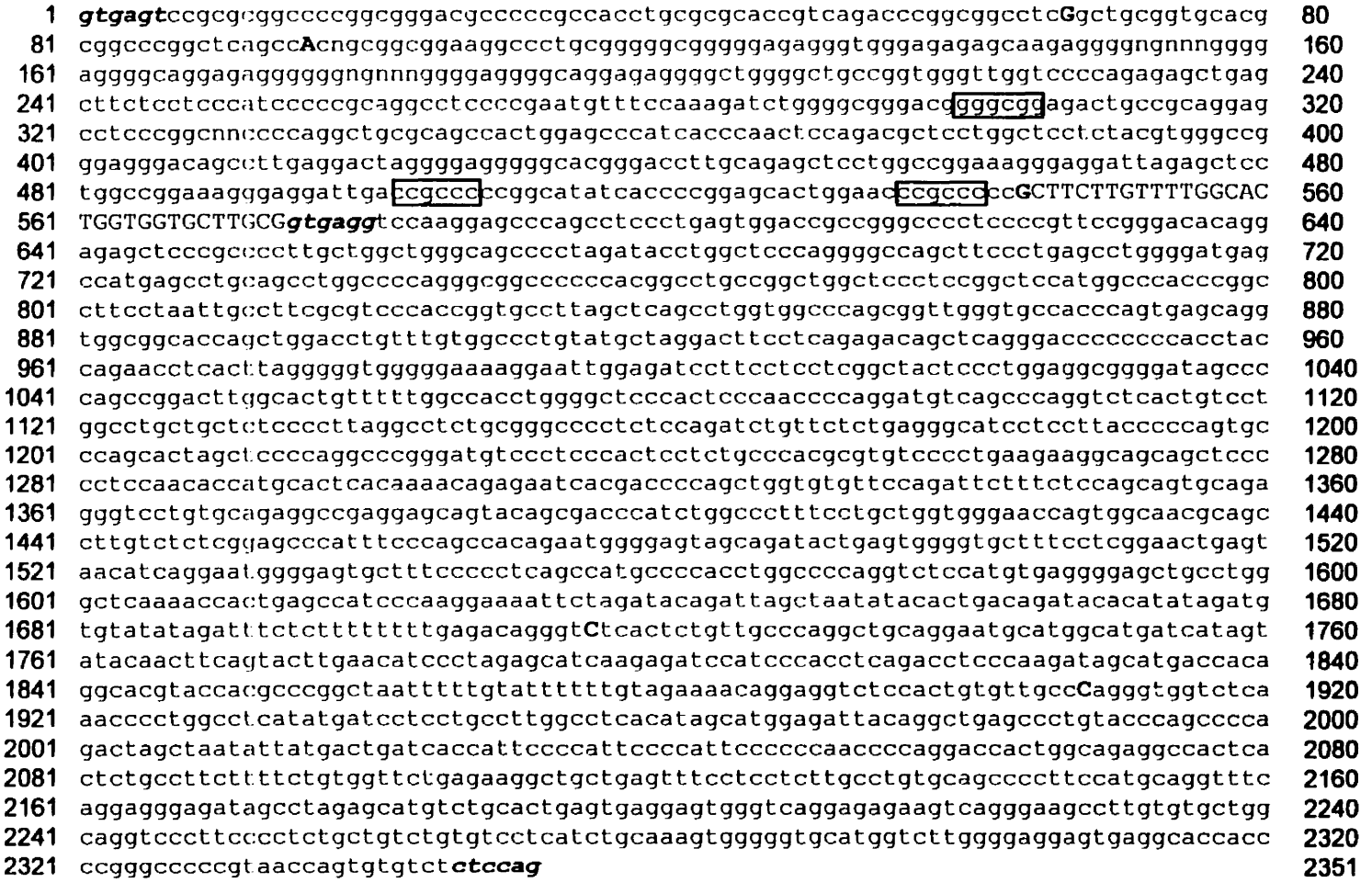


Table 3.2 Promoter Prediction in the Intron 1 of 9804 Gene[a]

Start[b]	End	Score	Promoter sequence[c]
27	77	0.70	gcccccgccacctgcgcgcaccgtcagacccggcggcctc**G**gctgcggtg
56	106	0.85	ccggcggcctcggctgcggtgcacgcggcccggctcagcc**A**cngcggcgg
450	500	0.97	gcccccggcatatcaccccggagcactggaacccgcccccc**G**cttcttgtt
1621	1671	0.72	tagatgtgtatatagatttctctttttttgagacagggt**C**tcactctgt
1854	1864	0.72	gtatttttgtagaaaacaggaggtctccactgtgttgcc**C**agggtggtc

[a] Predictions were produced in an EPPNN analysis (Reese and Eeckman, 1995).

[b] Positions corresponding to the numbering in Figure 3.6.

[c] The boldface uppercase letters indicate the potential transcription initiation sites.

3.5 Alternative Usage of Exon IA And IB of TSA-1

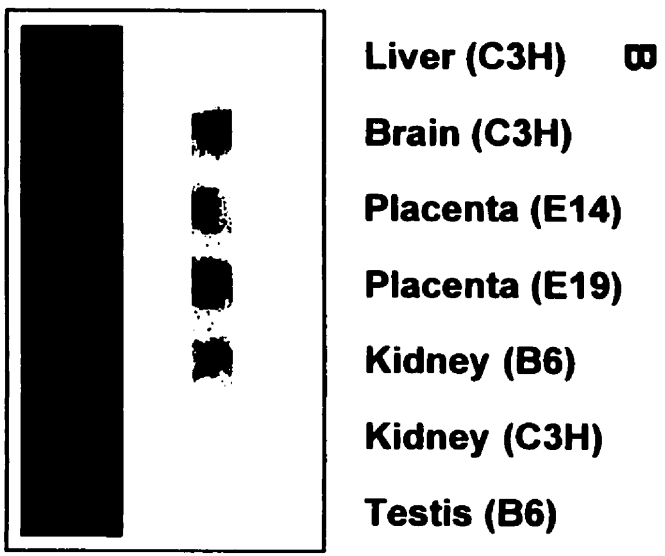
Mouse TSA-1 genomic sequence was recently reported (Classon and Coverdale, 1996), which identified a typical four exon organization of Ly-6 genes. However, our EST database search revealed a stretch of novel mouse placenta cDNA sequence (GenBank accession number AA023213) 5' to exon II of TSA-1, at the place of reported TSA-1 exon I. Alignment of the cDNA sequence with the known TSA-1 genomic sequence (EMBL/GenBank accession number U47737/U47850) located the novel cDNA sequence 315 bp 5' to the known TSA-1 exon I. For convenience, the previously reported exon I was named exon IA, while the novel cDNA sequence was called exon IB. To investigate whether exon I is alternatively utilized in different tissues or stages of development, we initiated a Northern blot analysis of mouse tissues with probes prepared from cDNA (pan-TSA-1) , and probes specific for exon IA (TSA-1XIA) and exon IB (TSA-1XIB). When detected by pan-TSA-1, the mRNA had a broad distribution in almost all the tissues tested (Fig. 3.2). With the TSA-1XIA probe, a basically identical tissue distribution pattern was observed (Fig. 3.7A). In contrast, the TSA-1XIB usage was found to be more limited and detected distinctively only in the brain, C57BL/6 kidney and placenta (Fig. 3.7B). Considering that the exon IB signal is much weaker than that of exon IA, it is evident that exon IA is preferentially selected in most tissues, while exon IB is activated under certain circumstances.

3.6 Discussion

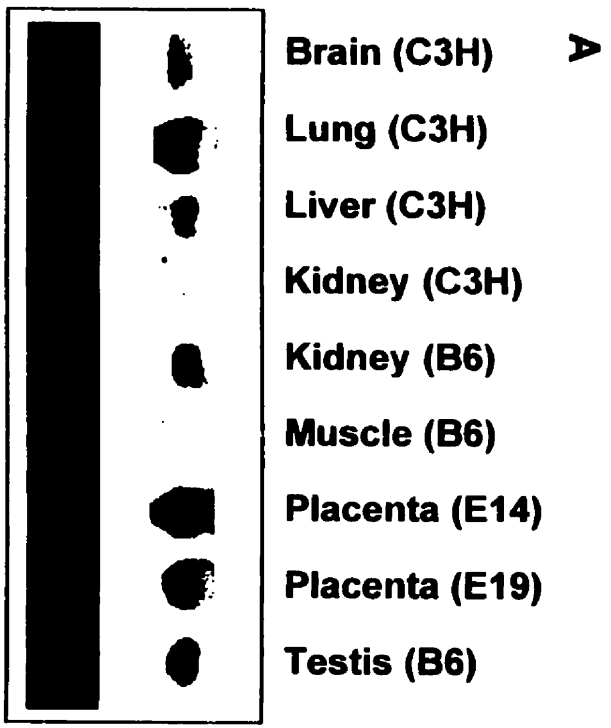
By comparison of human 9804 and mouse *TSA-1* gene expression in the Northern blot analysis, it is evident that both messages are broadly distributed in a variety of tissues. Another parallel between TSA-1/Sca-2 and 9804 mRNA expression is the similarity of expression levels in certain tissues. For example, low levels are expressed in both testis and heart while high levels are found in liver and lung. Expression of both genes can be detected in kidney, spleen and brain. Furthermore, both human and mouse adrenals express very high levels of the corresponding RNAs. However, a discrepancy between the expression of 9804 and TSA-1 was observed in our Northern analysis. Although, TSA-1 is mainly expressed on peripheral B cells and little on T cells, 9804 is neither expressed on the B

Figure 3.7 Use of Alternative First Exon for TSA-1 Transcription in Different Mouse Tissues

The same RNA preparations as in Figure 3.2 were analysed. The top panel (A) shows the Northern blot with exon IA probe (TSA1XIAR oligonucleotide) and bottom one (B) with exon IB cDNA probe. The cDNA sequence corresponds to the whole exon IB (Fig. 3.8), generated by PCR amplification with TSA1X1BR/T7 primer pair and subsequently cloned into pBluescript II KS^+ vector.


Liver (C3H)
Brain (C3H)
Placenta (E14)
Placenta (E19)
Kidney (B6)
Kidney (C3H)
Testis (B6)

B


Brain (C3H)
Lung (C3H)
Liver (C3H)
Kidney (C3H)
Kidney (B6)
Muscle (B6)
Placenta (E14)
Placenta (E19)
Testis (B6)

A

lymphoblast cell lines Daudi and Raji, nor is the expression inducible by IFNs or ATRA in these cells (see Chapter 4). In contrast, it was expressed at a moderate level on the normal PBL and T cell lymphoma Jurkat. Since tumor cells have undergone a series of biological changes, we do not know whether the data from Daudi and Raji are any indication of 9804 expression in normal B lymphocytes. Further studies of 9804 expression on different cell types will be better performed by using FACS analysis once Abs to 9804 are available, or by *in situ* hybridization in normal cells or tissues sections. Overall, our data from Northern analyses are consistent with the notion that 9804 is a functional homologue of mouse TSA-1/Sca-2.

A role of TSA-1 in early intrathymic T cell development has been proposed (MacNeil *et al.*, 1993; Randle *et al.*, 1993). But the broad tissue distribution and high level expression of the human *9804* and mouse *TSA-1* genes in some of the nonlymphoid tissues suggest that their function may not be limited to hematopoiesis. Among the nonlymphoid tissues, human and mouse adrenals have the most impressively high levels of 9804 and TSA-1 expression. It is tempting to speculate that 9804 and TSA-1 are involved in some physiological processes in the adrenal gland. However we have no direct data to support this notion and it will be pertinent to investigate which cell type(s) in the adrenal gland express 9804. The level of 9804 RNA expression is generally elevated in most tumor cell lines examined in this study as compared with their normal counterparts. The neuroblastoma cell line SK-N-MC, the epidermoid carcinoma cell lines A431 and CaSki, and the myeloid cell lines KG-1, KG-1a, TF-1, and U937 all showed a significantly increased 9804 expression compared with the CNS, skin (Fig. 3.1, Fig. 3.3) and granulocytes (data not shown). Furthermore, within the myeloid leukemias, the most immature one, KG-1, showed a much higher level of 9804 RNA expression than the most mature one, HL60, in which granulocytic cell differentiation can be observed in a certain percentage of cultures (Gallagher *et al.*, 1979). It has been suggested from previous studies that Ly-6 might be a marker of tumor-associated membrane alterations in NIH 3T3 cells (Ivanyi *et al.*, 1986) and the expression of Ly-6A/E was upregulated on highly tumorigenic variants of polyoma virus-transformed Balb/c 3T3 cells as compared with weakly tumorigenic variants (Katz *et al.*, 1994). *In vivo* studies indicated that the Ly-6^{hi}

cells exerted a significantly higher tumorigenicity and metastatic phenotype than Ly-6^{lo} cells (Eshel *et al.*, 1995). In our Northern studies, the level of 9804 mRNA expression increased in most tumor cell lines and decreased with increasing grade of tumor cell differentiation in myeloid leukemias. These observations also suggest that 9804 might be associated with tumorigenesis and could be used as a marker for the grade of tumor cell differentiation.

Alternative exon usage is a widespread phenomenon in higher eukaryotes (Feng *et al.*, 1997, Agarwal *et al.* 1996, Mahnke-Zizelman *et al.*, 1996, Busslinger *et al.*, 1996, Hu *et al.*, 1996, Gachter *et al.*, 1996). This process occurs along with an alternative splicing mechanism to regulate gene expression in a tissue or cell specific manner (Chabot, 1996; Gallego *et al.*, 1996). Ly-6 is a multigene family of molecules expressed in variety of tissues or cells. The functional forms of the proteins are encoded by multiple closely related genes rather than by alternative splicing mechanism, though alternative splicing has been described in exon I of Ly-6E (LeClair *et al.*, 1986, McGrew *et al.*, 1991), Ly-6G.1 and, probably, Ly-6F.1 (Fleming *et al.*, 1993a). No multiple exon usage has been described in Ly-6 genes thus far. Now we report a second mRNA species of TSA-1 containing a 5' sequence encoded by a novel exon I (1B) located 315 bp 5' to the known TSA-1 exon I (IA). This mRNA species was only found in brain, placenta and C57BL/6 kidney tissues, while the proximate exon I (exon IA) is preferentially utilized in most of the tissues including placenta, brain and kidney. This observation implies that tissue specific factors may be involved in the selection of the exon IB promoter. Further studies will be required to ascertain which cell types preferentially use exon IB, such as *in situ* hybridization. The functional relevance of the alternative exon I itself is unclear as either exon I is completely nontranslated. However the 5' untranslated region may regulate the gene expression by controlling mRNA stability (Bonnieu *et al.* 1988; Moore *et al.*, 1988).

Interestingly, a potential alternate exon I usage was also noticed in *9804* gene expression. From our S1 nuclease assay, a strongly labeled protected fragment of 294 bp was detected in A431 but not in KG-1a cells (Fig. 2.8). Comparison of the expression of the 9804 RNA and 9804 exon I in these cell lines showed a significantly decreased 9804 exon I expression in KG-1a cells (Fig. 3.5). With the intron 1 sequence, a promoter was

predicted by EPPNN with a high prediction score (Table 3.2). The transcription initiation site is at the nucleotide 543 of the intron 1 (Fig. 3.6), but no apparent transcription initiation site directly from the exon II was predicted. The inability to detect an S1 nuclease protected fragment when KG-1a cell RNA was used may therefore indicate that 9804 transcription is sometimes initiated from another promoter such as the one predicted with high score in the intron 1. We have made constructs of genomic fragments from both 5' flanking region of the exon I and from intron 1 in a luciferase reporter vector in order to investigate this further.

Characterization of the tissue distribution of the TSA-1 and 9804 RNA revealed that while expressed in a variety of hematopoietic cells, they are among the Ly-6 genes most widely expressed also in nonhematopoietic tissues. Although widely expressed, 9804 transcription is regulated during cell differentiation and immune responses as will be demonstrated in the next chapter.

CHAPTER 4

REGULATION OF 9804 TRANSCRIPT LEVELS

4.1 Introduction

Ly-6 molecules have been implicated in cellular activation and immune responses. Mitogen activation of T or B cells leads to increased expression of Ly-6A/E (Kirmura *et al.*, 1980; Rock *et al.*, 1986, Reiser *et al.*, 1986b; Palfree *et al.*, 1986a; Kirmura *et al.*, 1984). The expression of Ly-6C is also increased on mitogen-stimulated T cells (Takei *et al.*, 1980; Kirmura *et al.*, 1984). Although not expressed in resting T cells, TSA-1 is induced on T cells treated with ConA (Godfrey *et al.*, 1988; Izon *et al.*, 1996).

The expression of Ly-6 molecules is regulated by cytokines, particularly IFNs. *In vitro* treatment of purified resting T cells with IFN-α/β results in a strong enhancement of Ly-6A/E, and to a lesser extent with IFN-γ, at both protein and RNA levels (Dumont *et al*, 1986). Ly-6C expression on T cells is also enhanced by IFN-α/β but not by IFN-γ (Rock *et al.*, 1986). Similar induction of Ly-6A/E by IL-1 was observed in several T cell lines (LeClair *et al.*, 1989). TNF synergistically acts with IFN-γ on this effect in T cells, thymocytes, bone marrow cells but not B cells (Malek *et al.*, 1989). *In vivo* treatment of mice with either IFN-α/β, IFN-γ or the IFN inducer poly(I:C) upregulates Ly-6A/E expression on T cells (Dumont *et al.*, 1986b), B cells, B cell tumors and bone marrow cells (Malek *et al.*, 1989). Endogenous IFN-γ production appears to be responsible at least partially for the upregulation of Ly-6 on mitogen ConA or IL-1-activated T cells as the induced expression is blocked by mAb to IFN-γ (Dumont *et al.*, 1988; LeClair *et al.*, 1989). In T cells, the induction of Ly-6 expression by IFNs occurs at a dose which does not elicit an apparent enhancement of other important T cell surface molecules, such as H-2, β2m, Thy-1, Ly-1 and Lyt-2 (Dumont *et al.*, 1986). In addition, Ly-6 expression is also strongly induced by IFNs in a variety of nonlymphoid tissues/cells, including mouse bone marrow or thymic stromal cells or cell lines (Godfrey *et al.*, 1988; Izon *et al.*, 1996), renal tubular epithelium and vascular endothelium (Blake *et al.*, 1993), central nervous system (Cray *et al.*, 1990), osteosarcomas (Horowitz *et al.*, 1994). The IFN inducibility

of Ly-6 in cellular activation may also indicate a more general role for these molecules in immune modulation than T cell activation.

While studying the regulated expression of the 9804 gene by cytokines, particularly IFNs, the 9804 gene was reported to be a retinoic acid inducible gene, RIG-E (Mao *et al.*, 1996). The RIG-E cDNA was cloned from a library derived from the APL cell line NB4 during ATRA-induced differentiation. ATRA has a potent effect on the differentiation of normal or leukemia promyeloid cells causing these cells to preferentially differentiate towards the granulocytic lineage, and has been used successfully in establishing complete remission in APL patients (Breitman *et al.*, 1980; Breitman *et al.*, 1981; Huang *et al.*, 1988; Castaigne *et al.*, 1990). As one of a few genes whose expression are highly inducible by ATRA, 9804 is likely to be involved in this cellular differentiation process. In addition to ISRE, a potential RARE was also found in the 5' flanking region of the 9804 gene (see Chapter 2). To further investigate the conditions under which 9804 function is likely to be relevant, the regulated expression of the 9804 gene by cytokines and ATRA was studied in several different cell types and is presented in this chapter.

4.2 Effect of cytokines/ATRA on 9804 RNA expression in cell lines

To test if the human structural homologue of mouse TSA-1 is modulated by cytokines, and if ATRA functions in cell types other than NB4, 9804 expression was studied by Northern blot analysis. A panel of 13 human recombinant cytokines were applied to the cultures of an array of human tumor cell lines representing myeloid (U937, NB4, HL-60, TF-1 and KG-1), lymphoid (Jurkat and Raji), glioblastoma (U373MG) and leiomyosarcoma (THB88) cell types. Except for Raji, all the cell lines expressed 9804 RNA although at different levels (Fig. 4.1). After 20 hrs treatment under the concentrations applied (Table 4.1), the majority of the cytokines (IL-1, IL-2, IL-11, SCF, M-CSF, GM-CSF, IL-3, IL-4, G-CSF, IFN-γ, IL-4 and IL-5) by themselves did not elevate or suppress 9804 mRNA levels. But IFN-α did induce a strong enhancement of 9804 mRNA in most of these cell lines (Fig. 4.1). The most dramatic induction was observed in the glioblastoma U373MG and lieomyosarcoma HTB88 cell lines that had

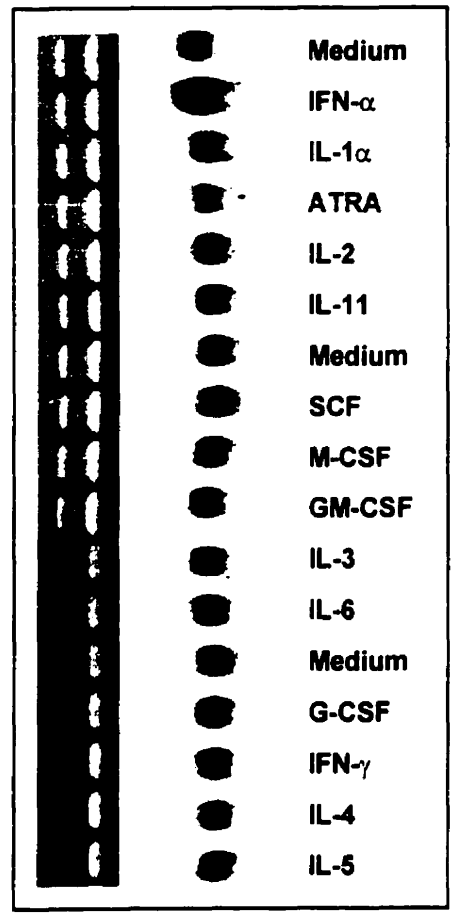
Table 4.1 Cells And Reagents Used in The 9804 Transcription Studies

CELLS/CELL LINES		REAGENTS	CONCENTRATION
U937	histiocytic lymphoma	**(Schering Canada Inc.*)**	
Jurkat	T lymphoma	IFN-α-2b	1,000 units/ml
TF-1	erythroleukemia	**(Genetics Institute, Inc. USA*)**	
Raji	B lymphoma	IL-1α	10 units/ml
NB4	acute promyelocytic leukemia	IL-2	2.5 units/ml
HTB88	leiomyosarcoma	IL-11	1,000 ng/ml
KG-1	acute myelogeneous leukemia	SCF	10 units/ml
U373MG	glioblastoma	M-CSF	100 ng/ml
HL-60	promyelocytic leukemia	GM-CSF	50 ng/ml
PBMC	peripheral blood mononuclear cells	IL-3	100 units/ml
		IL-6	100 units/ml
REAGENTS	**CONCENTRATION**	G-CSF	1,000 units/ml
		IFN-γ	100 units/ml
(Sigma)		IL-4	100 units/ml
ATRA	1μM	IL-5	50 ng/ml

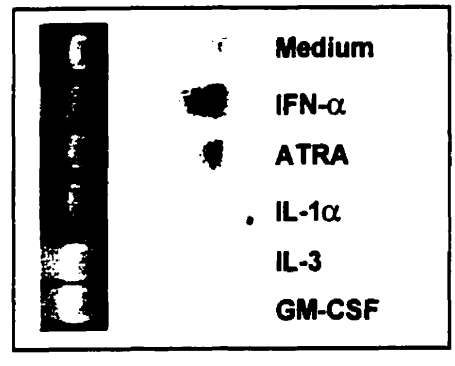
* All are human recombinant cytokines.

Figure 4.1 Effect of Cytokines/ATRA on 9804 RNA Expression in Cell Lines

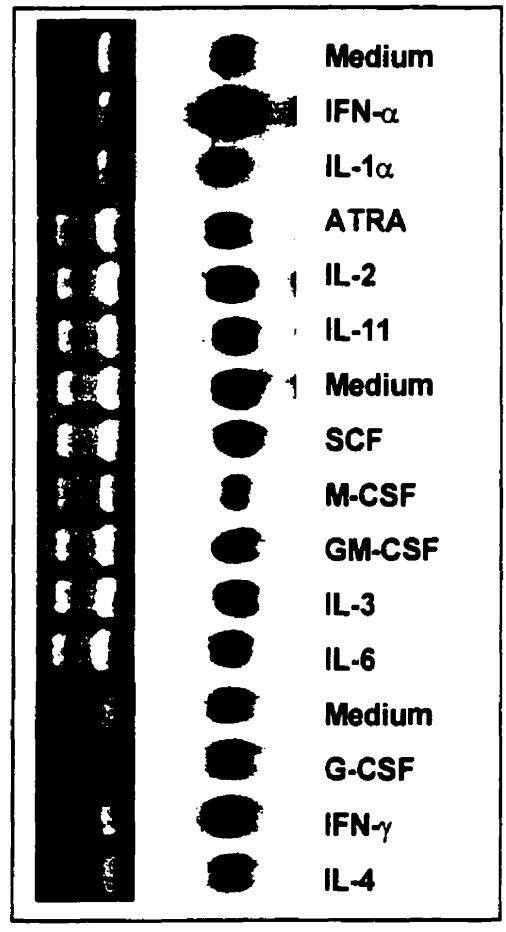
Cells of different origins (A to D: myeloid, E and F: Lymphoid, G: glioblastoma and H: leiomyosarcoma) were cultured at a density of 1.5 x 10^6/ml for 20 hours in the presence of cytokines and ATRA at concentrations given in Table 4.1. Total RNA (20 μg/lane) from each sample isolated by modified method of Chirgwin *et al.* (1979), were electrophoresed, blotted and probed with ^{32}P random priming labeled 9804 cDNA. Top panels are the autoradiographs and bottom panels are the ethidium bromide stained 28s and/or 18s rRNA to show equal loading. The size of single 9804 mRNA species is 1.3 kb.


Medium
IFN-α
IL-1α
ATRA
IL-2
IL-11
Medium
SCF
M-CSF
GM-CSF
IL-3
IL-6
Medium
G-CSF
IFN-γ
IL-4
IL-5

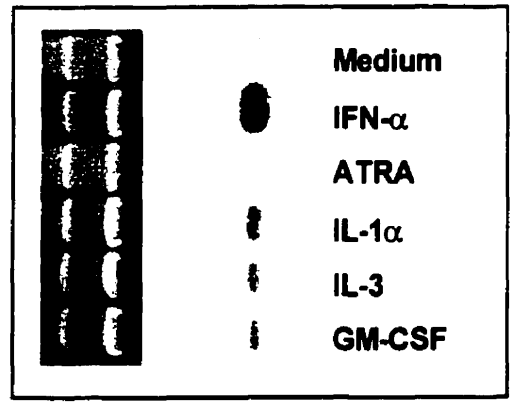
A. U937


Medium
IFN-α
ATRA
IL-1α
IL-3
GM-CSF

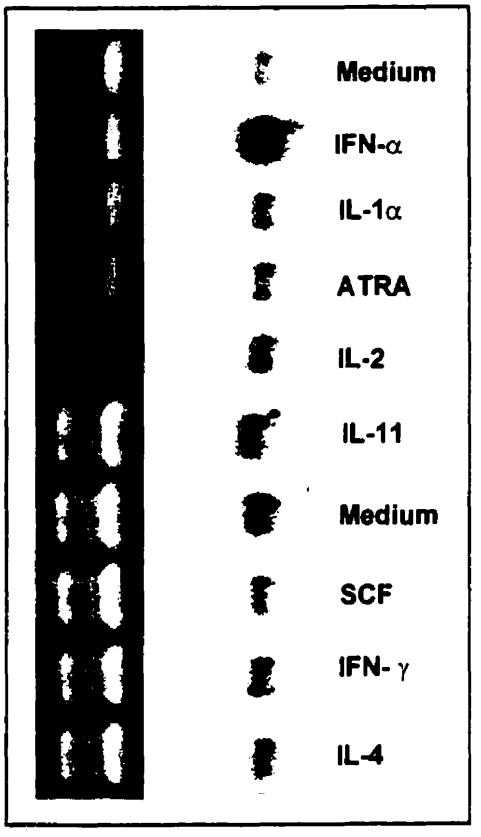
B. NB4


Medium
IFN-α
IL-1α
ATRA
IL-2
IL-11
Medium
SCF
M-CSF
GM-CSF
IL-3
IL-6
Medium
G-CSF
IFN-γ
IL-4

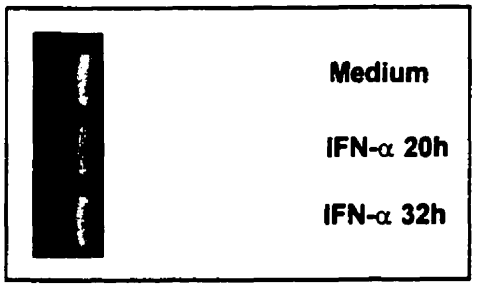
C. TF-1


Medium
IFN-α
ATRA
IL-1α
IL-3
GM-CSF

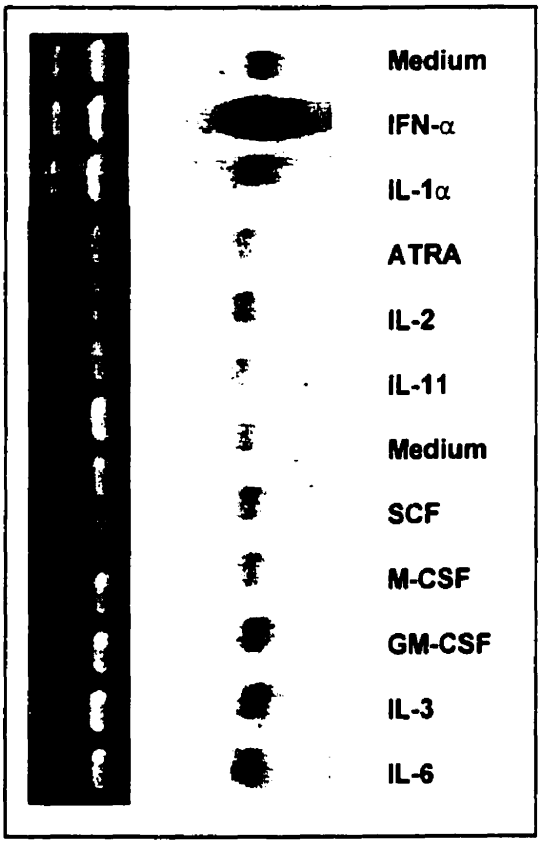
D. KG-1



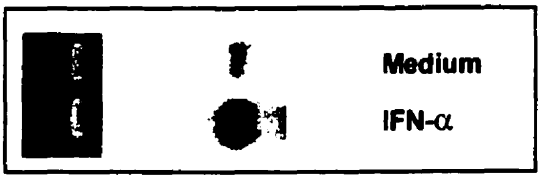
E. Jurkat



F. Raji



G. U373MG



H. HTB88

Figure 4.2 Induction of 9804 RNA by 48 hrs Induction with ATRA

The cells were cultured at a density of 1 x 10^6/ml with (+) or without (-) the addition of 1μM of ATRA (Sigma). Cells were then harvested after 48 hrs for RNA isolation. Total RNA samples (20 μg/lane) were separated on the gel, blotted and probed with ^{32}P random priming labeled 9804 cDNA.

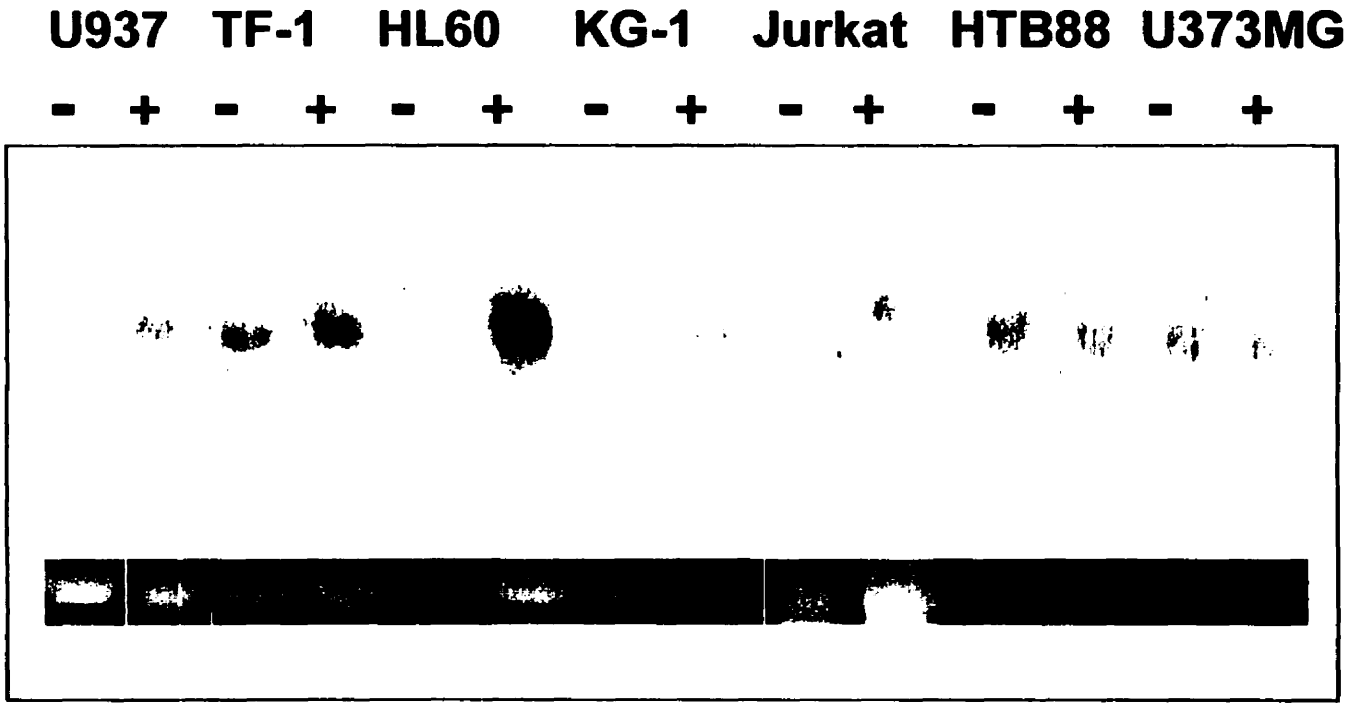
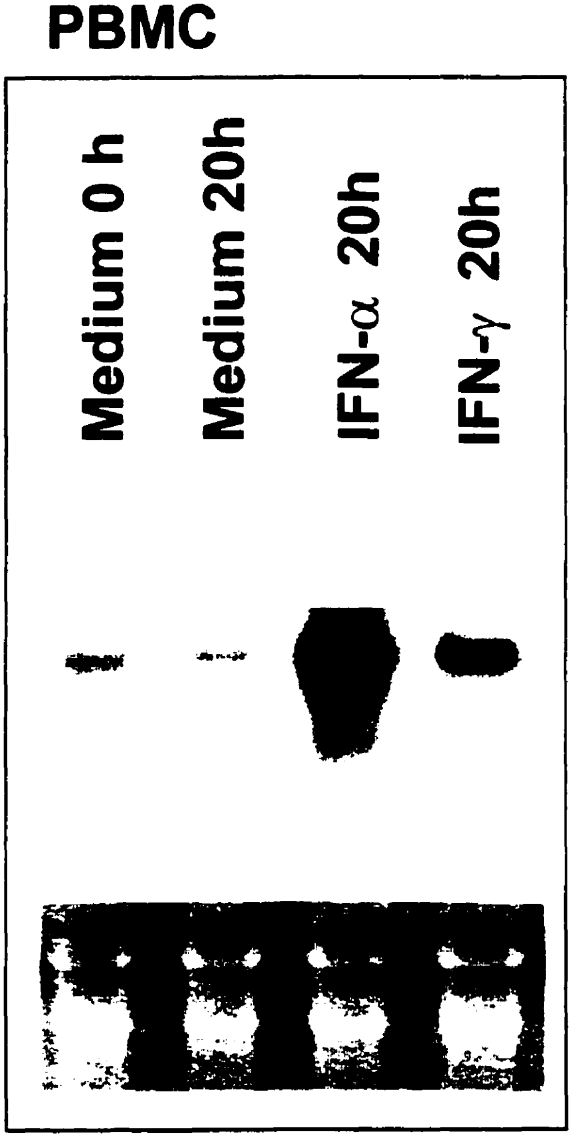

U937
TF-1
HL60
KG-1
Jurkat
HTB88
U373MG
- + - + - + - + - + - + - + - +

Figure 4.3 IFN Induction of 9804 RNA Expression in Normal PBMC

The mononuclear cells were isolated from heparinized peripheral venous blood of a healthy donor using Lympholyte®-H (Cedarlane Laboratory Ltd.) following manufacturer's instruction. The PBMC were plated at a density of 1.5 x 10^6/ml with or without IFN-α or IFN-γ and harvested at the time points indicated in the figure. Total RNA samples (15 μg/lane) were electrophoresed, blotted and probed with ^{32}P random priming labeled 9804 cDNA.


PBMC
Medium 0 h
Medium 20h
IFN-α 20h
IFN-γ 20h

relatively low basal level 9804 mRNA (Fig. 4.1H). However, 9804 mRNA was not induced by IFN-α in all cell lines. The B cell lines Raji (Fig. 4.1F) and Daudi (not shown) were not responsive even after prolonged exposure (32 hrs) to IFN-α.

In contrast to IFN-α, 20 hours of ATRA treatment at a concentration of 1 μM only induced a low level of 9804 expression in NB4 cells, but not in other cell lines tested including U937, TF-1, Jurkat, U373MG, HL-60, and KG-1 (Fig. 4.1). However, an apparent enhancement of 9804 RNA expression in HL-60 and a marginal induction in TF-1 and U937 were detected after 48 hrs exposure to ATRA (Fig. 4.2). Clearly, the induction of 9804 mRNA expression by ATRA is a phenomenon limited only to a few myeloid cell lines.

4.3 9804 RNA Induction by IFN in Normal PBMC

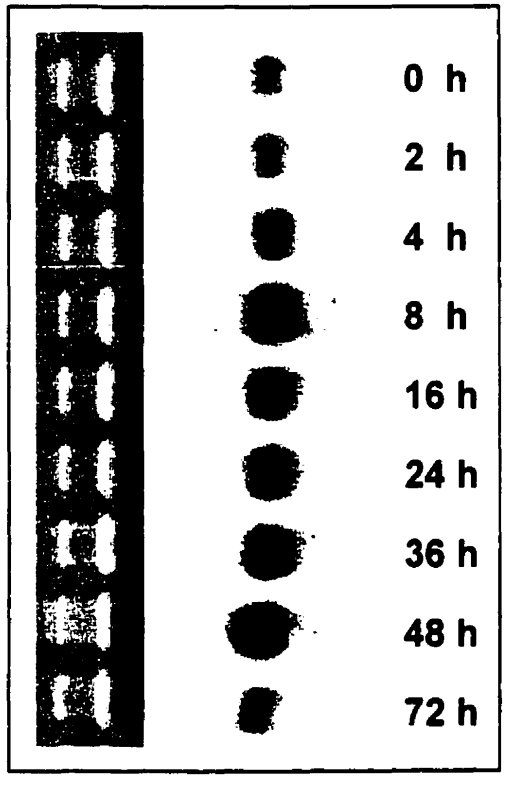
To study the induction of 9804 by IFNs in normal lymphocytes, peripheral blood mononuclear cells (PBMC) were isolated from a healthy donor and subjected to *in vitro* treatment with IFN-α and IFN-γ. IFN-α induced a strong 9804 mRNA expression in the PBMC after 20 hrs (Fig. 4.3). Interestingly, IFN-γ also induced 9804 mRNA expression although to a much lesser extent than IFN-α (Fig. 4.3). Presumably, T cells and granulocytes are major contributors to this increased level, since we know that cell lines of these types respond well, but since IFN-α had no effect on 9804 expression in Raji and Daudi, we can not assume that 9804 is induced in normal B cells by this cytokine. Clearly, studies with purified T or B cells are required since the unresponsiveness of Raji and Daudi to the IFNs might reflect an anomaly existing in these tumor cell lines. Indeed, 9804 (human TSA-1) expression was detected in cDNA libraries prepared from transformed B cell lines MRC5 and BC (Capon *et* al., 1996).

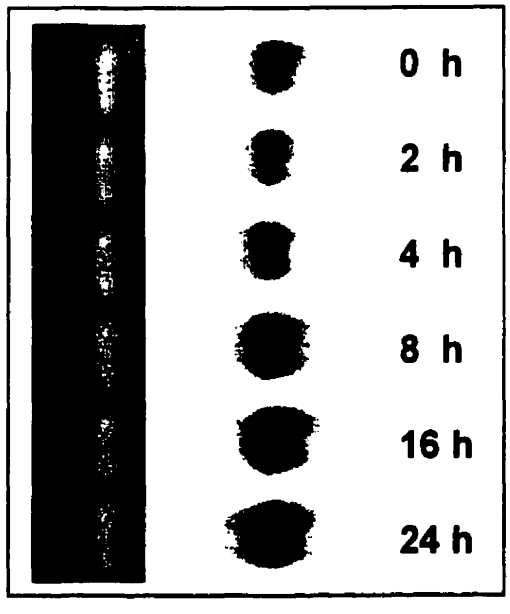
4.4 Time course of 9804 mRNA induction by IFN-α/ATRA

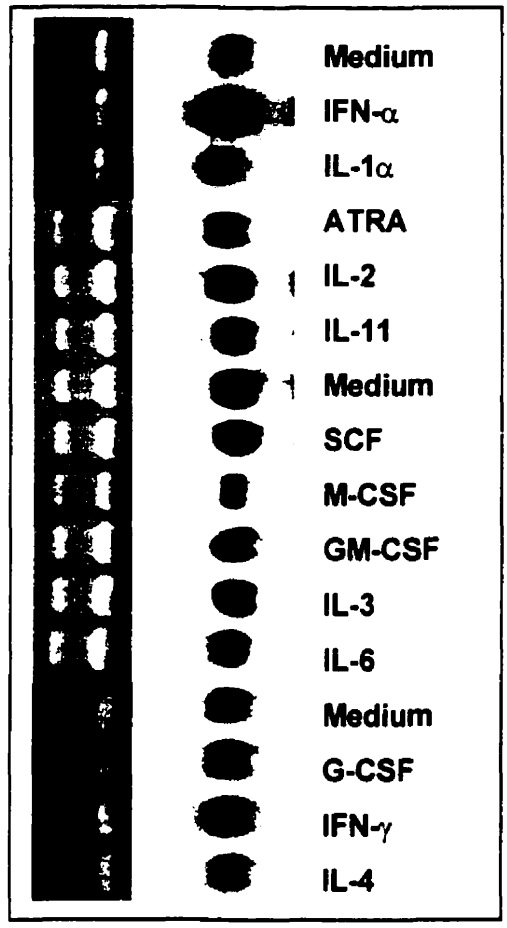
The expression of IFN-inducible genes requires transcription activation in response to IFN-receptor interaction on the cell surface and signaling through the Jak-STAT pathway (Darnell *et al.*, 1994). This process involves the activation of preexisting cytoplasmic proteins by phosphorylation and is a relatively early event. Some genes

Figure 4.4 Time Course of 9804 RNA Induction by IFN-α/ATRA in Cell Lines

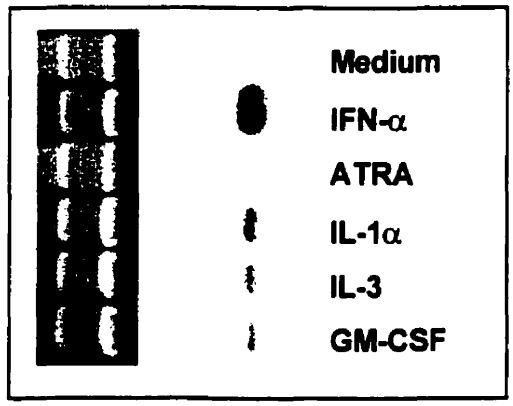
Jurkat (A), U937 (B), HL-60 (C) and NB4 (D) cells were cultured at a density of 2 x 10^6/ml with 1,000 IU/ml IFN-α and NB4 (E) cells with 1 μM ATRA. The cells were harvested for RNA isolation at the time points indicated in the figures. For the time points beyond 36 hrs, cells were fed with fresh medium with IFN-α or ATRA at 36 hrs. Total RNA samples (20 μg/lane) were used for Northern blot analyses with ^{32}P random priming labeled 9804 cDNA probe.


A. Jurkat
0 h
2 h
4 h
8 h
16 h
24 h
36 h
48 h
72 h


B. U937
0 h
2 h
4 h
8 h
16 h
24 h


Medium
IFN-α
IL-1α
ATRA
IL-2
IL-11
Medium
SCF
M-CSF
GM-CSF
IL-3
IL-6
Medium
G-CSF
IFN-γ
IL-4

C. TF-1


Medium
IFN-α
ATRA
IL-1α
IL-3
GM-CSF

D. KG-1

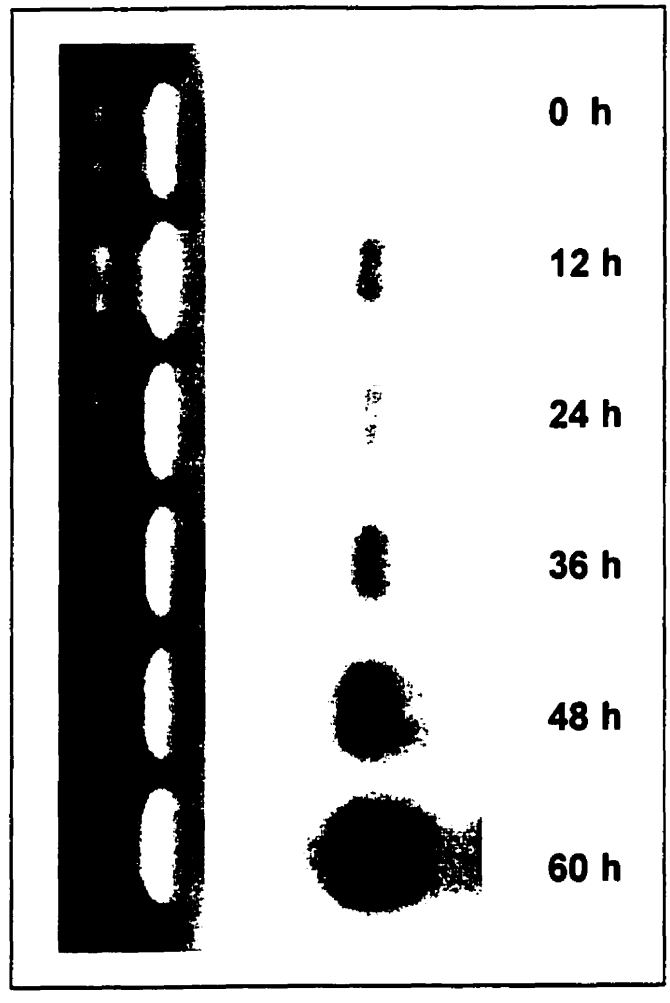

E. NB4
0 h
12 h
24 h
36 h
48 h
60 h

Figure 4.5 Effect of Cycloheximide/Actinomycin D on IFN-α Induction of 9804 RNA

U937 cells were cultured at a density of 2 x 10^6/ml with 1,000 IU/ml IFN-α for 12 or 20 hours. Cycloheximide (CHX; 35 μg/ml) was added to the cultures 15 min prior to the addition of IFN-α. Actinomycin D (Act. D; 3.5 μg/ml) was added to the cultures after 6 or 10 hours treatment with IFN-α in the 12 or 20 hrs cultures, respectively. Total RNA samples (10 μg/lane) were separated on the gel, blotted and hybridized with ^{32}P random priming labeled 9804 cDNA probe.

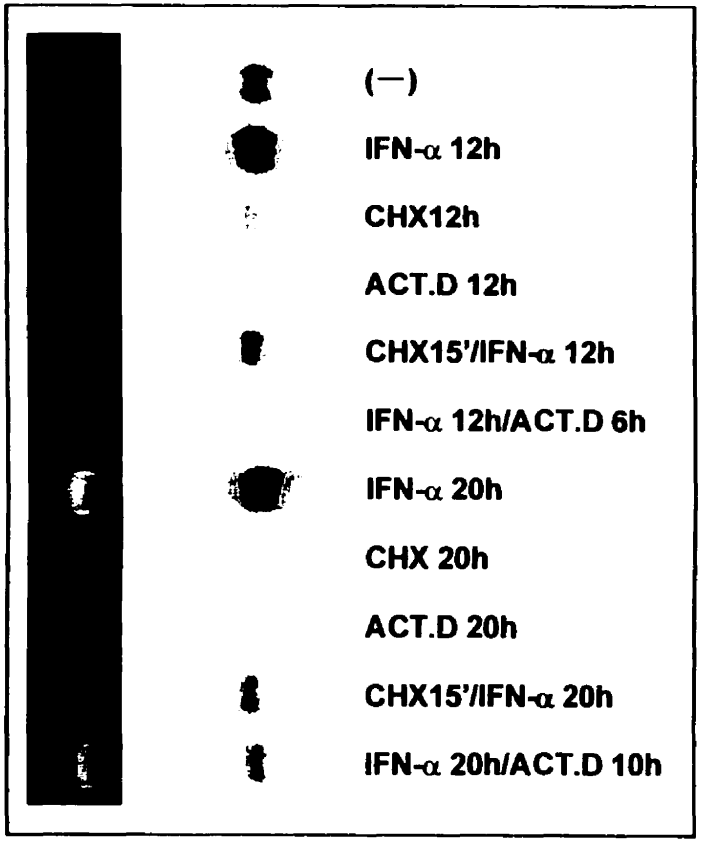
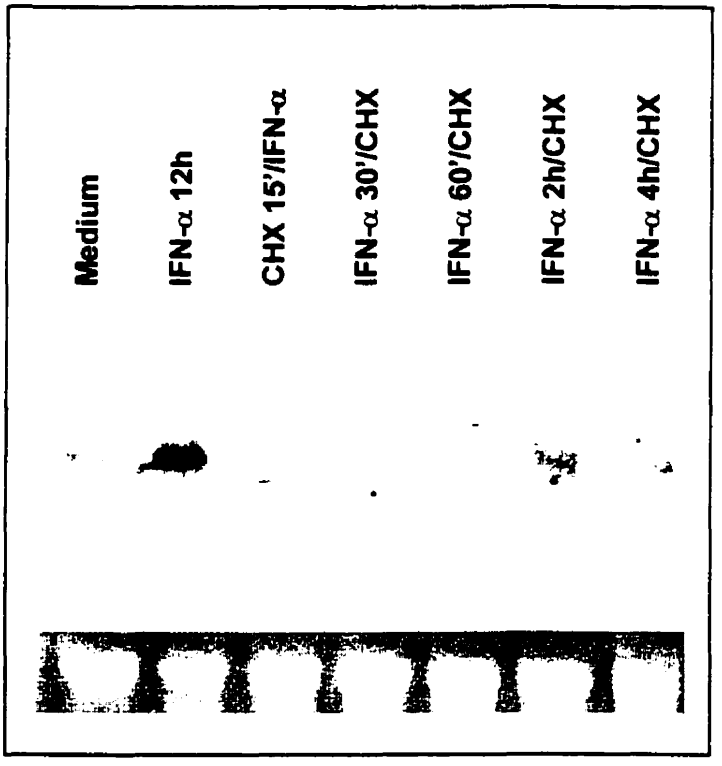

U937
(—)
IFN-α 12h
CHX12h
ACT.D 12h
CHX15'/IFN-α 12h
IFN-α 12h/ACT.D 6h
IFN-α 20h
CHX 20h
ACT.D 20h
CHX15'/IFN-α 20h
IFN-α 20h/ACT.D 10h

Figure 4.6 Time Course of Cycloheximide Effect on 9804

Jurkat cells were cultured at a density of 2 x 10^6/ml with 1,000 IU/ml IFN-α for 12 hrs. Cycloheximide was added 15 min prior to, 30 min, 60 min, 2 hrs or 4 hrs after the addition of IFN-α. 10 μg/lane of total RNA samples were used for Northern blot with ^{32}P random priming labeled 9804 cDNA probe.


Jurkat
Medium
IFN-α 12h
CHX 15'/IFN-α
IFN-α 30'/CHX
IFN-α 60'/CHX
IFN-α 2h/CHX
IFN-α 4h/CHX

respond quickly to retinoic acid. However, induction of cellular differentiation by ATRA is slower, as synthesis of a series of regulatory proteins and differentiation-specific proteins is required (Olsson *et al.*, 1996). The time course of 9804 induction by either IFN-α or ATRA was studied with cell lines Jurkat, U937, HL-60 and NB4 or NB4 cells. The induction by IFN-α started from 4 hrs (Jurkat) to 12 hrs (NB4) and peaked at 8 hrs (Jurkat) to 36 hrs (HL-60 and NB4) (Fig. 4.4A to D). With respect to ATRA, the induction of 9804 expression in the NB4 cells is a relatively late event. It was clearly established only at 36 hrs and reached a peak after 60 hrs of ATRA treatment (Fig. 4.4E).

4.5 The mode of 9804 induction by IFN-α

To see whether protein synthesis is required for 9804 induction by IFN-α, or if mRNA stabilization contributed to the elevated 9804 message levels, either the protein synthesis inhibitor, cyclohexamide (CHX) or the RNA transcription inhibitor actinomycin D (Act.D) was included in the cultures of U937 cells. Addition of CHX to the culture for 12 or 20 hrs inhibited both basal level and IFN-α-induced 9804 mRNA expression if CHX was added 15 min prior to IFN (Fig. 4.5). This indicated that novel protein synthesis was required for 9804 induction by IFN-α. A further time course of inhibition was performed in Jurkat cells by treating the cells with CHX 15 min before or 30 min to 240 min after IFN-α induction. The inhibitory effect of CHX was observed when CHX was added 15 min prior to or within 2 hrs of IFN-α induction (Fig. 4.6). The results suggested that IFN-α induced the expression of the 9804 gene indirectly through protein factor(s) synthesized within 2 hrs of IFN-α addition, although IFN-α may also act directly in a cooperative process.

Twelve or 20 hrs of Act.D treatment of the U937 cultures completely abolished 9804 mRNA expression (Fig. 4.5). A low level of 9804 mRNA expression was observed if Act. D was added 10 hrs after IFN-α induction in the 20 hrs culture (Fig. 4.5). This low level expression was not seen when Act. D was added 6 hrs after IFN-α induction in the 12 hrs culture. This may indicate an early induction of 9804 mRNA after only 8 hrs

of IFN-α exposure in the U937 cells as was evident in the time course of 9804 induction (Fig. 4.4B). Therefore, the results of the inclusion of Act. D in the culture suggested that IFN-α induced 9804 mRNA is likely at the level of transcription. However, further studies with longer time course for mRNA stability and RNA run-on assay for the transcription will be needed.

4.6 Summary and discussion

Among a panel of thirteen human recombinant cytokines, IFN-α induced 9804 mRNA expression in a variety of human cell lines and normal human PBMC. IFN-γ did not do so except for a slight enhancement in PBMC. In this respect, the control of 9804 expression resembles that of mouse Ly-6C, which is induced by IFN-α/β but not IFN-γ. The mechanism for induction of gene transcription by IFN-α and IFN-γ has been reasonably well understood. The extracellular signal is transduced through the Jak-STAT pathways (Darnell *et al.*, 1994). The IFN-receptor interaction at the cell surface leads to the activation of kinases of the Jak family, that phosphorylate preexisting cytoplasmic proteins called STATs (signal transducers and activators of transcription). The phosporylated STAT proteins translocate to the nucleus where they form complexes which are transcription factors that bind to specific DNA elements, and direct transcription of the targeted genes. DNA response elements for IFN-α and IFN-γ have been characterized in Ly-6A/E and Ly-6C genes. An IFN-α-stimulated response element (ISRE) has been defined in the promoter region of the Ly-6C gene (Bothwell *et al.*, 1988) and an IFN-γ activation site (GAS) in that of the Ly-6A/E gene (Khan *et al.*, 1990). The ISRE in the Ly-6C promoter is responsible IFN-α/β inducibility. The GAS site in the Ly-6A/E gene is required and sufficient for transcription activation by both IFN-α and IFN-γ (Khan *et al.*, 1993).

As described in Chapter 2, a potential ISRE was found in the 5' flanking region of the 9804 gene that did not contain a GAS site. It corresponds well to the observation that 9804 expression is induced by IFN-α but not IFN-γ. However, inhibition of 9804 expression by CHX points to the requirement for new protein synthesis which occurs within 2 hrs of IFN-α treatment, suggesting that either 9804 transcription activation by

IFN-α is a purely indirect process or that another IFN-inducible protein factor(s) is involved cooperatively in this process. Characterization of the 9804 promoter by means of reporter assays and DNA foot-printing of the 5' flanking region of the 9804 gene with nuclear extracts obtained at various times post-treatment will provide further evidence for the IFN-α's mode of action in 9804 expression.

In contrast to the relatively general phenomenon of IFN-α-induced 9804 expression, induction by ATRA is limited to a few myeloid leukemias. It is also a less intensive and a relatively late event (36-48 hrs).

Retinoic acids exert their pleiotropic biological effects by interacting with their receptors (RARs) that belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors including the receptors for steroids, retinoids, thyroid hormones and vitamin D_3 (VDR) (Mangelsdorf *et al.*, 1995). ATRA also induces *in vitro* differentiation of leukemia or solid tumor cell lines, and can lead to a complete remission in patients with APL (Breitman *et al.*, 1981; Huang *et al.*, 1988; Castaigne *et al.*, 1990). Cytogenetically, APL is characterized by the presence of a non-random translocation t(15;17) involving *RARα* on chromosome 17 and *PML* on chromosome 15. *PML* encodes a zinc finger transcriptional factor (de Thé *et al.*, 1991) which has been shown to be a growth repressor (Mu *et al.*, 1994; Liu *et al.*, 1995). The PML-RARα fusion protein has been implicated in both pathogenesis and effective treatment with RA of this disease. On the one hand, PML-RARα may interfere with the normal signaling pathways of RARα and PML in cell differentiation. On the other hand, PML-RARα renders leukemic cells sensitive to ATRA-induced differentiation, although the mechanism involved has not been identified. Indeed, NB4 is the only permanent *in vitro* APL cell line with t(15;17) which is highly sensitive to RA-induced granulocytic differentiation (Lanotte *et al.*, 1991). In the U937 myeloid precursor cells which lost the capacity to differentiation under the action of different stimuli (vitamin D_3 and TGF-β1), expression of PML-RARα protein enhanced the sensitivity of the cells to RA (Grignani *et al.*, 1993). NB4 variants that lose the expression of PML-RARα protein also fail to differentiate in response to RA (Dermime *et al.*, 1993).

As one of a few genes highly induced by ATRA in NB4 cells, 9804 has been thought to be involved in the process of ATRA-induced granulocytic differentiation of APL. Although two potential RARE half sites were predicted in the 5'-flanking region of the 9804 gene as described in Chapter 2, it is still to be determined whether ATRA interacts directly with the 9804 promoter to drive gene transcription. However, the time course of ATRA-induction of 9804 mRNA indicated a late event (36-48 hrs) and probably, therefore, an indirect process. This is consistent with the fact that the granulocytic differentiation of NB4 cells does not become apparent until 72 hrs of ATRA induction (Lanotte *et al.*, 1991). It remains to be seen, therefore, whether the 9804 protein actually participates in further driving the differentiation process, or is merely a consequence of generating a more differentiated phenotype. Our ongoing luciferase reporter study with the sequences from 5' flanking region of 9804 will help to address the question of whether the retinoic acid receptor participates directly in 9804 induction.

Although most cytokines produced no obvious effect on 9804 mRNA levels in the cell types tested, it is possible that combinations of such regulatory factors may be effective. Coordination and cross-talk between signaling pathways is common. In particular, interaction between IFN and RA pathways has been observed in several cases. RA upregulates STAT proteins or related proteins (e.g. ISGF-3, GAF and IRF-1) in IFN-unresponsive cells, rendering the cells responsive to IFN-α and IFN-γ (Kolla *et al.*, 1996; Matikainen *et al.*, 1996). On the other hand, IFN-γ mediates an increase in RARγ and a suppression of RA-mediated cytoplasmic retinoic acid binding protein type II (CRABPII) in breast cancer cells (Widschwndter *et al.*, 1995). The induction of 9804 mRNA by both IFN-α and ATRA in our experiments suggested we should investigate the posibility of a synergistic effect of the two reagents. It will be clinically important to study more thoroughly the induction of 9804 by the combination of ATRA and IFN-α in suboptimal concentrations, since RA-resistance (Chen *et al.*, 1991) and RA-syndrome (Vahdat *et al.*, 1994) have been major drawbacks in the treatment of APL patients with RA. Furthermore, genetic and biochemical characterization of the induction of 9804 expression by ATRA and IFN-α during myeloid cell differentiation will further our understanding of the control of 9804 expression during this process. If 9804 expression

is connected with the development of a signaling pathway, that helps to drive differentiation process. Antibodies to 9804 may activate this pathway and influence the rate and degree of differentiation.

CHAPTER 5

RECOMBINANT Ly-6 DOMAIN PROTEIN EXPRESSION IN BACULOVIRUS SYSTEM

5.1 Introduction

Characterization of the structure and expression of the 9804 gene in this thesis revealed a human homologue of the murine Ly-6 gene family which is involved in hematopoiesis (Bamezai *et al.*, 1995), cell adhesion (Johnson *et al.*, 1993) and lymphocyte activation (Rock *et al.*, 1986, Malek *et al.*, 1986, Yeh *et al.*, 1987). The 9804 gene is transcriptionally active in a broad range of tissues and cell lines of different lineages. The transcription is dramatically upregulated by IFN-α in normal human PBMC and most of the cell lines tested. Also interestingly, the gene is induced by ATRA during the differentiation of several myeloid leukemia cell lines. Detection of the 9804 expression at the protein level will be important to explore the functional properties of the gene product in IFN or RA-related processes. Antibodies (Abs) specific for the 9804 protein are therefore desired in this respect. In fact, Ly-6 Abs have been useful tools in the functional studies, since crosslinking Ly-6 mAbs on lymphocyte surfaces can induce cell signaling and activation in the presence of certain costimulatory factors. Anti-receptor antibodies often mimic many of the changes induced in response to the physiological ligand. Although Ly-6 ligands have not been identified, evidence for their presence has been obtained from several studies (Johnson *et al.*, 1993, Bamezai *et al.*, 1995a, Bamezai *et al.*, 1995b). Searching for the ligands will be an important part of the Ly-6 functional studies. For the purpose of 9804 Ab production and ligand studies, recombinant 9804 and other Ly-6 proteins in an appropriately folded conformation and in quantities were required.

The baculovirus system has been widely used for expression of heterologous proteins in cultured insect cells. The system has the advantage of providing a high yield of recombinant proteins with post-translational modification. O- and N-glycosylation has been found in the human recombinant protein expression (Domingo and Trowbridge, 1988; 1990; Chen *et al.*, 1991). The recombinant human CD59 of the Ly-6 superfamily

Figure 5.1 BOMIGF-IL-3 in Baculovirus Expression Vector pBlueBacIII

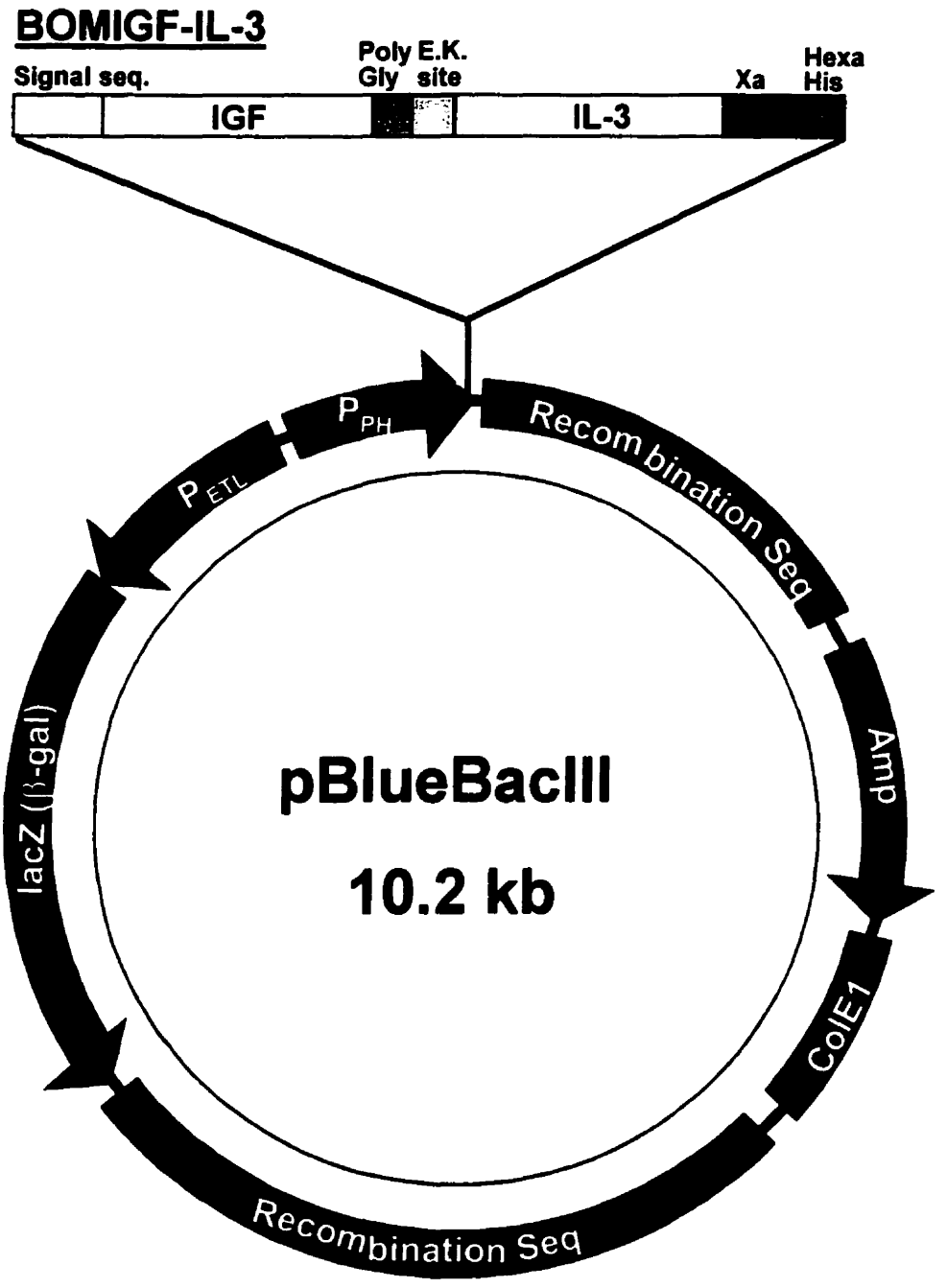
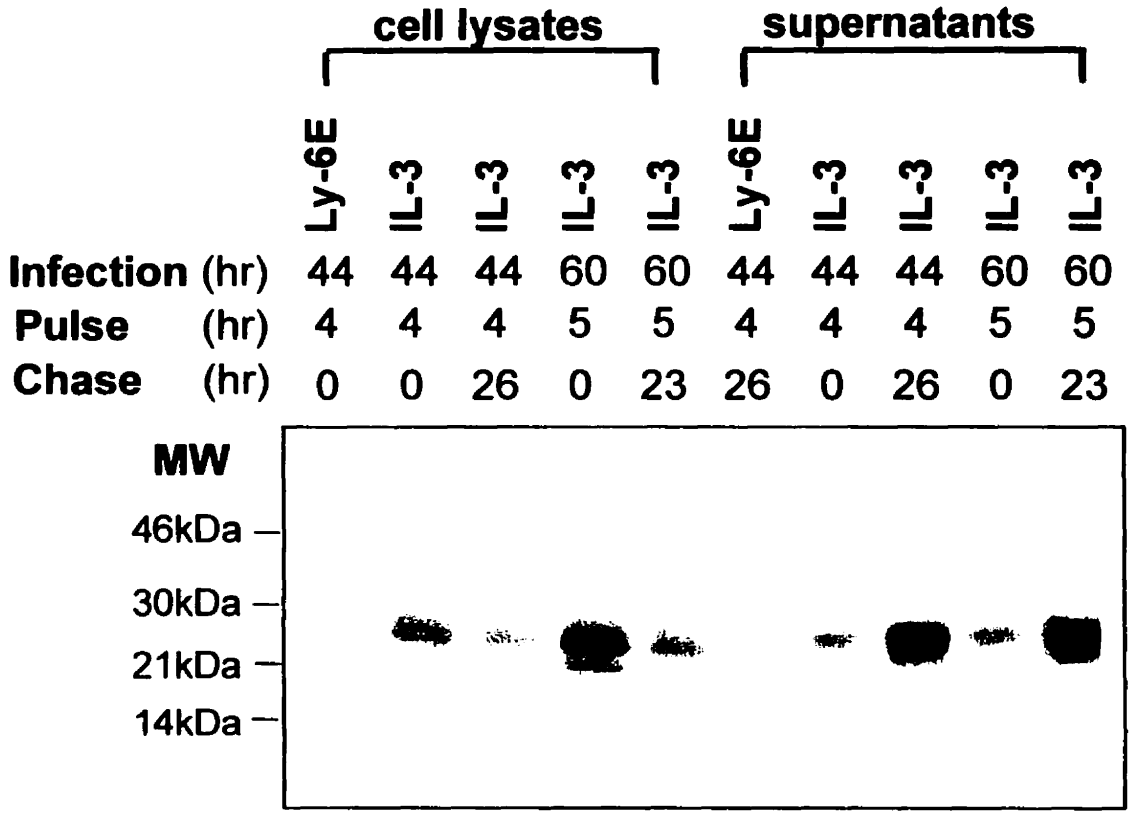

BOMIGF-IL-3
Signal seq.
Poly Gly
E.K. site
Xa
Hexa His
IGF
IL-3
P PH
Recombination Seq
P ETL
Amp
lacZ (β-gal)
ColE1
Recombination Seq
pBlueBacIII
10.2 kb

Figure 5.2 Immunoprecipitation of BOMIGF-IL-3

Sf9 cells were infected with recombinant BOMIGF-IL-3 virus (the construct is shown on the bottom panel), labeled with ^{35}S-cysteine and chased for the times indicated. The cell lysates and culture media were immunoadsorbed on anti-human IL-3 mAb coated plastic microwells. The recombinant BOMIGF-Ly-6 was used as negative control.


cell lysates
supernatants
Ly-6E
IL-3
IL-3
IL-3
IL-3
Ly-6E
IL-3
IL-3
IL-3
IL-3
Infection (hr) 44 44 44 60 60 44 44 44 60 60
Pulse (hr) 4 4 4 5 5 4 4 4 5 5
Chase (hr) 0 0 26 0 23 26 0 26 0 23
MW
46kDa
30kDa
21kDa
14kDa

has been expressed abundantly in insect cells using baculovirus vectors in a GPI-anchored (Davies and Morgan, 1993) or soluble form (Sugita *et al*, 1994). Both forms of the recombinants were shown to retain their intrinsic action of protecting cells from lysis by human complement.

The baculovirus system was also developed and used successfully for expression of a secreted fusion protein having both IGFII and IL-3 domains by Dr. Congote's group in the Endocrine Laboratory, McGill University (DiFalco *et al.*, 1997; DiFalco and Congote 1997). IGFII gene was fused to the sequence for N-terminal bombyxin signal peptide, and placed under the polyhedrin promoter of the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) expression vector pBlueBacIII (Fig. 5.1) to generate BOMIGF vector. The chimeric fusion product could therefore be directed efficiently by the insect signal peptide into secretary pathway in the *Spodoptera frugiperda* insect cell line Sf9 during viral infection. The vector was also designed to include cloning sites and codons for an enterokinase cleavage site at C-terminal end of the IGFII domain (Fig. 5.1). These two features of the BOMIGF vector allow the generation of a series of secreted IGFII-fusion proteins, while the C-terminal domain of the fusion protein can be released by enterokinase cleavage. Furthermore, the fusion protein can be detected by a IGFII bioassay (Congote *et al.*, 1987). We demonstrated the efficiency of this system by studying the expression and secretion of the IGFII-IL-3 fusion protein. The fusion was detected by immunoadsorption to mAb against human IL-3 bound to the plastic microwells, followed by SDS-PAGE and autoradiography. The recombinant protein was produced abundantly after 48 hrs infection, and the amount of the labeled secreted protein increased after a chase with complete medium (Fig. 5.2). The detection of IGFII-IL-3 in the baculovirus expression system provided an indication of the efficiency of producing secreted proteins with this type of fusion construct. We therefore proceeded to engineer viruses for expression of IGFII-Ly-6 domain fusion proteins. One had 9804 domain we could produce antigen for immunization. Others had Ly-6E or Ly-6C domains, and might be of use in ligand and functional studies in mouse. Appropriate folding of the Ly-6 domain can be monitored with a known mAb SK70.49, which is specific for Ly-6E.1 and conformation sensitive (Kirmura *et al.*, 1984).

Figure 5.3 Constructs of BOMIGF-Ly-6 Fusion Genes

DNA fragments encoding the Ly-6 domains of Ly-6E and 9804 were generated by PCR using primers that affect addition of restriction sites for cloning, enterokinase (E.K.) cleavage site, and replacement of the hydrophobic tail, required for GPI anchoring, with 6 amino acids GYSKSN. The *Nco* I and *Nru* I fragments from Ly-6E and 9804 PCR clones, respectively, were ligated into the corresponding restriction sites of the BOMIGF vector to replace IL-3 and generate the fusion genes of BOMIGF-Ly-6E and BOMIGF-9804.

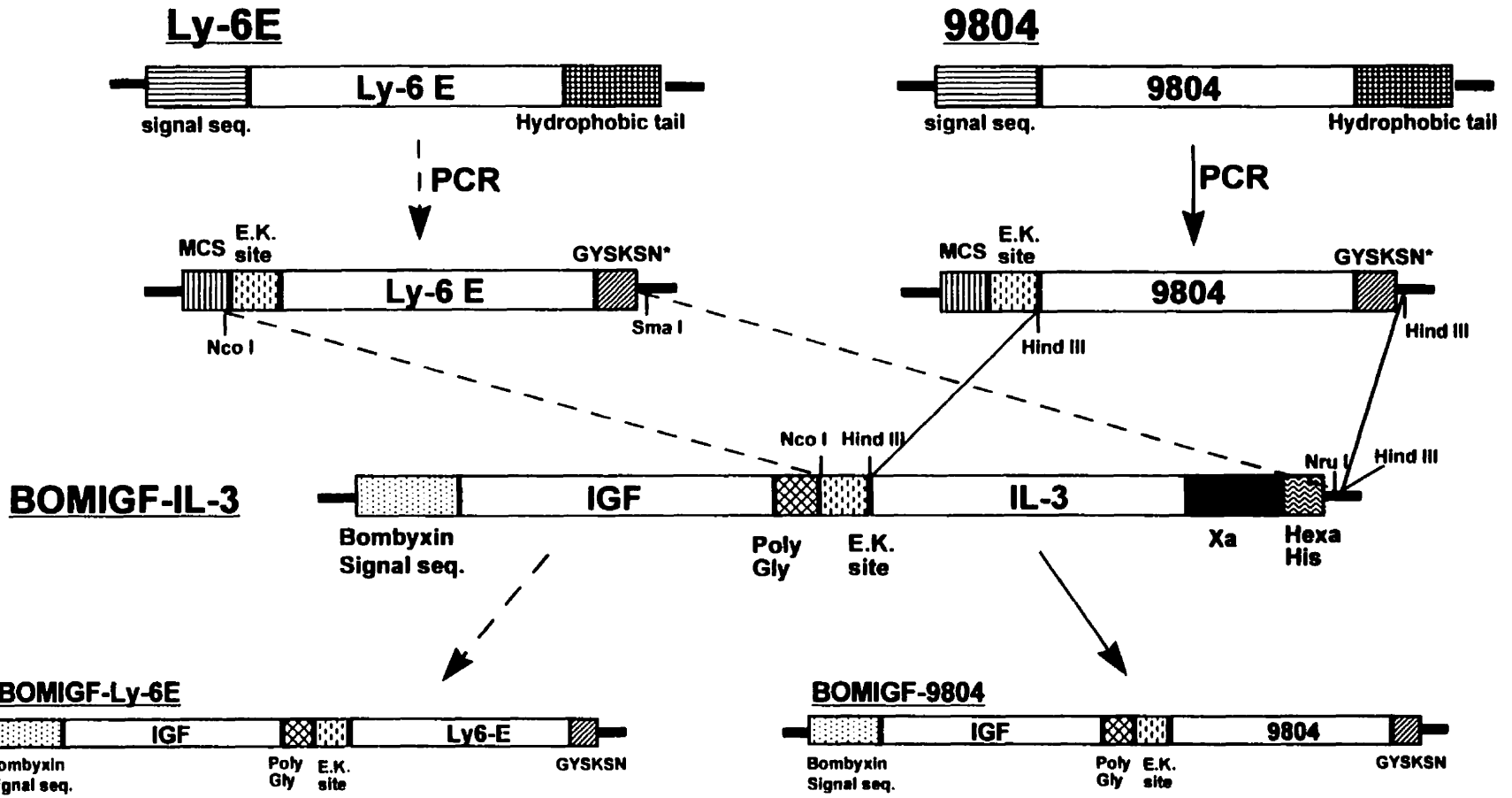

Ly-6E
Ly-6 E
signal seq.
Hydrophobic tail
PCR
MCS
E.K. site
GYSKSN*
Ly-6 E
Nco I
Sma I
9804
9804
signal seq.
Hydrophobic tail
PCR
MCS
E.K. site
GYSKSN*
9804
Hind III
Hind III
BOMIGF-IL-3
Nco I
Hind III
Nru I
Hind III
IGF
IL-3
Bombyxin Signal seq.
Poly Gly
E.K. site
Xa
Hexa His
BOMIGF-Ly-6E
IGF
Ly6-E
Bombyxin Signal seq.
Poly Gly
E.K. site
GYSKSN
BOMIGF-9804
IGF
9804
Bombyxin Signal seq.
Poly Gly
E.K. site
GYSKSN

Table 5.1 Oligonucleotides Used in the Ly-6 Recombinant Protein Expression*

Oligonucleotide	Direction	Nucleotide sequence
9804BVF	forward	5' GATGACAAGCTTATGTGCTTCTCC 3'
9804J12R	reverse	5' TACTCGAGCCCT(G/C)ATTGCACAGAAAGCT 3'
LY6BVE	forward	5' ATGACAAGCTTGAGTGTTACCAG 3'
BVLY6C	forward	5' GATGACAAGCTTCAGTGCTACGAG 3'
T7	reverse	5' AATACGACTCACTATAG 3'
LY6BVJ	forward	5' TGGCCATGCAATTCGAAGGACGATTGATGACAAGCTT 3'
J12RSOL	reverse	5' TCGAGTCAATTGTACTTACTC CGAGCCCT 3'

* conditions for fragment amplification by PCR are given in the Experimental Procedures.

5.2 Construction of The BOMIGF-9804 And BOMIGF-Ly-6 Fusion Genes

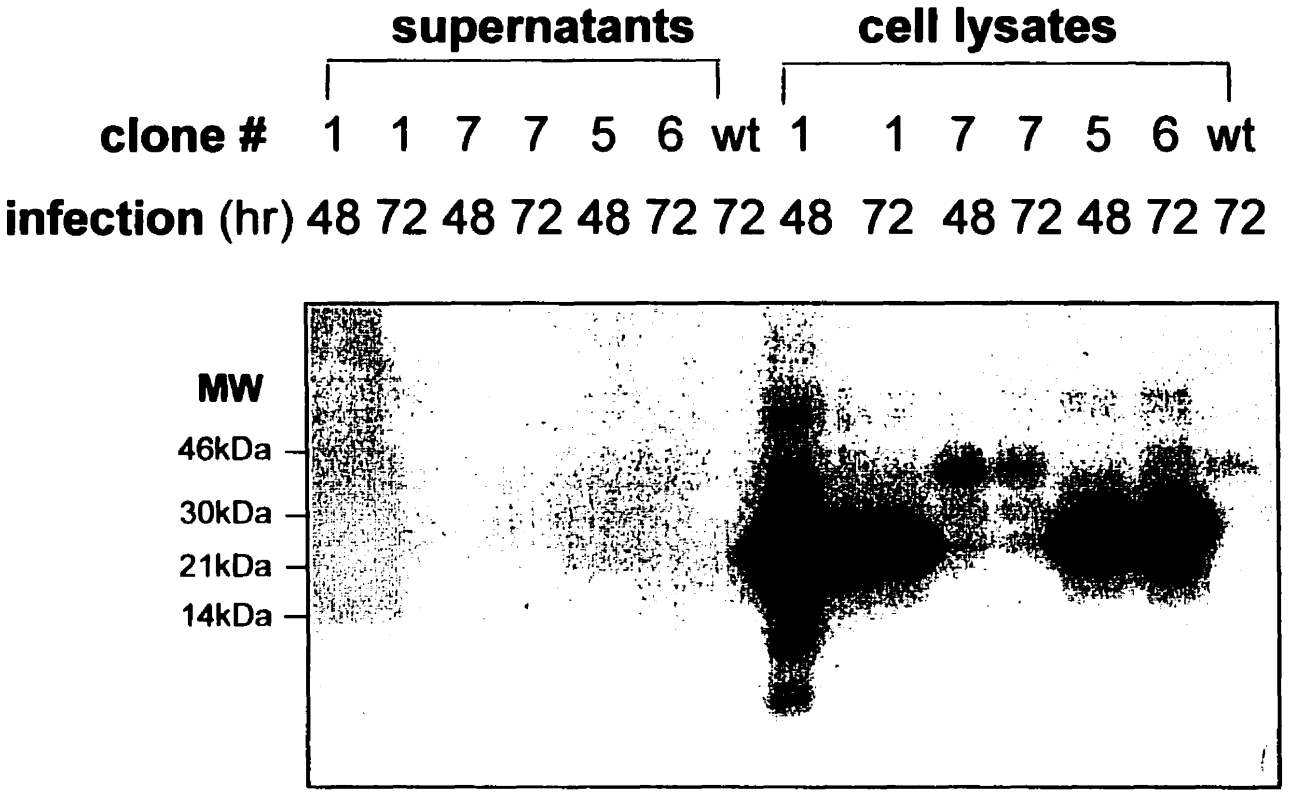
To create fusion genes for 9804, Ly-6A/E and Ly-6C with IGFII, primer pairs were designed in order to introduce the appropriate in-frame link of Ly-6 sequence with IGFII and to substitute the short C-terminal sequence for the long hydrophobic GPI signal sequence. The primers 9804BVF/9804J12R, Ly6BVE/T7 AND BVLY6C/T7 (see Table 5.1), were first used to amplify the coding regions of 9804, Ly-6A/E and Ly-6C genes, respectively. Second primer pairs, Ly6BVJ/J12RSOL and Ly6SOL for 9804 and Ly-6, respectively, were applied to introduce a polylinker and sequence for enterokinase cleavage site at 5' end and codons for six amino acids, GYSKSN, at 3' end to replace that for C-terminal hydrophobic tail. The constructs were shuttled through pBluescript II KS$^-$ vector to facilitate sequence confirmation and subcloning into the expression vector. The *Hind* III fragment of 9804 construct or *Not* I/*Sma* I fragments of Ly-6A/E and C constructs were then excised and adapted into *Hind* III or *Not* I/*Nru* I digested pBlueBacIII-BOMIGF vector to generate BOMIGF-9804 or BOMBIGF-Ly-6 fusion genes, so that Ly-6 modules would be placed immediately downstream of the enterokinase cleavage site (Fig. 5.3 showing 9804 and Ly-6E). This should allow the option of the proteolytic release of the Ly-6 domains once the fusion proteins have been produced. The fusion genes were checked by DNA sequencing to confirm the placement of Ly-6 portions in frame to BOMIGF. Although a point mutation, most likely introduced by *Taq* DNA polymerase during PCR, was present in BOMIGF-Ly-6A, (a K to E replacement at amino acid position 66 of the Ly-6 domain), others were correct and used for expression.

5.3 Construction And Isolation of The Recombinant Baculoviruses

Plasmids containing BOMIGF-9804 and BOMIGF-Ly-6 fusion genes were prepared and cotransfected with the linear AcMNPV DNA into Sf9 cells by cationic liposome mediated method (Invitrogen). Recombinant viruses were thus generated through a homologous recombination process. These were selected on the basis of β-galactosidase expression and purified by plaque assay transfection (see Experimental procedures). Single blue plaques containing recombinant viruses were picked and

Figure 5.4 Immunoprecipitation of BOMIGF-Ly-6E

Sf9 cells were infected with clones #1, #5, #6, #7 of the recombinant BOMIGF-Ly-6E and wild type baculoviruses. 48 and 72 hours postinfection, the cells were metabolically labeled with ^{35}S-cysteine for 4 hours and chased for 7 hours. The cell lysates and supernatants were immunoprecipitated with mAb SK70.94 (anti-Ly-6E), resolved on the SDS-PAGE gel and autoradiographed. Low range molecular weight standards (GIBCO BRL) were loaded on the gel for the protein size comparison.


supernatants
cell lysates
clone # 1 1 7 7 5 6 wt 1 1 7 7 5 6 wt
infection (hr) 48 72 48 72 48 72 72 48 72 48 72 48 72 72
MW
46kDa
30kDa
21kDa
14kDa

amplified for high titer virus stocks. The titers were determined by plaque assay as between 10^8 to 10^9 plaque forming unit (pfu) /ml. Further expression of the recombinant fusion proteins was carried out from the high titer virus stocks.

5.4 Expression And Analysis of The Fusion Proteins in Sf9 Insect Cells

Sf9 insect cells were plated in the culture dishes at a seeding density of 1,000/mm^2 or cultured in spinner flask as a suspension culture at a density of 2 X 10^6/ml. Log phase growing cells were infected with virus stocks at a multiplicity of infection (MOI) of 10. The cells were incubated for 2 to 3 days at 27 °C before proceeding to further assays for the fusion protein expression.

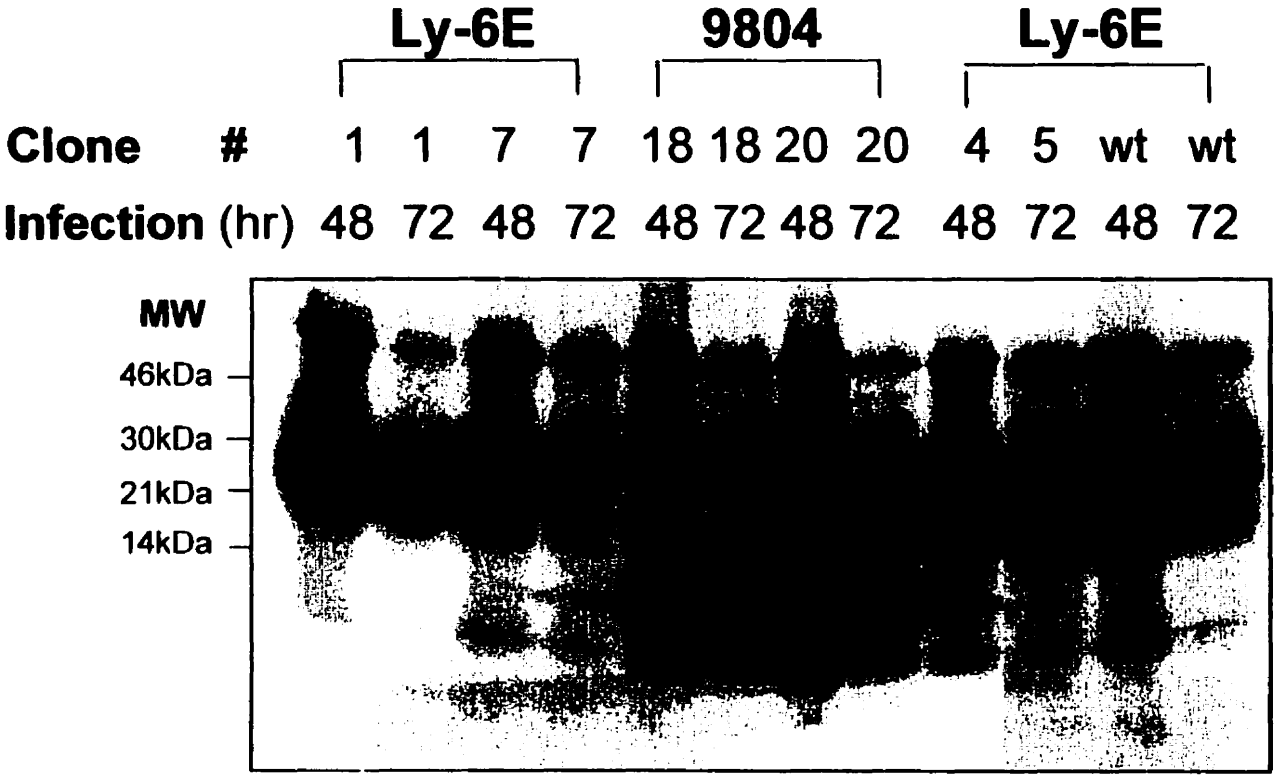
5.4.1 IGFII Growth Factor Assay of The Fusion Proteins

The expression of the IGF-Ly6 or IGF-9804 fusion proteins was first analyzed by a growth factor assay for the thymidine incorporation in the bovine fetal liver cells (Congote *et al.*, 1989). Briefly, Sf9 cells were infected with the recombinant viruses for 72 hrs. The culture supernatants were chromatographed with Sep Pak C_{18} column and gel filtration HPLC. The gel filtration fractions were dried by speed vacuum, redissolved and tested in the bioassay. Samples were added to a culture of bovine fetal liver cells for 18 hrs, pulse labeled for one hour ^{3}H-thymidine and TCA precipitatable thymidine incorporation measured by scintillation counting..

Among the clones tested (#1 to 6 of the BOMIGF-Ly-6A, #1 of the BONIGF-Ly-6E, #1 BOMIGF-Ly-6C and #2 to 3 of the BOMIGF-9804), no IGFII growth factor activity was found by thymidine incorporation. We had assumed that the recombinant fusion proteins would be efficiently secreted like the IGFII-IL-3. From these results, however, it appeared that either there were no recombinant fusion proteins produced, the IGFII moiety was not active in these fusion proteins, or they were not secreted into the medium. To determine whether the recombinant proteins were produced or secreted, we focused on the Ly-6E fusion protein and detected it after biosynthetic labeling with ^{35}S-cysteine by immunoprecipitation with mAb SK70.94.

Figure 5.5 SDS-PAGE of BOMIGF-Ly-6 Fusion Proteins

Sf9 cells were infected with recombinant BOMIGF-Ly-6E and BOMIGF-9804 viruses for 48 and/or 72 hours followed by ^{35}S-cysteine labeling 4 hours and chasing 7 hours. The cells were lysed with buffer containing the detergent Triton X-100 and sodium deoxycholate. Samples were applied to the SDS-PAGE and autoradiographed. Wild type (wt) was included as control. Low range molecular weight standards (GIBCO BRL) were loaded on the gel as protein size markers.


Ly-6E
9804
Ly-6E
Clone #
1 1 7 7 18 18 20 20 4 5 wt wt
Infection (hr)
48 72 48 72 48 72 48 72 48 72 48 72
MW
46kDa
30kDa
21kDa
14kDa

5.4.2 Immunopricipitation of BOMIGF-Ly-6E Fusion Proteins

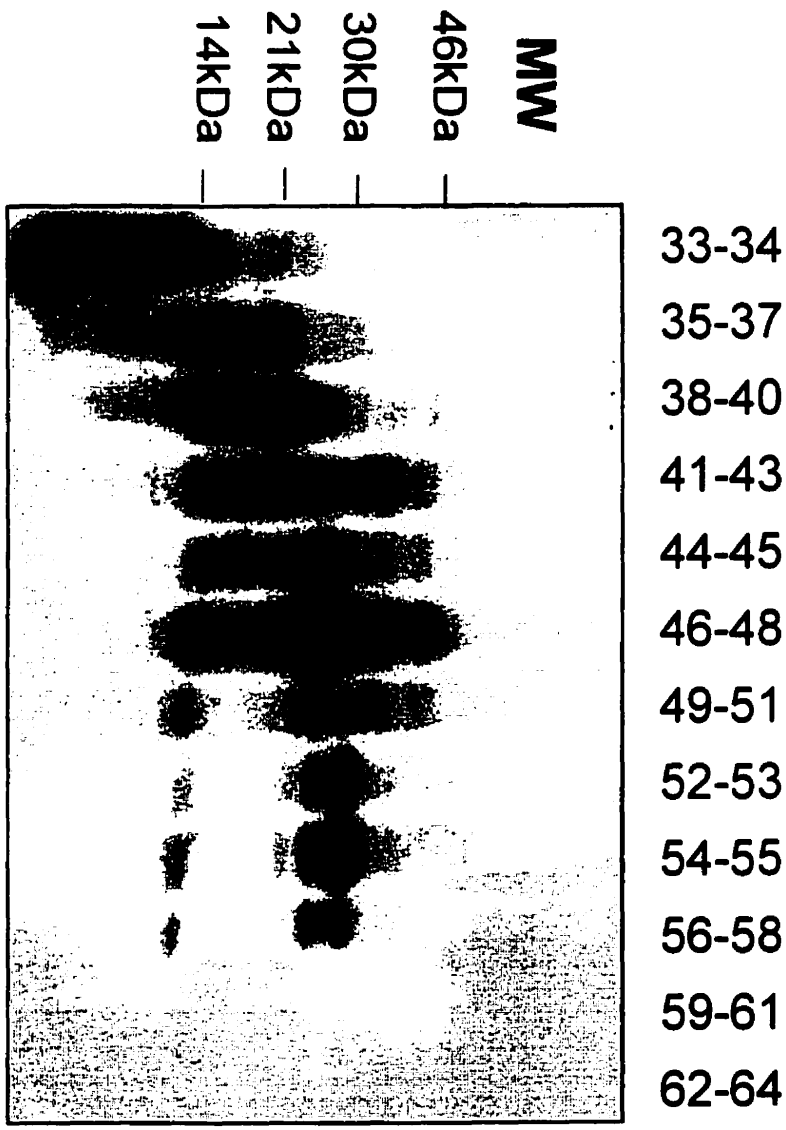
Sf9 (3 X 10^6) cells were plated in a 60 mm^2 culture dish and infected with the BOMIGF-Ly-6E recombinant virus stock after cell attachment. The infected cells were incubated at 27 °C for 48 or 72 hrs, then were labeled with 3 µl of [^{35}S]-cysteine (1078 Ci/mmol, 10.35 mCi/ml, ICN) in cysteine-free SF-900 SFM medium (GIBCO BRL) for 4 hrs followed by 2 hrs chasing by addition of complete medium. Culture supernatants were collected and cells lysed by addition of lysis buffer (0.1 M Tris-HCl, pH 8, 0.15 M NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, 5 mM iodoacetamide, 2 mM EDTA, 1 mM PMSF).

The culture supernatant and cell lysate were pre-cleared with protein A agarose (GIBCO BRL) before the Ly-6E.1-specific mAb SK70.94 was added. Interaction was allowed at 4 °C over night with gentle shaking. Protein A agarose were added to precipitate the mAb-bound antigens. The precipitated samples were boiled in the sample buffer for 5 min. and applied to a stepping gel of reducing tricine-SDS-PAGE. The gel was dried and autoradiographed.

A strongly labeled band of 28 kDa was shown in immunoprecipitates from extracts from cells infected for 48 and 72 hrs with BOMIGF-Ly-6E clones 1, 5 and 6, but in neither case from culture supernatants (Fig. 5.4). No corresponding signal was detected from supernatants or from the cell lysate of the wild type control or clone 7 (Fig. 5.4). Therefore this recombinant IGF-Ly-6E fusion protein was produced by the recombinant virus infected cells but not wild type virus infected cells, but the protein was not secreted. This also was the case for the other Ly-6 domain fusion proteins since they could be seen after SDS-PAGE and autoradiograpy in whole cell lysates as dominant bands, but not in the media (see below). Presumably they remained cell-associated within the endoplasmic reticulum or the early Golgi compartment. Nevertheless, the Ly-6E domain in the fusion protein appeared to be appropriately folded since it could be detected by SK70.94, a mAb which is conformation sensitive and detects specifically the Ly-6E antigen in a nondenatured form.

Figure 5.6 SDS-PAGE of BOMIGF-9804 HPLC Fractions

C_4 Vydac reverse phase column HPLC was performed on an extract from Sf9 cells infected with clone #20 of BOMBIGF-9804 (acid extraction medium). with small amount of acid extract from the ^{35}S-cysteine labeled Sf9 cell infected with clone #18 of material of clone 18 BOMBIGF-9804 as a tracer. Two to 3 HPLC fractions were pooled, dried and applied to the tricine SDS-PAGE. No labeled materials eluted from fractions 1 to 32 (not shown). Samples from 33 to 64 are shown in the figure.


MW
46kDa
30kDa
21kDa
14kDa
33-34
35-37
38-40
41-43
44-45
46-48
49-51
52-53
54-55
56-58
59-61
62-64

5.4.3 Metabolic Labeling of BOMIGF-9804 And BOMIGF-Ly6 Fusion Proteins

As 9804 and Ly-6 are cysteine-rich proteins, we expect them to be among the most strongly labeled proteins when the infected insect cells are grown in the presence of [^{35}S]-cysteine. Furthermore, as overexpressed proteins, they should be clearly detected above the background of other cell proteins. [^{35}S]-cysteine labeling of the cells was performed. 3 X 10^6 Sf9 cells were plated per 60 mm^2 culture dish and infected with the recombinant viruses of BOMIGF-9804 and BOIGF-Ly-6E or wild type at a MOI of 10. At 48 or 72 hours postinfection, the complete medium was replaced with 5 ml of cysteine-free Sf-900 SFM medium and the cells were labeled for 4 hours with 3 μl of [^{35}S]-cysteine (1078 Ci/mmol, 10.35 mCi/ml, ICN), followed by overnight (approximately 16 hours) chasing with complete medium. The cells were pelleted and disrupted with the lysis buffer. Samples were analyzed by SDS-PAGE on a step gradient gel using the tricine-buffered system (Schägger and von Jagow, 1987). The gels were dried before autoradiography.

One representative experiment of metabolic labeling of IGFII-9804 and IGFII-Ly-6E fusion proteins is presented in Figure 5.5. At 48 or 72 hours postinfection, a strongly labeled band of about 28 kDa was detected specifically in clones 1, 4, 5 and 6 of BOMIGF-Ly-6E. However, no band at 28 kDa was seem in BOMIGF-9804 infected cell lysates, instead, a 15 kDa band was detected in clones 18 and 20 with equivalent intensity to that of 28 kDa in BOMIFG-Ly-6E. No corresponding high intensity band of 15 or 28 kDa was detected from 48 and 72 hrs infections of wild type virus. Clone 7 of the BOMIGF-Ly-6E was defective in production of immunoprecipitatable Ly-6E fusion protein, as previously demonstrated in Figure 5.4. In Figure 5.5 we see no evidence of cysteine-rich recombinant protein synthesis upon infection of this clone. Interestingly, the size of strongly labeled band of 15 kDa detected from the BOMIGF-9804 is about half of the size of the expected fusion proteins. A likely source of such a product is a degradation of the fusion protein between the two domains. This could be at the enterokinase cleavage site which was engineered between two domains for the purpose of releasing the Ly-6 domains from the fusion proteins. Since both IGFII and Ly-6 moieties

are cysteine-rich and similar in size, they might have been equally well labeled, cleaved into 15 kDa fragments and resolved as double bands very close to each other on the gel (Fig. 5.5). The protein cleavage could have occurred intracellularly or during the solubilization of the proteins for gel electrophoresis. In any case, we expected the Ly-6 domains to be intact and proceeded to purify them while looking for possible reasons for the degradation.

5.4.4 HPLC Purification of The 9804 Fusion Proteins

In order to generate Abs specific to 9804 protein, we first tried to purify the recombinant 9804 protein in a useful amount. HPLC was chosen as a powerful and convenient procedure. The infection was performed in a spinner flask with 50 ml of the Sf9 cells in a density of 2 X 10^6/ml medium. The cells were infected with BOMIGF-9804 recombinant virus clone #20 at an MOI of 10. Meanwhile, 3 X 10^6 Sf9 cells were plated in a 60 mm dish, infected with BOMIGF-9804 recombinant clone #18 and metabolically labeled with [^{35}S]-cysteine as described in the section 5.3. At 72 hrs postinfection, both labeled and unlabeled cells were collected by centrifugation at 1,100 rpm for 10 minutes. The cell pellets were then extracted in an acid extraction medium (1 M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and 1% (v/v) TFA) by homogenizing with Dounce Tissue Grinder. The cell extracts were cleared by centrifugation and subjected to reversed-phase extraction with C_{18} Sep Pak cartridges (Waters). The cartridge was eluted with acetonitrile-water (80:20, v/v), containing 0.1% (v/v) TFA and the eluate dried under speed vacuum. The samples from labeled and unlabeled cell extracts were mixed and applied to C_4 Vydac reversed phase HPLC. Acetonitrile fractions were collected, CPM measured, and samples subject SDS-PAGE analysis. On both CPM readouts and SDS-PAGE of HPLC fractions, the most strongly labeled component was eluted in fractions 46 to 48 with an approximate size of 28 kDa, as shown on the autoradiograph of the tricine-SDS-PAGE (Fig. 5.6). The size is similar to that of IGF-Ly-6E fusion protein detected by immunoprecipitation with mAb SK70.94. Therefore, with acid extraction, the 9804 fusion protein remained nondegraded. This implies that the proteinase activity encountered in the BOMIGF-9804 metabolic labeling experiment may have been a result

of the extraction method. Although IGF-9804 fusion protein was not secreted, it was expressed abundantly.

5.5 Discussion:

To produce quantities of soluble 9804 and Ly-6 proteins for immunization, functional and ligand studies, we employed a baculovirus expression system which had been successfully used to generate soluble IGFII-IL-3 protein (DiFalco *et al*, 1997; DiFalco and Fernando 1997). In this system, the gene for IL-3 was ligated in frame to that for human IGFII to generate the fusion gene which was placed under the control of baculovirus strong later promoter P_{PH}. An enterokinase cleavage site (DDDDK) was introduced between the two domains. The secretion of the fusion protein (BOMIGF-IL-3) is directed by an insect signal sequence fused to the N-terminus of IGFII. Expression of the fusion protein can be monitored by a bioassay of IGFII activity (Congote *et al.*, 1989) and in IL-3 ELISA, while the IL-3 moiety can be released by enterokinase cleavage between two domains of the fusion protein.

Sequences encoding 9804 and Ly-6 were inserted into the BOMIGF vector in the place of IL-3. Meanwhile the C-terminal signal sequence for GPI-attachment of the Ly-6 domains was replaced with a short tail GYSKSN. The 9804 and Ly-6 fusion proteins were, therefore, expected to be secreted and detectable in the IGFII bioassay. No antibody is available to 9804 yet, but appropriate folding of Ly-6E domains expressed in this way was predicted on the basis of the expression of Ly-6 E which was detected by mAb SK70.94.

IGF-Ly-6E and IGF-9804 were expressed abundantly in the baculovirus expression system 72 hours postinfection. However, neither were secreted as they were not detected in the culture supernatants even after 16 hours chasing.

It has been shown that expression and secretion of mammalian proteins in baculovirus system is influenced by different signal peptides. Heterologous signal peptides might be inefficiently recognized by the insect cell protein translocation machinery (Jarvis *et al.*, 1993). But in these constructs, a bombyxin signal peptide was used, and has proved to be efficient in directing secretion of other IGFII fusion proteins.

The cell-dependent glycosylation or folding is also a major factor that determines the final *in vivo* biopotency and secretion of recombinant glycoproteins. In baculovirus expression system for tissue type plasminogen activator (t-PA) and soluble form of CD4 (sCD4) (Jarvis and Summers, 1989; Kupesch *et al.*, 1992), an incomplete secretion of the sCD4 has been observed, although the protein was abundantly produced and correctly folded. Treatment of insect cells with tunicamycin, a glycosylation inhibitor, prevented secretion of the recombinant proteins. However, the secretion process was resistant to endo-beta-N-acetyl-D-glucosamindse H (endo-H), which removes immature, high-mannose-type oligosaccharides. The results demonstrated that, in the baculovirus expression system, the addition of N-linked oligosaccharides is required for the secretion of recombinant proteins, but complete glycosylation or processing of N-linked olygosaccharides is not an absolute requirement for secretion or folding.

In the case of BOMIGF-9804 and BOMIGF-Ly-6E, an insect signal sequence was used in the system, and has been shown to function efficiently for some IGFII-fusion proteins, as we demonstrated with IGF-IL-3. Thus we did not expect any problem with signal peptide. In fact, Ly-6E, although not secreted, was correctly folded, indicating that signal peptide was functioning to direct the polypeptide into the endoplastic reticulum (ER) lumen. It appears that, like sCD4 or t-PA protein, intracellular retention of BOMIGF-9804 and BONIGF-Ly-6 was not caused by misfolding of the Ly-6 domain.

Two types of signals are thought to confer apical targeting specificity to cell surface proteins in polarized epithelial cells: N-linked oligosaccharide chains and GPI anchor (Fiedler and Simons, 1995; Scheiffele *et al.*, 1995; Brown *et al.*, 1989; Lisanti *et al.*, 1989; Lisanti *et al.*, 1990). Native GPI-anchored Thy-1 was localized to the apical surface of the epithelia, while truncating 22 out of 31 hydrophobic amino acids from the COOH-terminus of Thy-1 resulted in apical secretion of Thy-1 (Powell *et al.*, 1991). DAF lacking its COOH-terminal signal sequence is also secreted (Caras and Weddell, 1989), while a fusion protein of human growth hormone and decay accelerating factor with a mutated sequence (hGHDAF28) was retained in a pre-Golgi compartment and degraded (Wainwright and Field, 1997). In our case of the recombinant 9804 and Ly-6E proteins, the GPI anchor was removed. In the absence of a GPI anchor, N-glycan signal

may determine the fate of the recombinant proteins after synthesis in the ER. Since the soluble forms of the recombinant 9804 and Ly-6E proteins in our experiments also lack any glycosylation site, it is possible that the recombinant proteins are retained in the pre-Golgi compartment as a consequence of missing appropriate sorting signal. CD59 does have an N-glycosylation site in the Ly-6 domain and has been efficiently secreted in a baculovirus expression system (Sugita *et al.*, 1994). In our other attempts to express soluble Ly-6 or 9804 proteins, the intracellular retention of the recombinant soluble Ly-6E proteins or a 9804 domain fused to IgG heavy chain was also observed with mammalian transfections (unpublished data). Incorporation of potential glycosylation site in the recombinant 9804 and Ly-6 proteins in future studies will provide evidence for a role of glycosylation in the secretion of the recombinant proteins.

Purification of the recombinant proteins from cells could be less convenient than from medium. Nevertheless, bioactive Ly-6-related proteins could be obtained through carefully selecting the purification strategies, since the proteins were abundantly expressed in the baculovirus system, and the Ly-6 domains of the fusion proteins are most likely correctly folded and their biological function retained.

CHAPTER 6
SUMMARY AND DISCUSSION

6.1 Summary

Ly-6 molecules were initially identified in mouse (McKenzie *et al.*, 1977; Woody *et al.*, 1977; Feeney & Hämmerling, 1976 as a result of immunogenetic, immunochemical and biochemical studies in this species. The *Ly-6* genes and the gene products have been reasonably well characterized (reviewed by Korty, 1989; Gumley *et al.*, 1995; Palfree 1996). In particular, Ly-6 are cell surface GPI-anchored proteins (Reiser *et al.*, 1986; LeClair *et al.*, 1986; Hämmelburger *et al.*, 1987), whose expression is controlled by a gene complex of at least 18 tightly linked genes on mouse chromosome 15 (Kamiura *et al.*, 1991). Cloning of Ly-6 cDNAs and characterization of the chromosomal genes have revealed a multigene family with a characteristic four exon organization (Bothwell *et al.*, 1988; McGrew and Rock, 1991; Stanford, 1992; Fleming *et al.*, 1993; Gumley, *et al.*, 1995). The encoded peptides have characteristic placement of 10 cysteine residues within the mature proteins and both N- and C-terminal consensus sequences. Sequence analysis of Ly-6-related proteins has revealed a structural relationship of Ly-6 proteins with neurotoxins. The cysteine and other conserved amino acid residues within Ly-6 domains have been identified to be important for the folding of the proteins into a 3D structure similar to that of neurotoxins as typified by α-bungarotoxin (Fleming *et al.*, 1993a).

Based on unique tissue distribution pattern during hematopoiesis (Spangrude *et al.*, 1988; Stout *et al.*, 1975; Eckhardt *et al.*, 1980; Godfrey *et al.*, 1992; Randle *et al.*, 1993; Bamezai *et al.*, 1995), Ly-6 molecules are expected to play roles in cell differentiation. *In vitro* studies with mAbs specific to Ly-6 have shown that cross-linking of Ly-6 molecules, in the presence of appropriate second signals, could induce lymphocyte signaling and activation as measured by elevated $[Ca^{++}]_i$, tyrosine phosphorylation, cytokine production and cell proliferation (Flood *et al.*, 1985; Malek *et al.*, 1986; Ortega *et al.*, 1986; Rock *et al.*, 1986; Yeh *et al.*, 1987; Havran *et al.*, 1988). The observations imply that crosslinking of Ly-6 molecules by their natural ligands can affect intracellular signaling. Several lines of evidence point to their involvement in

direct cell-cell contact through ligands on cell surfaces which promote intercellular adhesion or signal interactions (Bamezai *et al.*, 1995a; Bamezai *et al.*, 1995b; Johnson *et al.*, 1993; Brakenhoff *et al.*, 1995 and Schrijvers et al., 1991). The actual physiological function of Ly-6 molecules have yet to be elucidated.

Outside the mouse species, Ly-6 homologues have been identified in rat (RK6, 10 and 11) (Friedman *et al.*, 1990), and very recently in chicken (ChSca-2) (GenBank accession number: L34554, CHKSCA2A). More distant Ly-6 homologues were also found in squid (Sgp-2) (Willian *et al.*, 1988), human (CD59 and uPAR) (Davies *et al,* 1989; Sugita *et al.*, 1989; Sawada *et al.*, 1989; Nielsen *et al.*, 1988; Roldan *et al.*, 1990), but the significance of Ly-6 in other species including human was largely unknown. Because of the conservation of the Ly-6-related genes in a wide range of the species and a potential role for Ly-6 molecules in immune responses, homologous *Ly-6* genes in human species were expected. My first objective was to identify *Ly-6* genes in species other than rodents, in particular in human, and characterize them and their products.

Preliminary screening by Southern blotting of chromosomal DNAs from wide range of species revealed several fragments in rabbit cross-hybridizing with the Ly-6A probe and single band patterns only with the ThB probe in guinea pig (see Appendix). The cloning and sequencing of the cross-hybridizing fragments were started. But data from the first clones did no yield any sequences related to *Ly-6* genes. During the course of this project, there was a major breakthrough in the Ly-6 field. A human Ly-6 cDNA, named 9804, was discovered by our collaborators E. Cohen and B. Landgraf in CytoMed, Mass. and became available for further study. The preceding chapters of this thesis described the characterization of *9804* gene structure and expression. This chapter will summarize the major findings of the research, discuss the immunological/physiological significance of some of the results and prospect several avenues for future research.

6.2 *9804* (hTSA-1) Is A Human Gene Apparently Homologous with Mouse *TSA-1*

To characterize the *9804* gene structure, a human genomic cosmid clone which hybridized with 9804 cDNA was fragmented with restriction endonucleases. The fragments hybridizing with 9804 cDNA were subcloned into the pBluescript II KS^{+-}

vector. The complete gene was characterized by isolation and sequencing of several overlapping gene fragments. The *9804* gene has the typical four exon organization of the Ly-6 family. The 9804 cDNA sequence is contained in three coding exons (II to IV), while exon I is entirely non-coding. The intron/exon boundaries were found to corresponded well with those of other *Ly-6* genes (see Chapter 2). Database searches with 9804 cDNA sequence revealed a similarity with mouse Ly-6 with highest score (60% in nucleotide and 63% in amino acid identity) to TSA-1. Furthermore, like mouse *Ly-6* genes, the 9804 promoter lacked TATA or CAAT consensus sequences but contains several GC boxes.

For physical mapping of the *9804* gene, DNA from BIOS-MAP human-hamster hybrid cell panel 1 was initially screened by PCR with *9804*-specific primers. The bands amplified were only from hybrids containing chromosome 8. Further studies were performed by D. Wells using our primer pairs on human chromosome 8-related human-hamster cell hybrids (CL-17, 3;8/4-1, MC2F, 21q$^-$ and TL/UC), YACs in the distal portion of a large YAC contig (Chen *et al.*, 1996) extending into 8q24, and a human chromosome 8 cosmid library. Final mapping of the *9804* gene was completed by fluorescent *in situ* hybridization with a positive cosmid clone (c101F1) on the human-hamster hybrid CL-17. The refined studies localized *9804* gene on chromosome 8 at the region 8q24.3 (telomeric to *c-myc)* as a single copy in the human genome (see Chapter 2). The mapping of *9804* gene to the 8q24.3 region is significant since the region is homologous to the mouse *Ly-6* locus on chromosome 15. One would expect, therefore, additional human Ly-6-related genes in the vicinity of the *9804* gene. This is further supported by a recent report of the discovery and mapping of another human Ly-6 homologue, E48, to the some region on chromosome 8.

Northern analyses of 9804 expression (see Chapter 3) revealed a broad tissue distribution pattern in normal fetal and adult tissues or cells, and cell lines of diverse lineages. The highest expression levels in adults are found in lung, kidney, ovary, PBMC and liver. The same high levels are evident in fetal spleen and adrenal. Low levels are present in the heart, muscle, thymus, adult spleen and fetal liver. However, difference has been noticed that, while TSA-1 is mainly expressed on peripheral B cells and little on T

cells, 9804 is neither expressed nor induced by IFNs or ATRA on the B cell lines Raji and Daudi, but it is expressed at a moderate level on the normal PBL and T cell line Jurkat. The fine distribution of 9804 on different cell types should be determined by FACS or *in situ* hybridization. Overall, the broad tissue distribution and the similarity of gene expression in certain tissues are parallel to that observed in mouse TSA-1. These data are consistent with the notion that 9804 is a functional homologue of mouse TSA-1.

6.3 *9804* Is An IFN-α- As Well As Retinoic Acid-Inducible Gene

Among a panel of thirteen human recombinant cytokines, IFN-α induced 9804 mRNA expression in a variety of human cell lines and normal human PBMC. *9804* gene was also reported last year as a retinoic acid inducible gene, *RIG-E* (Mao *et al.*, 1996). Our data from the Northern blot analyses showed that elevation of 9804 mRNA levels in response to ATRA was restricted to myeloid leukemia cell lines (NB4, HL60 and U937), was relatively less intense and occurred as a later event (36-48 hrs). In contrast, 9804 expression is more strongly and rapidly (8-12 hrs) enhanced by IFN-α in normal PBMC and in a broad range of cell lines (Jurkat, U937, HL60, NB4, KG-1, TF-1, U373MG and HTB88). Results from inclusion of cyclohexamide and actinomycin D in the cultures indicated that new protein synthesis was required during the first 2 hrs of induction of 9804 by IFN-α and that the induction was likely at the level of transcription.

Analysis of the 5'-flanking region of the human *9804* gene revealed a very large number of putative response elements, as is generally the case in signal sequence scans of arbitrary DNA sequences. Most of these will not be of any physiological relevance. Interestingly, however, ISRE and RARE were also found in the 5' flanking region, and these may function in the induction of *9804* gene expression by IFN-α and ATRA (Chapter 4). Promoter studies with a luciferase reporter system are being performed in a series of constructs generated from the 5'-flanking region of the *9804* gene. Direct or indirect effects of IFN-α or ATRA on the gene expression is being further studied in the transient transfectants treated with IFN-α or ATRA.

The induction of *9804* gene by IFN-α and ATRA are both interesting when considering the circumstances under which *9804*/TSA-1 may function. The IFN-

inducibility of Ly-6 RNA and protein expression is found in other members of the Ly-6 family, particularly Ly-6A/E and Ly-6C (Dumont *et al.*, 1986a; Rock *et al.*, 1986). In addition to entrenching the relationship between 9804 and mouse Ly-6, the IFN-α inducibility of 9804 suggests a functional relevance of this molecule to immune regulation and anti-viral responses. The induction of *Ly-6* genes by IFNs has been well documented through cell surface receptor and cytoplasmic Jak-STAT phosphoproteins. On the other hand, retinoic acids exert their pleiotropic biological effects by interacting with their receptors (RARs) that belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors (Mangelsdorf *et al.*, 1995). ATRA induces *in vitro* differentiation of leukemia or solid tumor cell lines and a complete remission in patients with APL (Breitman *et al.*, 1980; Breitman *et al.*, 1981; Huang *et al.*, 1988; Castaigne *et al.*, 1990). As one of a few genes induced during cell differentiation, 9804 is potentially involved in the process of ATRA-induced cell differentiation, possibly allowing further signals for differentiation through inter-cellular interactions. The studies of the induction of 9804 expression with suboptimal concentrations of IFN-α or ATRA, or in combination have been started to investigate a possible synergistic effect of these two inducers. These studies will be clinically significant since RA-resistance and toxicity have been observed in the treatment of APL patients. 9804 expression may be indicative of a successful response to IFN and RA induced leukemia cell differentiation.

6.4 TSA-1 And 9804 Use Alternative Exon I for Mechanism of Transcription

During a routine dbEST search, a second exon I (exon IB) was found to be used in some transcripts of TSA-1 gene. This exon is further upstream of the initially identified exon I (exon IA). An alternative exon I usage was observed in TSA-1 by detecting the transcripts with either pan-TSA-1, exon IA or exon IB probes (see Chapter 3). Exon IA is used most of the time, while exon IB is only used by a few tissues such as brain, kidney (C57BL/6) and placenta.

This discovery alerted us to the possibility of alternative promoters for 9804 transcription. Already we had suspected that not all transcripts contained the 9804 exon I because the original 9804 cDNA, and many of dbEST entries for this gene did not have

any exon I sequence. Also in the S1 nuclease assay with reverse primer (9804X1R), we could not detect a protected fragment with RNA prepared from KG-1a cells (Fig. 2.8). Northern blot analyses with 9804X1R probe detected significantly lower level of 9804 mRNA than that was detected with the 9804 cDNA probe (without exon I sequence) in the KG-1a (Fig. 3.5). This may indicate that either an alternative exon I was used, as was observed in mouse TSA-1, or transcription is initiated from exon II. As TSA-1 and 9804 both have broad tissue distribution pattern, tissue- or cell-specific factors may be responsible for the different mechanisms of exon I usage.

6.5 Future Areas of Human Ly-6 Research

In the past few years, the discovery of human homologues of mouse *Ly-6* genes (*9804/RIG-E* and *E48*) has brought Ly-6 research into the human arena. The structural and sequence characterization of the human *Ly-6* genes comfirmed their membership of the Ly-6 family. A human Ly-6 multigene family is emerging with the evidence of the mapping of E48 and 9804/RIG-E genes to chromosome 8q24 at the same region syntenic with that of mouse chromosome 15 containing the *Ly-6* locus. A preliminary Southern blot result from Mao *et al.* (Mao *et al.*, 1996) has also shown a multiple cross-hybridization banding pattern in human genomic DNA with a RIG-E cDNA probe. It is to be expected, therefore, more human *Ly-6* genes will be identified within this region. As a consequence of this chromosome mapping work, several overlapping cosmids have been identified in the vicinity of the 9804 gene, these initiating the process of chromosomal walking which should lead to the isolation of other human Ly-6 genes.

It has been evident that E48 is involved in keratinocyte adhesion (Brankenhoff *et al.*, 1995). And a functional relevance of 9804 molecule during cell differentiation and immune response has been suggested by the observations of the induction of 9804 RNA expression by IFN-α and ATRA. It is also intriguing that 9804 cDNA was identified as a potential costimulatory factor in an *in vitro* system designed for T cell activation studies (Cohen *et al.*, submitted). The structural similarity with TSA-1 and other Ly-6 molecules suggested that 9804 protein is most likely a cell surface receptor with GPI-attachment. To study the functional model and ligands for 9804 molecule, specific Abs and

recombinant soluble 9804 proteins are desired. We have been expressing and purifying soluble 9804 and other mouse Ly-6 molecules from the baculovirus system for the purpose of immunizing animals and for ligand studies. Although the recombinant proteins were not secreted, which means they must be purified from a higher level of irrelevant proteins, they were expressed abundantly and to the extent we were able to asses it in the case of Ly-6E. They are likely appropriately folded. The purification steps performed so far yield a high level of enrichment of the recombinant proteins from the background of other cysteine containing proteins. Further work is required with a larger volume of starting material before we can asses purity with respect to protein in general.

To further characterize the 9804 promoter and the response elements for IFN-α and ATRA induction, promoter reporter assay will be continued with the fragments from the 5'-flanking region of the 9804 gene in the luciferase expression vector. In addition, gel retardation and foot-printing within the promoter region will be performed to identify tissue-specific transcription factors involved in the induction of the *9804* gene by ATRA or IFNs. Identification of response elements or transcription regulatory factors will help us understand the signaling of and cross-talk between IFN-α and RA pathways.

To identify the expression of the *9804* gene in particular cell types in complex tissues, such as the adrenal, *in situ* hybridization studies will be carried out. Detection of 9804 protein expression relies on the generation and use of 9804 Abs. They will be particularly useful for studying a tissue or cell type specificity and potential functions of 9804 molecules during hematopoiesis and cell activation.

In conclusion, we have characterized and mapped the first human Ly-6 homologue *9804* and clearly established the presence of a human Ly-6 multigene family. Further analysis on the region 8q24 of the human chromosome 8 will lead to the identification of additional genes of the human *Ly-6* multigene family in the vicinity of *9804*. From the sequence analysis and expression studies, we defined 9804 as a structural as well as functional homologue of mouse TSA-1. The strong induction of 9804 in several cell types by IFN-α indicated an important function of the molecule in immune responses. We therefore expect that study during immune responses will be the most fruitful approach to understand one of its functions. Further studies during myeloid cell

differentiation is important to our understanding of 9804, and Ly-6 in general, in the fundamental principles of cellular development and activation.

CHAPTER 7

EXPERIMENTAL PROCEDURES

7.1 Isolation of DNA And RNA

7.1.1 High Molecular Weight DNA Isolation

Freshly obtained tissue (1-2g) from various species was chopped into about 10 mm^3, washed in ice cold Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, pH 7.4) and ground on dry ice to powder. This was resuspended in 2 ml of a 10 mM Tris-HCl, pH 7.6, 50 mM NaCl solution, followed by the addition of 12 ml of 10 mM Tris-HCl pH 7.6, 50 mM NaCl, 0.2% SDS containing freshly added proteinase K (200 μg/ml) and mixed gently. Following incubation at 42 °C for 16 hrs with gentle agitation to digest the proteins, the DNA was extracted with an equal volume of water-saturated phenol-chloroform-isoamyl alcohol (24:24:1). Extractions were performed in screw capped polypropylene tubes by gentle rocking for 10 min to obtain a complete emulsion, centrifuging at 600 x g (using a Beckman JS-4.2 rotor in a Beckman J6-B centrifuge; Beckman Instruments Inc. Fullerton, CA) for 3 min, and recovering the aqueous phase. The extraction was repeated once with phenol-chloroform-isoamyl alcohol (24:24:1) and twice with chloroform-isoamyl alcohol (24:1). The DNA was precipitated from the aqueous phase by adding 0.02 volume of 3 M NaCl and 1 volume of isopropanol. The DNA was then removed with a glass rod, washed 3 times with 70% ethanol and resuspended in 1 mM Tris-HCl, pH 7.6, and 0.1 mM EDTA. The resuspended DNA was then dialyzed extensively in 1 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, at 4 °C until the DNA was fully dissolved. The concentration of the DNA was determined by measuring the absorbance at 260 nm (Hitachi U-2000 Spectrophotometer, Hitachi Instruments, Danbury).

7.1.2 Bacteriophage Lambda DNA Isolation

Bacteria were grown in LB medium plus 10 mM $MgCl_2$ and 0.2% maltose or NZY medium plus 0.2% maltose at 37 °C to mid-log phase (OD_{590} = 0.6) and infected with phage lysate at a MOI (multiplicity of infection) of 0.1. The bacteria and phages

were mixed well and left stationary for 10 min at 37 °C to permit effective adsorption of phage to the bacteria. The infected cells were cultured at 37 °C with vigorously shaking until lysis was obvious (4-5 hrs postinfection). Solid NaCl was added to 1 M, and dissolved by mixing. The cultures was centrifuged at 5,000 x g for 20 min at 4 °C (JA-20 rotor, Beckman J2-21 centrifuge) to pellet cell debris. Polyethylene glycol (PEG, Sigma PEG 6000) was added to a final concentration of 1.25 M. The mixture was stirred until the PEG was completely dissolved (>1 hr) and then centrifuged again at 5,000 x g for 20 min at 4 °C to pellet the phage particles. The precipitated phage was resuspended in TNM (0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl, 0.01 M $MgCl_2$), overlayed on top of a two step CsCl (5 M and 3 M in TNM) gradient in an SW27 Ultra clear centrifuge tubes and centrifuged in the SW27 rotor for 90 min at 24,000 rpm at 15 °C. The band of the phage between the 3 M and 5 M solutions was collected and extracted twice with phenol (equilibrated with 0.2 M Tris-HCl, pH 7.6) and 4 times with chloroform. The aqueous phase containing purified phage DNA was then dialyzed vs. TE buffer (0.01 M Tris-HCl, pH 8.0, 0.001 M EDTA) for 1-2 days at 4 °C with 3 changes of buffer. The phage DNA was precipitated with 2.5 volume of 100% ethanol, and the DNA removed with a glass rod, washed with 70% ethanol, and allowed residue ethanol to evaporate before redissolving DNA with H_2O. The concentration was determined by measuring the absorbance 260 nm (Hitachi U2000 Spectrophotometer).

7.1.3 Plasmid/Cosmid DNA Isolation

Plasmid/cosmid preparation was performed based on a modified alkaline lysis method (Feliciello and Chinali, 1993) or using QIAGEN Plasmid Maxi column following the manufacturer's instruction. Mini-prep by alkaline lysis method is described below while a large scale plasmid preparation was carried out essentially by scaling up this protocol and using the Backman T2-21 for centrifugation.

A single bacterial colony was inoculated in 5 ml of LB medium supplemented with 0.2% glucose containing appropriate antibiotic and grown overnight at 37 °C with vigorously shaking. The cells are pelleted then resuspended and washed with cold STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH8.0) by centrifugation at 1,000 x

g for 5 min. 250 µl of ice cold solution I (50 mM glucose, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) was added and votexed well. 500 µl of freshly prepared solution II (0.2 N NaOH-1% SDS) was then added and mixed immediately by inversion of the tube which was kept on ice for 5 min. 750 µl of ice cold solution III (4 M potassium acetate-2 M acetic aced) was added with immediate and vigorous shaking of the tube to mix the contents. The tube was kept on ice for 10 min followed by centrifugation at 12,000g for 5 min at 4 °C. The supernatant was transferred to a clean tube and the DNA precipitated by addition of 0.7 ml of isopropanol and centrifugation for 5 min at 12,000g. The DNA pellet was resuspended in 250 µl of TE containing 10 µg/ml of RNase, votexed and left at room temperature for 10 min. The plasmid/cosmid DNA was precipitated by addition of 300 µl of 88% isopropanol-0.2 M potassium acetate, incubated at RT for 10 min and recovered by centrifugation at 12,000g for 15 min. The pellet was resuspend in 50 µl of TE and stored at -20 °C.

7.1.4 Total RNA Extraction

Total cellular RNA was isolated from cell lines and tissues by a modification of Chirgwin's guanidinium/CsCl ultracentrifugation method (Chirgwin *et al.*, 1979). Briefly, fresh or frozen cell pellet (2 x 10^7 to 10^8 cells) was lysed by addition of 2.5 ml of guanidine working solution (4 M guanidine isothiocyanate, 26.4 mM sodium citrate, pH 7.0, 0.5% N-lauroylsarcosine, 93 mM 2-mecaptoethanol, final pH 5.6). Tissue (1 to 2 gm) was homogenized in 5 ml guanidine working solution without sarkosyl which was added after homogenization. The tissue homogenate was centrifuged at 10,000 rpm, 12 °C for 20 min to obtain supernatant. The cell lysate or supernatant from the tissue was sheered through 21G needle for 10 times and overlayed onto 1.2 ml CsCl in an ultracentrifuge tube. The tubes were centrifuged at 35,000 rpm, (SW60 rotor, Beckman J2-21 centrifuge) for 15 hrs at 18 °C. The resulting pellet was then dissolved in dH_2O and precipitated twice at -70 °C by addition of 0.1 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol. The RNA was recovered by centrifugation in a microcentrifuge at maximum speed for 15 min, rinsed in 70% ethanol and resuspended in 12 to 50µl of diethyl pyrocarbonate (DEPC)-treated dH_2O. The concentration was

determined by measuring the absorbance at 260 nm (Hitachi U-2000 Spectrophotometer).

7.1.5 Single Stranded Phage DNA Preparation

Single colonies of XL-1 Blue bacteria containing cloned DNA fragments in pBluescript II KS^{+-} vectors, selected on tetracycline and ampicillin plates, were inoculated in 4 ml of LB medium containing ampicillin and grown over night at 37 °C. The over night culture was inoculated into 50 ml of superbloth (35g Bacto-tryptone, 20g Bacto-yeast extract, 5g NaCl per liter, pH 7.5) and grown to OD_{600} = 0.3. Help phage (M13 KO7) was added at MOI = 20 and the culture continued for 8 hrs. The culture was heated to 65 °C and clarified by centrifugation at 17,000g for 5 min. 1.2 ml supernatant was transferred to each fresh tube and precipitated by addition of 300 µl 20% PEG at room temperature for 15 min. DNA was recovered by centrifugation at 11,000g for 20 min and the pellet was resuspended in 300 µl of TE, pH 8.0. The suspension was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) twice and chloroform: isoamyl alcohol (24:1) once. Single stranded DNA was precipitated with 0.67 volume of 7.5 M NH_4AC, pH 7.5 and 2.5 volume of 100% ethanol on ice for 15 min and recovered by centrifugation at 11,000 rpm, 4 °C for 20 min. The pellet was washed with 70% cold ethanol, dried and dissolved in 20 µl of TE and analyzed by electrophoresis in a 1% agarose gel. The DNA concentration was determined by measuring the absorbance at 260 nm (Hitachi U-2000 Spectrophotometer).

7.1.6 Preparation of Insert Fragments

cDNA inserts used for ligation or as probes in Northern or Southern blot analyses were excised from plasmids by restriction digestion singly or in combination accordingly, and run on a 1% agarose gel in TAE buffer. The fragments were visualized by viewing the ethidium bromide stained gel on a UV transilluminator. DNA fragments were sliced and isolated from the gel by either GeneClean Kit (InvitroGene) according to manufacture's instruction or electroeluted in the dialysis tubing, phenol:chloroform: isoamyl:alcohol (24:24:1) extracted and ethanol precipitated. The DNA concentration

was estimated by running an aliquot on a mini-agarose gel against standard linear DNA of known concentration.

7.2 Radiolabeling of The Probes

7.2.1 End-labeling of Oligonucleotides

Oligonucleotides were all synthesized in the Sheldom Biotechnology Center, McGill University. Those used in the primer extension and S1 nuclease assay and as probes in Northern blot analyses were end-labeled in a reaction of 50 pmol oligonucleotide, 2 μl of γ-ATP [^{32}P] (7,000 Ci/mmol, 166 Ci/ml, ICN), 1 x OPA^{+} buffer (Pharmacia), 1 mM spermidine and 10 units T4 polynucleotide kinase (GIBCO, BRL) at 37 °C for 30 min. The reaction was applied to a 1 ml G-50 Sephadex spun column (home-made in a 1 ml syringe). The flow through containing the labeled DNA was collected, and 2 μl was counted to determine incorporation of label.

7.2.2 Random-Primer Labeling of cDNA Probes

The purified cDNA inserts were labeled using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instruction. Briefly, 30 ng insert in 9 μl of H_2O was heated at 100 °C to denature the double stranded DNA and quickly chilled on ice. 1 μl of each dATP, dGTP and dTTP, 2 μl of reaction mix which contained reaction buffer and random hexamer oligonucleotide, 5 μl of ^{32}P-dCTP (300 mCi/mmol) and 1 μl of Klenow fragment were added. The reaction was carried out at 37 °C for 30 min and the enzyme activity was inactivated in 50 mM EDTA at 65 °C for 10 min. The labeled probe was precipitated at -70 °C with 6μl of 3 M sodium acetate, 120 μl of ethanol and 8μl of tRNA carrier. The labeling efficiency was determined by measuring CPM.

7.2.3 Generation of Single-Stranded Probes for S1 Nuclease Assay

Single-stranded probes for use in nuclease protection analysis were generated either by PCR amplification or primed synthesis from single stranded DNA template with Klenow fragment of *E. coli* DNA polymerase I. For PCR amplification, plasmid

P26 (see Figure 2.3) was cut with restriction enzyme *Bgl* II and used as template for linear PCR with T7 primer for 40 cycles (denaturing at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 72 °C for 1 min). The first PCR product of 1,500 nucleotides was gel purified and used as template in the asymmetric PCR. Reverse primer 9804X1R (Table 2.2), corresponding to the 3' end of the 9804 exon I, was used in a 40 cycled-PCR (denaturing at 94 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 1 min) with an uniform incorporation of 2μl ^{32}P-dCTP (300 mCi/mmol) during the reactions. The PCR product was electrophoresed on 1% agarose gel and autoradiographed. The labeled band of approximately 1,300 nucleotides was excised from the gel and purified by electroelution and precipitation. The specificity of the probe generated was determined by CPM. For the alternative procedure, single-stranded template SS16 was mixed with end-labeled 9804X1R in Klenow salt solution (50 mM NaCl, 10 mM Tris-HCl, pH 7.5 and 10 mM $MgCl_2$), heated at 85 °C for 5 min to denature and cooled down slowly to 37 °C over 30 min to allow primer annealing. 1 μl of each 20 mM dATP, dGTP and dTTP, 1 μl of 100 μM dCTP and 5 μl of [α-^{32}P]dCTP (300 mCi/mmol, ICN), 10 mM DTT and 5 units of Klenow fragment of *E. coli* DNA polymerase I were added to the reaction and incubated for 30 min at room temperature. 1 μl of a 20 mM unlabeled dATP was added to the reaction and further incubated at room temperature for 20 min to complete the reaction. The Klenow was inactivated by heating at 68 °C for 10 min, and the duplex was digested with *Pst* I after adjustment of the concentration of NaCl to 100 mM. The radiolabeled probe was separated from the larger unlabeled template by electrophoresis through a denaturing alkaline agarose gel (section 7.4.2), and recovered by excision of the labeled region from the gel and electroelution. The incorporation of α-[^{32}P]dCTP was determined.

7.3 Library Screening by Hybridization

7.3.1 Rabbit Genomic Library

A rabbit genomic library in the lambda DASH II vector (Stratagene, La Jolla, CA) consisting of 2.0 x 10^6 independent clones was screened with an Ly-6A cDNA probe (690 bp released from pBluescript II KS$^+$ by *Eco* RI digestion) labeled with ^{32}P by

random priming. Duplicate plaque lifts on nitrocellulose filters (Schleicher & Schuell, Keene, NH) were prehybridized in 4X SET (1X SET is 0.15 M sodium chloride, 20 mM Tris-HCl, pH 7.8, 1 mM EDTA), 10X Denhardt's (1X Denhardt's is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.1% sodium pyrophosphate and 0.1% SDS at 50 °C for 6 hrs. Hybridization was in 6X SSC (1X SSC is 0.15 M sodium chloride, 0.015 M Trisodium citrate, pH 7.0), 10X Denhart's, 0.05% pyrophosphate at 42 °C for 16 hrs. Filters were then washed with 6X SSC, 0.1% SDS at RT for 15 min, 6X SSC, 0.1% SDS at 42 °C for 15 min, 2X SSC, 0.1% SDS at 42 °C for 40 min, 1X SSC, 0.1% SDS at 42 °C for one hr, and placed at -70 °C with XAR-5 film (Eastman Kodak Co., Rochester, NY) and an intensifying screen for autoradiographic detection of the bound probe. Positive clones were plaque purified and λ DNA preparations were made. The DNA from positively hybridizing clones were digested with the restriction enzyme *Eco* RI, *Not* I or other enzymes (*Pst* I, *Hind* III, *Kpn* I, *Bst* XI, *Bgl* II, *Xba* I, *Sac* I, *Bam* HI, *Sac* I, *Xho* I, *Sma* I, *Pvu* II, *Apa* I). The resulting fragments were then subcloned into pBluescript II KS$^+$. The nucleotide sequences of the cloned genomic fragments were obtained by double stranded dideoxy sequencing (T7 polymerase sequencing kit, Pharmacia).

7.3.2 Guinea Pig Genomic Library

A guinea pig genomic library in EMBL-3 (Clontech, Palo Alto, CA) containing 1.4 x 10^6 independent clones were probed with a ThB cDNA (700 bp *Hind* III/*Pst* I double digested fragment from pThB-A (Gumley *et al.*, 1992). labeled with ^{32}P by random priming. Prehybridization was performed in a hybridization buffer (4X SET, 10X Denhart's, 0.1% SDS, 0.1% sodium pyrophosphate and 50 μg/ml salmon sperm DNA) at 55 °C for 6 hrs. Hybridization was performed under the same condition as described above for 16 hrs. The DNA from positive clones was digested with *Pst* I and other restriction enzymes (*Kpn* I, *Apa* I, *Xho* I, *Sal* I, *Hind* III, *Eco* RI, *Sma* I, *Bam* HI, *Xba* I, *Not* I, *Bst* XI, *Sac* I and *Sac* II). Resulting fragments were subcloned into pBluescript II KS$^+$. The genomic fragments were sequenced by double stranded dideoxy method (T7 polymerase sequencing kit, Pharmacia).

7.4 Gel Electrophoresis

7.4.1 Agarose Gel Electrophoresis of DNA

Electrophoretic separations were carried out in a horizontal electrophoresis apparatus (Model MPH or QSH Multi Purpose Gel Electrophoresis, IBI) in 0.7%, 1.0%, or 1.5% (w/v) agarose containing ethidium bromide (0.5 μg/ml) at 5-10 V/cm for 1-4 hr at ambient temperature. In the case of genomic digestions, electrophoresis was performed at a low voltage of 120V overnight in the High Resolution Horizontal Gel Electrophoresis Unit (Model HRH, IBI) with buffer recirculation and cooling. Electrophoresis buffer was either 1X TAE (50 mM Tris base, 30 mM sodium acetate, 10 mM EDTA, pH 8.0) or 1X TBE (90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA, pH 8.0). DNA was visualized in the gel by short wavelength (254 nm) ultraviolet transillumination (Model UVT 400-M, U.V. fluorescent Table, IBI) and photographed using Polaroid ® film, type 667 (Polaroid Corp. Cambridge, MA).

7.4.2 Alkaline Agarose Denaturing Gel Electrophoresis

1% (w/v) agarose in H_2O was dissolved in a microwave oven and cooled down to 60 °C. NaOH was added to 50 mM and EDTA (pH 8.0) to 1 mM before pouring the gel. DNA samples were prepared by ethanol precipitation and dissolved in 10-20 μl of 50 mM NaOH, 1 mM EDTA, and 0.2 volume of 6X alkaline loading buffer (300 mM NaOH, 18% Ficoll, 6 mM EDTA, pH 8.0, 0.15% Bromocresol green and 0.25% Xylene cyanol FF) was added. The samples were electrophoresed in the alkaline running buffer (50 mM NaOH and 1 mM EDTA).

7.4.3 Polyacrylamide Gel Electrophoresis of Proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in either a mini-step gradient or a tricine buffering system with a Mini-Protean Slab-gel™ apparatus (Bio-Rad). The mini-step gel was a modification of Laemmli system (Cleland *et al.*, 1977). Briefly the protein samples were resuspended in 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 0.00125% bromophenol blue, with

2.5 β-mercaptoethanol (reducing), or without β-mercaptoethanol (nonreducing), and heated for 5 min at 100 °C to denature proteins. The samples were then applied to a 0.75 to 1.0 mm thick polyacrylamide gel comprising a separation phase of three steps (11.5%, 14.2% 17.0% acrylamide:acrylaide, 375 mM Tris, pH 8.8, and 0.1% SDS) and stacking phase (5.1% acrylamide, 159.5 mM Tris, pH 6.8, 0.127% SDS) with a glycine electrod buffer (24.8 mM Tris, 192 mM glycine, 0.1%SDS at volt limit 200V, current 50mA. The tricine-SDS-PAGE was based on the method by Schägger and Von Jagow (1987). Briefly, the separation gel was prepared as 12.5% acrylamide and 0.7% acrylaide, 3.3% sucrose, 1 M Tris-HCl pH 8.45 and 0.1% SDS and stacking gel as 4.2% acrylamide, 0.23% acrylaide, 0.75 M Tris-HCl and 0.075% SDS. Protein samples were prepared as described in the mini-step gradient gel and electrophoresed with anode buffer of 0.2 M Tris-HCl and cathode buffer of 0.1 M Tris, 0.1 M tricine and 0.1% SDS.

7.5 DNA And RNA Hybridizations

7.5.1 Bacterial Colony Lifts And Hybridization

Bacteria were spreaded or subcultured in grid-form on an LB agar plates containing appropriate antibiotic. Nitrocellulose (Schleicher & Schuell, 0.45μm, 82mm) or Nylon (ICN, 1.2 μm, 82mm) discs were placed on the bacterial colonies for 1 min. The filters were peeled from the plates and placed in succession, colony side up, on Whatman 3MM papers saturated with i) 0.5 M NaOH, 1.5 M NaCl for 10 min, ii) 1.5 M NaCl, 0.5 M Tris-HCl pH 7.4 for 5 min and iii) 2X SSC for 2 min. The filters were allowed to dry at room temperature before being baked for 2 hrs at 80 °C in a vacuum oven. The filters were prehybridized in 5X SSC, 5X Denhardt's solution, 0.2% SDS at 65 °C for 3-8 hrs, then change the fresh hybridization buffer with ^{32}P labeled probe at 65 °C for 16 hrs. The filters were washed at 68 °C for 60 min each in 2x SSC, 0.1% SDS and in 0.5X SSC, 1% SDS, respectively, and exposed at -70 °C with Kodak XAR-5 film with an intensifying screen.

7.5.2 Southern Blot Hybridization

Following electrophoresis on an agarose gel (section 7.4.1), the DNA was transferred to nylon membranes (Zeta-Probe, BioRad) by capillary blotting with 0.4 M NaOH for 16 hrs. In the case of high molecular weight genomic DNA digestion's, the DNA in the gels were depurinated by soaking the gel in 0.25 M HCl for 15 min before transferring. The membranes were then baked for 30 min at 80 °C in a vacuum oven. The blots were prehybridized in buffer containing 0.5 M NaH_2PO_4, pH 7.2, 7% SDS and 1 mM EDTA at 60 °C for 6 hrs, followed by hybridization in the fresh hybridization buffer with ^{32}P-labeled probe at 60 °C for 16 hrs. The blots were washed at 60 °C, 2 times for 30 -60 min each, in the solution of 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS, and 2 times in 1 mM EDTA, 40 mM $NaHPO_4$, 1% SDS. Autoradiograms were obtained by exposing Kodak XAR-5 film or REFLECTION film (NEN-Dupont) to the blots at -70 °C with intensifying screens.

7.5.3 Northern Blot Analysis

Human multiple tissue northern (MTN) blots were purchased from Clonetech (Palo Alto) and processed following the manufacturer's instructions. Some RNA from adult and fetal tissues were kindly supplied by A. Bateman (Endocrine Laboratory, Royal Victoria Hospital, Montreal) and most prepared as described in section 7.1.4. 10 to 20 μg of total RNA was denatured for 15 min at 56 °C in the denaturing buffer (64.5% formamide, 1.3X MOPS and 22.5% formaldehyde), separated on denaturing 0.85% agarose gel containing 3% formaldehyde in 1X MOPS, and transferred onto Zeta-Probe nylon membrane (BIO-RAD) with 10X SSC by a capillary method. The filters were then air-dried and baked at 80 °C vacuum oven for 30 min before proceeding to hybridization. For hybridization with cDNA probe, the filters were prehybridized for 2 to 12 hrs at 63 °C in hybridization buffer (0.5 M sodium phosphate, pH 7.2, 7% sodium SDS, and 1 mM EDTA). 1 to 2 x 10^6 CPM ^{32}P-labeled probe (with Random Primed DNA Labeling Kit, Boehringer Mannheim Biochemica) was added per ml of the hybridization buffer, and incubated for 20 hrs at 63 °C. The blot was then washed twice at 60 °C with 40 mM $NaHPO_4$, pH 7.2, 5% SDS, and 1 mM EDTA, and twice at 60 °C with 40 mM $NaHPO_4$, pH 7.2, 1% SDS, and 1 mM EDTA. For hybridization with oligonucleotide probe, the filters were prehybridized at 50

°C for 2 to 12 hrs in the hybridization buffer (5X SSC, 20 mM NaH_2PO_4. pH 7.0, 7% SDS, 10X Denhardt's). About 1 ng of end-labeled oligonucleotide per ml of the hybridization solution was added, and incubated at 50 °C for 20 hrs. The blot was then washed twice at 50 °C for 30 min in 3X SSC, 10X Denhardt's, 5% SDS, 25 mM NaH_2PO_4, pH 7.5, and once at 50 °C for 30 min in 1X SSC and 1% SDS. The blots were exposed to Kodak XAR-5 film or REFLECTION film (NEN-Dupont) at -70 °C with intensifying screens.

7.6 DNA Sequencing Reactions

Nucleotide sequences were obtained using the double-strand dideoxy chain termination DNA sequencing method (Sanger *et al.*, 1977) with either ^{T7}Sequencing Kit (Pharmacia) or Fidelity DNA Sequencing System (Oncor, Inc. MD). Sequencing reactions were performed as described by the manufacturers. Sequencing primers were either M13, T7, T3, KS, SK or custom-synthesized 15 to 25-mers (Sheldon Biotechnology Center, Montreal, Quebec) with [α]-^{35}S dATP (1,326 Ci/mmol, NEN-DuPont Canada Inc, Mississauga, Ont.). DNA template for sequencing was denatured in 0.5 N NaOH for 10 min at RT or in 0.2 N NaOH for 30 min at 37 °C and purified by ethanol precipitation. Sequencing reactions were analyzed on 6.4% polyacrylamide, 7 M urea denaturing gels at constant power 35W, limit = 3,000V, 100 mA and 35 W in 1X TBE buffer with a Standard Thermoplate Sequencer Gel Electrophoresis Unit (Model STS-45, IBI). Following electrophoresis, gels were dried in a gel drier (Model 583 Gel Dryer, BIO-RAD) at 80 °C for 2 hrs, and autoradiographed at room temperature.

7.7 Mapping of 5' 9804 RNA

7.7.1 S1 Nuclease Assay with Single-Stranded DNA Probes

Single-stranded DNA probes were prepared by asymmetric PCR with reverse primer 9804X1R (section 7.2.3) or 9804X1R primer extension by Klenow DNA polymerase I from the DNA template, clone P26 (Figure 2.3). 20 μg of total RNA from A431 or KG-1a cell lines was precipitated with ethanol and recovered by centrifugation. The RNA pellet was redissolved in 30 μl of hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, pH 8.0, 0.4 M NaCl, 80% formamide). The radiolabeled single-stranded

DNA probe (~0.01 pmol) was added to the dissolved RNA. The mixture was incubated at 85 °C for 10 min, quickly transferred to a 30 °C water bath and incubated for 16 hrs. 300 µl of ice-cold nuclease S1 mapping buffer (0.28 M NaCl, 0.05 M sodium acetate, 4.5 mM $ZnSO_4$, 20 µg/ml single stranded DNA carrier and 100-1000 unites/ml nuclease S1) was added to each reaction and incubated at 37 °C for 2 hrs. The reaction was stopped by adding 80 µl of nuclease S1 stop mixture (4 M ammonium acetate, 50 mM EDTA, pH 8.0, 50 µg/ml tRNA) on ice and the nucleic acids recovered from the nuclease S1 digest by ethanol precipitation. The nucleic acids were heated at 95 °C for 5 min before being loaded to a 6.4% polyacrylamide/7 M urea gel. A sequencing reaction from the same DNA template and 9805X1R primer was run on the same gel as a control of size.

7.7.2 Primer Extension Analysis

To localize the transcription initiation site of 9804 gene, primers 9804X1R, 9804X2R, and 9804X3R (25-mer, 17-mer and 17-mer, see Table 2.2) were used, which corresponds to nucleotides 1219 to 1243, 3645 to 3651 and 3075 to 3091 of the human 9804 gene (Figure 2.4), respectively. 0.05-0.2 pmol primer, end-labeled with [γ] ^{32}P-ATP (7000Ci/mmol, ICN), was hybridized to 20 µg total RNA in a solution of 40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide. The reaction was heated to 85 °C for 10 min, slowly cooled to 50 °C over 60 min and incubated at 37 °C for 16 hrs. The nucleic acids were precipitated with ethanol, and resuspended in a solution of 50 mM Tris-HCl, pH 7.6, 60 mM KCl, 10 mM $MgCl_2$, 1 mM of each dATP, dCTP, dGTP and dTTP, 1 mM dithiothreitol, 1U/µl RNasin (Pharmacia). The primers were extended with 2U/µl AMV reverse transcriptase at 42 °C for 1 hr. The extended products were ethanol precipitated and recovered by centrifugation. It was resuspended in sequencing Stop Solution (0.3% each Bromophenol Blue and Xylene Cyanol FF, 10 mM EDTA, pH 7.5, and 97.5% deionized formamide), and analyzed on a 6.4% acrylamide/7 M urea sequencing gel.

7.8 Recombinant Proteins Expression in Baculovirus System

7.8.1 Transfection Sf9 Cells

2.5 x 10^6 log phase Sf9 cells (>98% viable) were seeded in complete TNM-FH in a 60 mm plate and allowed to attach for 3 hrs. The medium was aspirate and replaced with 2 ml of Graces medium. Mix 1 µg of linear AcMNPV DNA (Catalog # B825-03), 3 µg of plasmid DNA (BOMIGF-9804 or BOMIGF-Ly-6) and 20 µl of the Cationic Liposome solution in 1 ml of Grace's media (Catalog # B823-01) and incubated at room temperature for 15 min. The Graces medium in the plates was removed and the transfection mix added evenly. The plate was incubated on a slowly rocking platform (Bellco #774020020) at room temperature for 4 hrs. Following the incubation period, an additional 1 ml of complete TNM-FH medium was added to each 60 mm plate and incubated at 27 °C in a humidified environment for 48 hrs. After 48 hrs, the medium was collected from the tranfected plates and stored at 4 °C (virus stocks).

7.8.2 Infection of Sf9 Cells And Recombinant Protein Expression

Sf9 insect cells were plated with Sf-900 SFM II medium in the culture dishes at a seeding density of 1,000/mm^2 or cultured in spinner flask as a suspension culture at a density of 2 x 10^6/ml. Log phase growing cells were infected with virus stocks at a multiplicity of infection (MOI) of 10. In the plate infection, approximately 1 ml of virus stock/2.5 x 10^6 cells was added and incubated at 27 °C for 2.5 hrs with gentle rocking. After infection, additional 2 ml of the Sf 900 SFM II medium was added and cells were further incubated for 2 to 3 days at 27 °C. The culture supernatants were collected and cells harvested for assays of the fusion protein expression.

7.8.3 Plaque Assay for Purification And Titration of Recombinant Viruses

2.5 x 10^6 cells was evenly seeded in each 60 mm plate with 3 ml of complete medium and left at room temperature for 3 hrs until the cells attach to the plate. The medium was aspirated and cells infected with 1 ml of the virus stock from transfection (section 7.7.1) or infection (section 7.8.2) in series dilution's. The plates were left at room temperature with rocking for 1.5 hrs allowing the infection to complete. For each 60 mm plate, 4 ml of the plating mix (1 ml of Sf-900 SFM at 47 °C, 1 ml of autoclaved

2.5% Baculovirus agarose at 47 °C (Invitrogene) and 2 ml of SF-900 SFM at room temperature with 150 μg/ml of X-gal) was prepared. The media from the plates were aspirated and 4 ml of the plating mix added to each plate without trapping bubbles. After the agarose has been set, the plates were sealed in a plastic bag containing a plate of 0.25% Racol II and left at 27 °C for 7-10 days. The recombinant blue plaques were visualized by eye and under low power microscope. Single blue plaques were picked for further round of purification or virus stock preparation, and the number counted for the titration.

7.9 Luciferase Reporter Assays

Luciferase Assay Reagent (20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP, final pH 7.8) was prepared in aliquot without ATP and DTT in 5 ml tubes and stored light-tightly at -20 °C. Immediately before the assay, ATP and DTT were added to the concentration described above. 100 μl of the Luciferase Assay Reagent was dispensed into each tube, mixed with 10 μl of the cell lysate (with 1% (v/v) Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT) of the transfection. The reactions were applied immediately to the luminometery using LKB Wallac 1250 Luminometer at a light emission of 562 nm and the light output for 10 seconds at 25 °C was measured.

7.10 Transfection of The Expression Constructs

7.10.1 Electroporation

Electroporation was employed for transfection of suspension mammalian cultures. Cells were fed the day before transfection and washed with PBS. 7 x 10^6 cells were resuspended in 400 μl RPMI 1640 medium containing 10 mM glucose and 0.1 mM DTT. 30-40 μg plasmid DNA of interest in pLUC vector and 10 μg of β-gal-containing plasmid (pcDNA3) were added to the cell suspension, mixed and led sit at RT in an electroporation cuvette of 0.4-cm electrode gap for 5 min. The mixture was then exposed to a single pulse of 350V, 960μF and infinite internal resistance with the ElectroPorator (Invitrogen). The

cells were plated immediately after pulse in 5 ml of complete medium and incubated ≥14 hrs following selection and assays. X-gal staining (Current Protocols of Molecular Biology) was performed as the control of the transfection efficiency.

7.10.2 Lipofection

Lipofection was mainly used in adherent cultures. Cells were grown to 60% confluency in culture dishes. 2 μg of each plasmid DNA of interest and 2 μg of the β-galactosidase-containing plasmid were mixed in a final volume of 100 μl of H_2O. 3 μg liposome (Sialomed) or in DNA:liposome =1:1.5, was added and incubated at RT for 15 min, followed on ice for 2 min. The cells were washed with serum-free medium and replaced with 1 ml of OPTI-MEMI Reduced Serum Medium (GIBCO BRL, Life Technologies). The DNA/Liposome mix was added to each plate, followed by incubation at 37 °C for 8 hrs. The medium was aspirated and replaced with complete medium (supplemented with 10% FBS), and the cells further cultured for 12-24 hrs. X-gal staining (Current Protocols of Molecular Biology) was performed for the control of the transfection efficiency.

7.11 Preparation of Insect Cell Extracts

Cells were collected by centrifugation at 1,100 rpm for 10 min and the pellet extracted with an extraction medium consisting of 1 M hydrochloric acid, containing 5% (v/v) formic acid, 1% (w/v) sodium chloride and 1% (v/v) trifluoracetic acid (TFA) by homogenizing with a Dounce Tissue Grinder. This extract was subjected to reversed-phase extraction with C18 Sep Pak cartridges (Waters). The proteins were eluted from the cartridges with acetonitrile-water (80:20, v/v), containing 0.1% (v/v) TFA and speed vacuum dried.

7.12 High-performance Liquid Chromatography

The lyophilized insect cell extract was fractionated by reverse-phase HPLC using a Waters Assoc. (Milford, MA, U.S.A.) system. C_4 Vydac reverse column was used with a gradient elution with 2-77% solvent B (80% acetonitrile containing 0.1% TFA) over 60

min (flow rate 1.5 ml/min). Column elutes were monitored for UV absorbance at 210 and 280 nm by Waters Model 481 variable-wavelength detector, and Waters Model 441 fixed-wavelength detector, connected in series and fractions were collected. The β-emission of the eluted fractions were then determined by scintillation counting.

7.13 PBMC Isolation from Human Blood

Heparinized human peripheral blood was diluted with an equal volume of Hank's balance solution in 50 ml tubes. Half a volume of Lympholyte®-H solution (Cedarlane Laboratory Ltd., Ont. Canada) was introduced to the bottom of the tubes and the gradient was centrifuged for 20 min at 800 x g at room temperature. After centrifugation, the mononuclear cell layer at the interface was collected and transferred to a new tube. The cells were diluted with RPMI 1640 medium and centrifuged at 800 x g for 10 min. The cells were washed 2-3 times in medium and cell number was counted before further processing.

7.14 Computer Database Searches

Database searches were performed using the e-mail server blast@ ncbi.nlm. nih.gov. Clustal w sequence alignments were performed through Web site: http://dot. imgen.bcm.tmc.
edu:9331/multi-align/Options/clustalw.html (Thompson *et al.*, 1994). Polypeptide sequence distance analyses and phenogram construction were performed using the PHYLIP software package from Joe Felsenstein (download with the documentation from the PHYLIP home page at Web site http:// evolution.genetics.washington.edu/phlip.html).

PROTDIST analysis was performed on aligned amino acid sequences of Ly-6 domains using the Dayhof PAM. The resulting matrix then served as input to KITSCH for tree computation. Optimizations were performed using Global branch rearrangement, whereby input sequences were jumbled 24 times. Analysis of several other programs and options within the PHYLIP package using rational alignment choices resulted in slightly different trees. These differences occurred within the relative placement of the most

divergent Ly-6 superfamily sequences, but all were consistent in the groupings of the Ly-6 family members.

Promoter predictions utilized the programs Proscan Version 1.7 through web site: http://biamas.dcrt.nih.gov:80/molbio/proscan, and EPPNN (Eukaryotic Promoter Prediction by Neural Network) at web page http://www.hgc.lbl.gov/projects/promoter.html. Potential transcription regulatory elements were investigated by the use of Web Signal Scan Service: http://bimas.dcrt.nih.gov:80/molbio/signal/, and by inspection.

APPENDIX

CROSS-SPECIES SCREENING WITH MOUSE Ly-6A AND ThB

A.1. Background

Before the discovery of the 9804 gene, a major focus in this laboratory was the identification of human homologues of mouse Ly-6 genes in order to conduct parallel studies of Ly-6 function in both species. When the first mouse Ly-6 cDNA was cloned, it was immediately used to probe human DNA on Southern blots and in genomic libraries, but no cross-hybridization was detected. This was likely due to a high degree of sequence divergence between species. Cloning of homologous genes from rat supported this view, since a high level of Ly-6 sequence divergence was evident even between these two closely related species (Friedman *et al.*, 1989). Further attempts were made at homology cloning by use of oligonucleotide probe mixtures, and PCR with degenerate primers based upon what appeared to be the most conserved sequences in the Ly-6 coding regions, but these attempts were not quickly rewarded. The cDNA libraries probed or used as templates were monkey kidney (Clontech, Cat. # OL1005a), dog kidney (Clontech, Cat. # DL1004a), human bone-marrow (Clontech, Cat. # HL1058a) and human spleen (Clontech, Cat. # HL1039a). Considering that we were dealing with a quite large multigene family, and that the independently regulated Ly-6 genes encoded hematopoietic surface proteins that were involved in signaling events affecting cell development and lymphocyte activation, we felt confident of the existence of human homologues of at least some members of the mouse Ly-6 family. Ly-6-related molecules had been found in human and some other species. These include the complement inhibitor CD59, the receptor for urokinase plasminogen activator (uPAR), and the squid grain glycoprotein Sgp-2. None of these, however, satisfied sufficient criteria to be possible homologues of any members of the tightly linked Ly-6 family. I adopted the strategy of cloning Ly-6 homologues from species as distant as possible from mouse, but which could still be detected by cross-hybridization with mouse cDNA probes at moderate stringency, and then using these probes to step further through the species toward human. It was during the course of this work that the human 9804 clone became

Figure A.1 Southern Blot of Genomic DNA from Different Species with Mouse Ly-6A cDNA

20-30 μg of genomic DNA from various species as indicated on top of each lane was digested with *Eco* RI (E) restriction enzyme and electrophoresed on a high resolution gel apparatus. The blot was hybridized with ^{32}P-labeled mouse Ly-6A.2 cDNA probe and autoradiographed. The C57BL/6 mouse genomic DNA was included as positive control and the molecular markers for the size range.

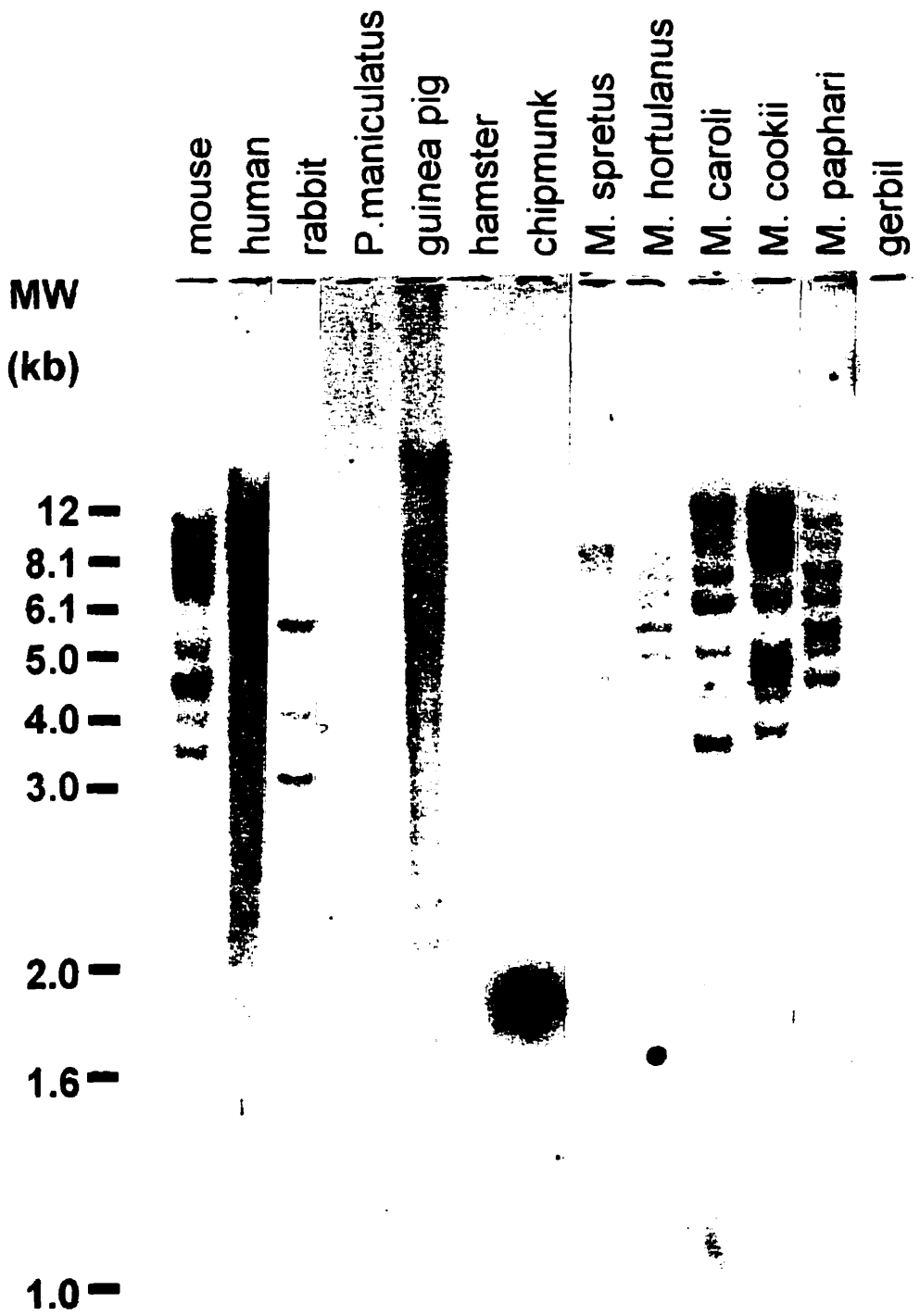

mouse
human
rabbit
P.maniculatus
guinea pig
hamster
chipmunk
M. spretus
M. hortulanus
M. caroli
M. cookii
M. paphari
gerbil
MW
(kb)
12
8.1
6.1
5.0
4.0
3.0
2.0
1.6
1.0

Figure A.2 Southern Blot of Genomic DNA from Different Species with Mouse ThB cDNA

20-30 μg of genomic DNA from various species as indicated on top of each lane was digested with Eco RI (E) restriction enzyme or with Eco RI (E), Pst I (P), Bam HI (B) and Hind III (H) in guinea pig DNA. The digestions were electrophoresed on a high resolution gel apparatus and gel blotted. The blots were hybridized with ^{32}P-labeled mouse ThB cDNA probe and autoradiographed. The C57BL/6 mouse genomic DNA was included as positive control and the molecular markers for the size range.

MW
(kb)
12–
8.1–
6.1–
4.0–
3.0–
2.0–
1.6–
1.0–
P.maniculatus (E)
hamster (E)
chipmunk (E)
M. spretus (E)
M. caroli (E)
M. saxicola (E)
gerbil (E)
mouse (E)
human(E)
rat (E)
rabbit (E)
guinea pig (E)
guinea pig (B)
guinea pig (P)
guinea pig (H)

available to us, and caused a shift in our attention. The cloning of Ly-6A-like sequences from rabbit, and ThB-like sequences from guinea pig, that I was working on at that time were put aside while I concentrated on the more important study of the human TSA-1-like gene. This work is reported here.

A.2. Cross-species Hybridization with Mouse Ly-6A

20-30 μg of high molecular weight DNA from various species was digested by Eco RI at 37 °C for 12-16 hrs and subjected to Southern blot analysis. The high resolution electrophoresis gel unit (Model HRH, IBI) was used to obtain a good separation. The DNA was transferred to a Zeta-Probe membrane (BioRad) and hybridized with a mouse Ly-6A cDNA probe (a 690 bp Eco RI fragment released from Ly-6A.2 cDNA-containing plasmid B18.KS$^+$ II). The hybridization was conducted in 0.5 M NaH_2PO_4 (pH 7.2), 7% SDS, and 1 mM EDTA at 58 °C, which was a reduced stringency for DNA/DNA hybridization allowing for approximately 20% nonhomology (Britten *et al.*, 1974; Beltz *et al.*, 1983). As expected (Fig. A.1), multiple bands similar to those seen in mouse were observed in all the *Mus* species tested (*M. spretus, M. hortulanus, M. caroli, and M. pahari*). Outside rodents, a crosshybridization was only detected in rabbit. The banding pattern was relatively simple with 3 to 4 bands between 3-6 kb. No signal was detected in the species of *P. maniculatus*, hamster, chipmunk and gerbil.

A.3. Cross-species Hybridization of Mouse ThB

20-30 μg of *Eco* RI-digested DNA from various species was separated on a 0.7% TBE agarose gel using a high resolution electrophoresis unit. The gel was blotted onto a Nylon membrane and hybridized with another mouse cDNA ThB (a 700 bp *Pst* I/*Hind* III fragment released from pThB-A (Gumley *et al.*, 1992). The hybridization and wash conditions were the same as those used in the Southern blot with mouse Ly-6A cDNA (section A.2). A single band was detected in all the *Mus* species including the control C57BL/6 mouse, *M. Spretus, M. caroli and M. saxicola* (Fig. A.2). Except in guinea pig, there was no cross-hybridization observed in other species tested in this study

Figure A.3 Restriction Digestion of Rabbit Phage Genomic Clones

10 μg of each phage DNA preparation from different clones was digested with *Eco* RI restriction enzyme and separated on 1% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was photographed to visualize the restriction patterns.

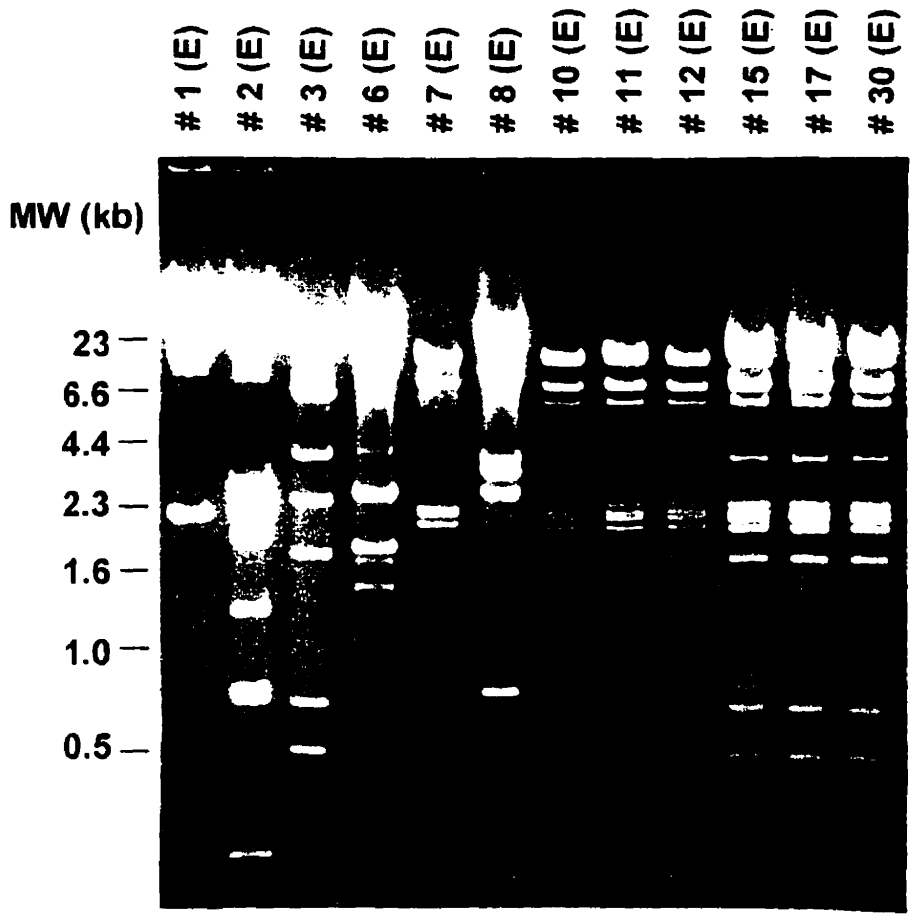

#1 (E)
#2 (E)
#3 (E)
#6 (E)
#7 (E)
#8 (E)
#10 (E)
#11 (E)
#12 (E)
#15 (E)
#17 (E)
#30 (E)
MW (kb)
23
6.6
4.4
2.3
1.6
1.0
0.5

Figure A.4 Southern Blot of The Rabbit Phage Genomic Clones

Representative rabbit phage genomic clones were digested with *Eco* RI and electrophoresed. The gel was blotted and hybridized with ^{32}P-labeled Ly-6A cDNA probe and autoradiographed. The restriction patterns were shown with the photograph of the ethidium bromide-stained gel (top panel) and the hybridized bands were shown in an autoradiograph (bottom panel).


#1
#2
#3
#6
#7
#8
#15
MW (kb)
12
6.0
3.0
1.6
1.0
0.5
MW (kb)
12
6.0
3.0
1.6
1.0
0.5

including *P. maniculatus*, hamster, chipmunk, human, rat and rabbit (Fig. A.2)., A single band was also detected in the Southern blot with different restriction digestions of guinea pig DNA. The banding pattern is similar to that of mouse *ThB* gene which does cross-hybridize with other Ly-6 genes.

A.4. Rabbit Genomic Library Screening And Sequencing

A New Zealand Whites rabbit genomic library (Catalog #: 945950, Stratagene) was screened by phage plaque hybridization with the Ly-6A cDNA probe in the buffer containing 6X SSC, 0.1% SDS and 0.05% pyrophosphate at 42 °C. The library consisted of 2 x 10^6 original plaques (clones) with an average insert size of 16 kb. Approximately 1.4 x 10^6 independent phage plaques were screened providing a 70% probability of picking up any particular genomic fragment. 40 positive plaques were isolated through three rounds of plaque hybridization, and phage DNA prepared for further analysis. The DNA from 12 clones was digested with various restriction enzymes. From the *Eco* RI digestion, 7 out of 12 clones showed different patterns (Fig. A.3). Southern blot hybridization (Fig. A.4) of the 7 rabbit genomic clones with mouse Ly-6A cDNA detected single or double inserts. In clones 6, 7 and 8, a strong smear was seen, which might indicate a repetitive sequence.

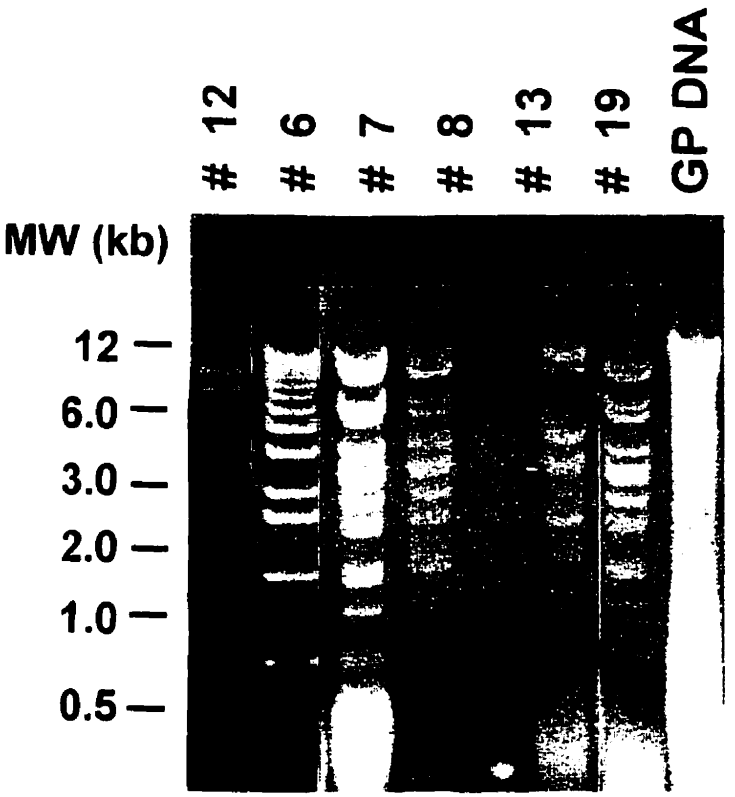
Eco RI fragments from some of the genomic clones were subcloned into the *Eco* RI site of pBluescript II KS$^+$ vector and partially sequenced. Blastn non-redundant database and EST database searches revealed a homology of some sequences from the original phage clones 1, 3, 15 and 17 with *L1* repetitive element or *Alu* repetitive sequences. No match was found with Ly-6-related sequence in the database for any of the clone sequences.

A.5. Guinea Pig Genomic Library Screening

A Hartley guinea pig genomic library (Catalog #: GL1002D, Clontech) was screened by phage plaque hybridization with the ThB cDNA probe in 4X SET, 10X Denhardt's, 0.1% SDS, 0.1% sodium pyrophosphate and 50 μg/ml denatured salmon sperm DNA at 55 °C. The library contains 1.4 x 10^6 plaques (clones) with an average

Figure A.5 Restriction Digestion and Southern Blot of Guinea Pig Phage Genomic Clones

10 μg of each phage DNA preparation from different clones was digested with Pst I restriction enzyme and electrophoresed on 1% agarose gel containing 0.5 μg/ml ethidium bromide. The gel was photographed to visualized the restriction patterns (top panel), blotted for the hybridization to ^{32}P-labeled ThB cDNA probe and autoradiographed (bottom panel). The high molecular weight genomic DNA of mouse was included as a positive control.


MW (kb)
12
6.0
3.0
2.0
1.0
0.5
12
6
7
8
13
19
GP DNA

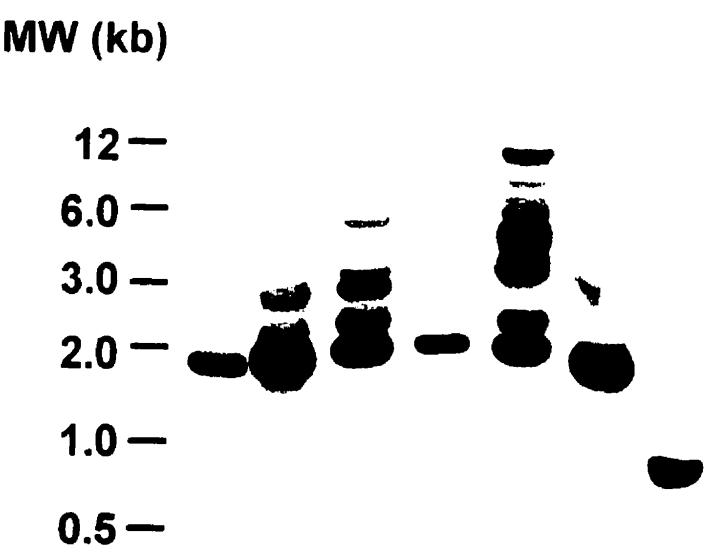
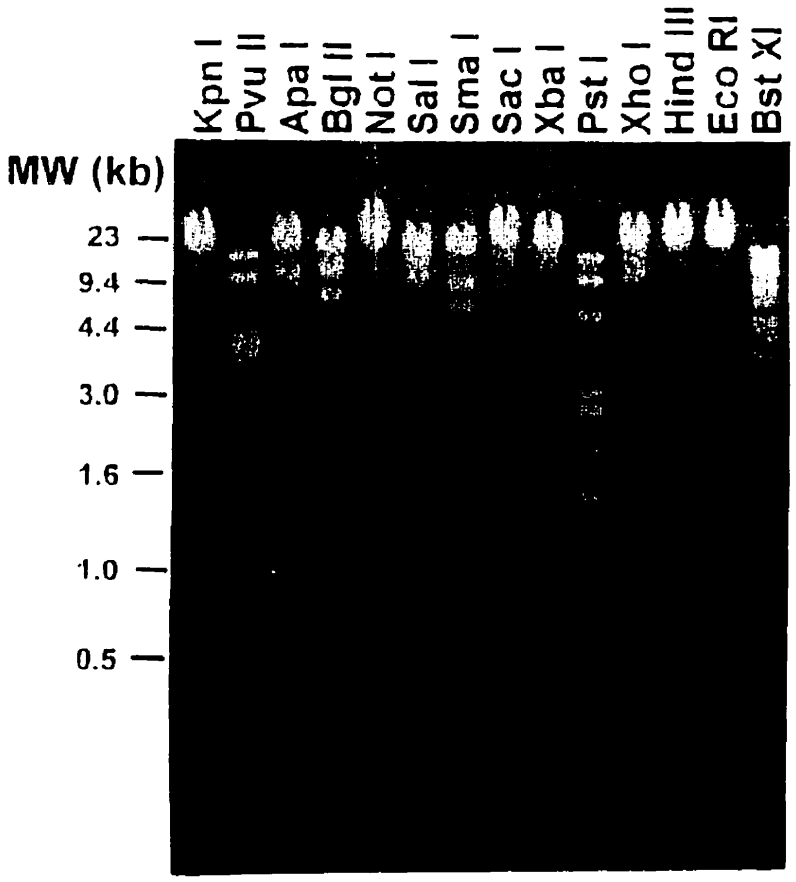
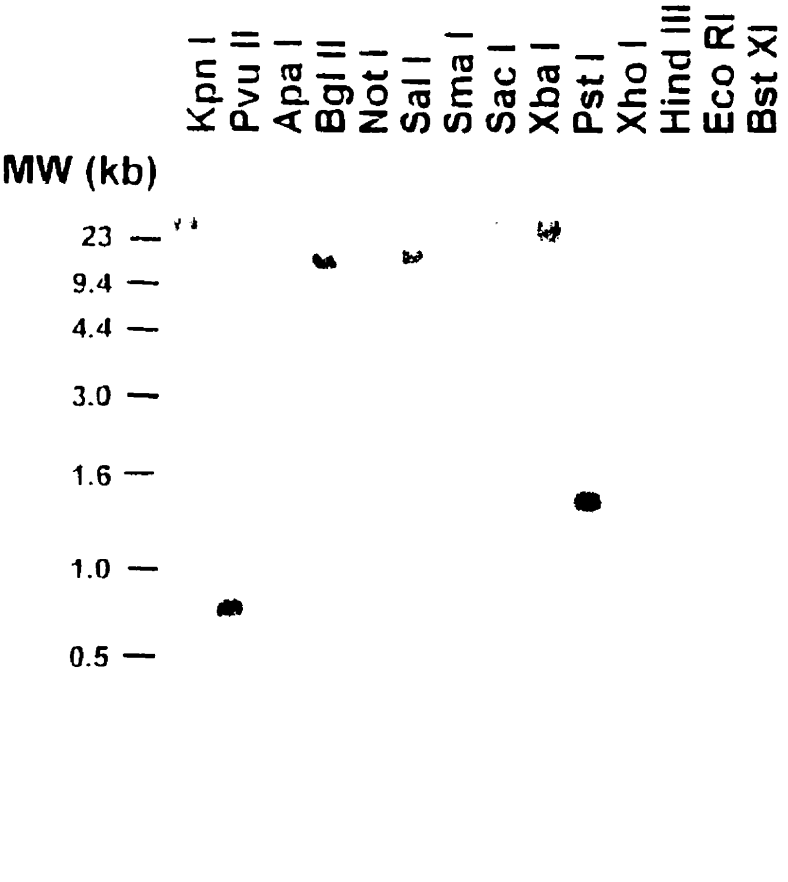

MW (kb)
12
6.0
3.0
2.0
1.0
0.5

Figure A.6 Southern Blot of The Guinea Pig Genomic Clone 12

10 μg of the Guinea pig genomic clone 12 was digested with a set of restriction enzymes as indicated on top of each lane (left panel). The digestions were electrophoresed on the gel and blotted for hybridization with ^{32}P-labeled ThB probe and autoradiographed (right panel).


Kpn I
Pvu II
Apa I
Bgl II
Not I
Sal I
Sma I
Sac I
Xba I
Pst I
Xho I
Hind III
Eco RI
Bst XI
MW (kb)
23
9.4
4.4
3.0
1.6
1.0
0.5


Kpn I
Pvu II
Apa I
Bgl II
Not I
Sal I
Sma I
Sac I
Xba I
Pst I
Xho I
Hind III
Eco RI
Bst XI
MW (kb)
23
9.4
4.4
3.0
1.6
1.0
0.5

insert size of 15 kb. Approximately 1.38 x 10^6 independent phage plaques were screened providing a 99% probability of picking up any particular genomic fragment. 10 positive clones were isolated through three rounds of plaque hybridization. A Southern blot of *Pst* I digested phage DNA was hybridized with the mouse ThB probe (Fig. A.5). Several crosshybridizing bands were seen from clones 6, 7 and 13, while a single band was observed from clones 12, 8, 19 and guinea pig genomic DNA. As a single ThB-like gene was expected, clone 12 was chosen for further analyses of restriction digestion and Southern blot analysis (Fig. A.6). The approximately 1.6 kb *Pst* I fragment was subcloned into pBluescript II KS^+ vector for sequence analysis. A positive subclone GP12.16.1 was completely sequenced. Alignment of the 1,615 bp GP12.16.1 sequence with mouse ThB and other Ly-6 sequences did not reveal any significant sequence homology and Blastn DNA database searches showed no other related sequence.

A.6. Discussion

Cross-hybridization to search for Ly-6 counterparts in most species other than mouse and rat has been unsuccessful due to a considerable divergence of Ly-6 genes between species. The recent discoveries of the human Ly-6 genes E48 and 9804/RIG-E were through non-direct cloning strategies.

The preliminary work of this thesis, using a cross-hybridization strategy in Southern blot analysis, demonstrated the presence of DNA sequences related to the Ly-6 family in rabbit and guinea pig. Under moderate hybridization stringency for approximately 20% nonhomology, multiple bands were detected in rabbit with mouse Ly-6A cDNA and single band in guinea pig with ThB cDNA. This corresponds well with the fact that mouse Ly-6 is a multigene family consisting of a large number of cross-hybridizing, tightly-linked genes on chromosome 15, and ThB and TSA-1 which do not cross-hybridize to other Ly-6 genes or to each other but are also tightly linked with the Ly-6 locus. It was presumed that the substantial sequence divergence prevented the detection of human Ly-6 genes under the hybridization conditions used here. However, the identification of the Ly-6 genes in rabbit and guinea pig was a breakthrough for identifying Ly-6 genes in species other than mouse and rat. Phylogenetically, rabbit and

guinea pig are out of rodentia and closer to human species. Identification of the Ly-6 genes from these two species would have been a useful step towards the discovery of novel human Ly-6 genes by providing probes for cross-hybridization cloning, and would have provide more data for locating the most conserved sequences within the genes.

Our Northern blot hybridization of rabbit and guinea pig RNA preparations from many tissues with mouse Ly-6A and ThB probes failed to detect any signal of expression (data not shown). Mouse Ly-6A is strongly induced by IFNs, and it may be that this gene is predominantly expressed only after induction in other species. The rabbit RNA analyzed came from normal tissues of a healthy rabbit, and may, therefore, have contained very low levels of the Ly-6-related mRNA. It is now known that ThB is most strongly expressed in skin, and its human homologue, E48, is exclusively found in skin. At the time of the Northern analysis of guinea pig tissues, we expected, based upon mouse ThB expression known at that time, that we would find the homologue expressed in thymus and other lymphoid tissues. Since we had no clue from the Northern blotting as to which tissue would contain the mRNA, in order to proceed with the cloning, genomic libraries were screened.

Despite a substantial effort, the sequencing data from the genomic clones did not yield any sequence related to Ly-6 genes. There are still more clones to analyze, however. It is likely that the low stringency led to many false positives. It is possible that the true positive clones of rabbit 'Ly-6A' and guinea pig 'ThB' are present in first picks from the libraries that have yet to be analyzed further. When we realized the first clones sequenced had to be false positive, we decided that the best way to proceed was to isolate hybridizing fragments from each clone and test them as probe on Southern blots. We would then only sequence those which gave band patterns like those obtained with the mouse Ly-6 probes. An alternative approach would be to make restricted libraries from fragments in the size range of the bands seen on Southern blots. Although some of the rabbit clones appeared to be hybridizing with mouse Ly-6A probes through repetitive sequences, there is no indication of hybridization to repetitive sequences on the Southern blot.

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