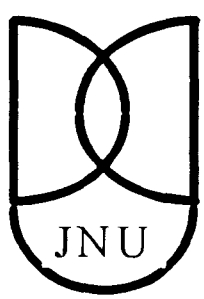
EXPRESSION OF RECOMBINANT INTERFERON REGULATORY FACTOR - 2 (IRF-2)

Thesis submitted to
Jawaharlal Nehru University
for the award of the Degree of

MASTER OF PHILOSOPHY

KRISHNA PRAKASH



SCHOOL OF LIFE SCIENCES
JAWAHARLAL NEHRU UNIVERSITY
NEW DELHI-110067
India
JULY, 2003

SCHOOL OF LIFE SCIENCES

Jawaharlal Nehru University

NEW DELHI-110067

CERTIFICATE

The research work included in this thesis entitled **"Expression of Recombinant Interferon Regulatory Factor-2 (IRF-2)"** has been carried out by Mr. Krishna Prakash in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or full for any other degree or diploma of any other university.

Krishna Prakash.

Krishna Prakash
Candidate

Prof. Alok Bhattacharya
(Dean)

Dr. P. C. Rath
(Supervisor)

Dedicated to my Grand Parents……

ACKNOWLEDGEMENTS

I am thankful to my supervisor Dr. P.C. Rath for allowing working with him. I am very grateful to him for his valuable guidence and constant encouragement throughout the progress of this work. I thank Dean, School of Life Sciences for providing me the necessary facilities. I am thankful to Dr. Gagan Bajaj, Ms. Menakshi Upreti and Mr. Raju U. Gadpayle who have constantly helped me in many ways for this work. I thank Jitendra, Rahul, Manoj, Subhash, Sanjay Khandelwal, Prasad K.P., Ganesh Baskey, Kamal Akhatar, Vikas Yadav, Amit Srivastava and all my classmates for their help. I thank my junior labmates Ritu and Amar for their kind help. I thank Late. Bhagat Ramjee, Chotelaljee and Ramkripal for their laboratory assistance and help. I thank Mr. Alexander, Mr. B.A Khan, Mr. S.K.Sharma, and Mr. S.K.Mishra for their technical support. I thank Mr. Saini for his excellent photography.

I am heartly thankful to my father and mother for their faith in me and for their moral support and encouragement.

Financial assistance from the Council of Scientific and Industrial Research (C.S.I.R.) in the form of Junior Research Fellowship (JRF) awarded to me is fully acknowledged.

(Krishna Prakash)

Abbreviations used

μg	microgram	DRAF	double stranded RNA activated factor
β-gal	β-Galactosidase	dsRNA	double stranded RNA
μl	microlitre	DTT	dithiothreitol
$^{-/-}$	Knock out	EBV	Epstein-Barr virus
^{0}C	Degree celcius	EDTA	Ethylenediamine tetraacetic acid
2′-5′ OAS	Oligo Adenylate Synthase	EGFR	Epidermal growth factor receptor
a.a.	Amino acid	EICE	Ets and IRF bindng sites
$A_{260/280}$	Absorbance at 260/280 nm wavelength	eIF-2	Eukaryotic Initiation Factor-2
APS	Ammonium Per Sulphate	EST	Expressed Sequence Tags
ATP	Adensine triphosphate	G_1	First growth phase
bHLH	Basic Helix-loop-helix	GAF/AAF	IFN-γ activated factor/ IFN-α-activated factor
bp	Base pairs	GAS	IFN-γ activation site
BSA	Bovine Serum Albumin	G-CSF	Granulocytic-colony stimulating factor
c.f.u.	Colony forming units	HAT	Histone acetyltransferase
CAP	Catabolite Activator Protein	HCV	Hepatitis C virus
CAS	Cellular Apoptosis Susceptibility	HHC	Human hepatocellular carcinomas
CREB	Cyclic-AMP response element binding protein	HHV-8	Human Herpes Virus-8
		HIV	Human Immunodeficiency Virus
CCE	Cell Cycle Element	HIV-1 LTR	HIV-1 Long Terminal Repeat
cDNA	Complementary Deoxyribose Nucleic Acid	HNF-3γ	Hepatocyte Nuclear Factor-3γ

CIA	Collagen-induced arthritis	hrs	hours
CML	Chronic Myelogenous Leukaemia	HTH	Helix-turn-helix
Con A	Concanavalin A	IAD	IRF association domain
Cox-2	Cyclooxegenase-2	ICS	Interferon Consensus Sequence
CII TA	Class II trabnsactivator	IFN	Interferon
CTL	Cytotoxic T lymphocyte	IFNAR	IFN - alpha Receptor
DBD	DNA Binding Domain	IFNGR	IFN-gamma Receptor
DNA	Deoxyribose Nucleic Acid	IPCS	IRF-1 p53 common sequence
IRF-E	IFN Regulatory Factor Element	nm	nanometre
IRFs	Interferon Regulatory Factors	nmole	nanomole
IRS	IRF Recognition Sequence	NPM	Nucleophosmin
ISGF	Interferon Stimulated Gene Factor	nt	nucleotide
ISRE	IFN Stimulated Response Element	ODN	Oligodeoxynucleotide
Kb	Kilo base	PACAP	pituitary adenylate cyclase-activating polypeptide
KCl	Potassium Chloride	PBL	peripheral blood lymphocytes
kd	kilodalton	PBS	Phosphate Bufered Saline
KHSV	Kaposi's sarcoma-associated herpes virus	PCAF	p300/CBP-associated factor
L	Litre	PCR	Polymerase Chain Reaction
LMCV	lymphocytic choriomenigitis virus	PEST	Pro, Glu, Ser, and Thr sequence motif
LMP-2	Low molecular weight polypeptide-2	Pip/LSI RF/ICS AT	PU.1 interacting Partner/Lymphoid specific IRF/ IFN consensus sequence binding protein in activated T-cell
LPS	Lipopolysaccharide	PKR	dsRNA dependent protein kinase
M	Molar	PMA	phorbol myristate acetate
MEF	Mouse embryonic fibroblast	pmole	picomole

mg	milligram	Poly (I.C.)	polyinosinic- polycytidilic acid
$MgSO_4$	Magnisium Sulphate	pRB	Retinoblastma protein
MHC	Major histocompatibility complex	PRD	Positive Regulatory Domain
ml	millilitre	PRL-R	Prolactin-Receptor
mM	millimolar	ROI	reactive oxygen intermediates
$MnCl_2$	Manganese Chloride	rpm	Rotations per minute
MOPS	3-[N-morpholino]propanesulphonic acid	RT	Room temperature
mRNA	messenger RNA	RT-PCR	Reverse Transcription-PCR
Na_2CO_3	Sodium Carbonate	SDS	Sodium dodecyl sulphate
NaCl	Sodium Chloride	SDS-PAGE	SDS-polyacrylamide gel electrophoresis
$NaHCO_3$	Sodium Bicarbonate	Sec.	Second
NDV	Newcastle Disease Virus	SH2	Src Homology 2
NES	Nuclear export signal	SLRP	small leucine-rich proteoglycan
NF-κB	Nuclear Factor kappa -B	STAT	Signal Transducers and Activators of Transcription
ng	Nanogram	TAP-1	Transporter associated with antigen processing-1
NK cell	Natural Killer cell	TLR	Toll Like Receptor
U	Unit	TNF-α	Tumor Necrosis Factor-α
VCAM-1	Vascular Cell Adhesion Molecule-1	TPA	12-O-tetradecanoylphorbol-13-acetate
vIRF	Viral IRF	UV	UltraViolet
Vol.	Volume		
X-Gal	5-bromo-4-chloro-3-indoyl-β-$_D$-galactopyranoside		

CONTENTS

1. Summary 1-1

2. Introduction 2-6

3. Review of literature 7-14
3.1 Discovery of IRF-2 07
3.2 The IRF family of transcriptional factors 07
3.3 Structure of IRF-2 09
3.4 Functions of IRF-2 10
3.5 Physiological role of IRF-2 11-1

4. Statement of problem: aims and objectives: 15-16
(a) To express recombinant IRF-2 in E.coli.
(b) To determine DNA binding activity of GST-IRF-2

5. Materials and Methods: 17-39

5.1 Materials 17

5.2. Reagents 18-25

5.3 Methods 26-39
5.3.1. Maintenance of *E.coli* strains: glycerol stocks. 26
(a) Bacterial culture growing in liquid media. 26
(b) Bacterial cultures on agar plates. 26
(c) Stab cultures. 26
5.3.2. Preparation of competent cells. 26
5.3.3. Transformation using competent cells. 27
5.3.4. Plasmid isolation: 27
(A) plasmid isolation from 50 ml. Bacterial culture. 27
5.3.5. Restriction digestion of plasmid DNA. 29
5.3.6. Purification of DNA fragments through low melt agarose. 29
5.3.7. Cloning of murine cDNA of IRF-2. 30
5.3.8. Insert preparation. 31
5.3.9 construction of GST-IRF-2 expression clones. 38
(A) pGEX-2TK-IRF-2 construct. 39
5.3.10. Expression of GST-IRF-2 39
(A) Induction of recombinant murine IRF-2. 39
(B) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 39
(C) Fixing staining of SDS-PAGE gels using comassie Brilliant blue R-250. 39

6. Results. 40-42

7. Discussion. 43-44

8. Conclusion. 45

9. References. 46-55

SUMMARY

1. SUMMARY:

Interferons Regulatory Factors (IRFs) are mammalian transcription factors of Interferons as well as Interferon inducible genes, involved in regulation of cell cycle, antioncogenesis, immunomodulation, control of cell proliferation. So far, 10 members of IRFs have been known. They are IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, IRF-8, IRF-9, IRF-10 and vIRFs (made by virus HSV).

IRF-2 is basically oncogenic in nature, it antagonises IRF-1 functions. Knockout mice for IRF-2 gene showed defect in Bone marrow suppression hematopoesis and B-cell lymphopoeisis, they died following Lymophocytic Choriomeningitis Virus (LCMV) infections (Mastsuyama *et al.*,1993). IRF-2 is functionally just opposite of IRF-1. Interestingly, one could have thought that as IRF-1 knockout mice showed lack of $CD8^+$ T cells and low cytotoxicity of N_K cells, the IRF-2 mice could restore it. But it does not happen so the reason being unknown. However, IRF-2 binds at ISRE sequence in viral infected cell.

Functionally, IRF-2 is repressor of IRF-1-mediated gene expression. Structure of IRF-2 has Repressor Domain (325-349aa); it plays a role in repression of transcription. In fact, IRF-2 also possesses Transactivation domain, it causes expression of H4 gene, VCAM-1 gene. Thus, it is a repressor as well as an activator for transcription.

Its DNA Binding Domain from 1-115 a.a governs function of IRF-2. N-terminal deletion of this domain makes IRF-2 inactive. There is five-tryptophan (W) repeats conserved along the IRF family. Mutations in the W repeats greatly inhibit its DNA binding activity as well as function. Hence DBD is very important for IRF-2.

My work is on IRF-2, its DNA Binding Domain. I have planed to clone the cDNA of IRF-2 gene in *E. coli* for expression of IRF-2 and show DNA binding activity of the recombinant protein by EMSA technique. This study is first step towards analysis of IRF-2 function *in vitro*.

INTRODUCTION

2. INTRODUCTION:

Interferon (*IFN*) is multifunctional secretory glycoprotein cytokines of approximately 20 kDa. They have physiological role in mammalian cells. IFNs have antiviral, antineoplastic, antiproliferative and apoptotic properties (Stark *et al.*, 1998). There are two types of IFNs, namely, Type I (IFNα/β) and Type II IFN (γ). IFNα/β is usually secreted by virus infected cells where as IFN (γ) is secreted by activated T cell and Natural killer (NK) cells for innate immunity. IFNs stimulate JAK- STAT signal transduction pathway (Darnell *et al.*, 1994) where as the STAT family of transcription factors activate transcription of IFNs-stimulated genes. IFNs also activate a group of transcription factors called Interferon Regulatory Factors (IRFs) (Kroger *et al.*, 2002). IRFs have helix-turn-helix (HTH) motif, tryptophan (w) repeats in their DNA binding Domain (DBD). They regulate transcription of many mammalian genes induced by cytokines and other agents (Taniguchi *et al.*, 1999). So, far ten members of the IRF family have been reported, they are IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, ICSBP (IRF-8), IRF-9/p48/ISGF3γ,IRF-10 and vIRF (coded by human herpes virus) (Taniguchi *et al.*, 2001). IRFs1/2 recognise certain variants of a consensus hexanucleotide sequence (GAAANN, where N any nucleotide) present in promoters of mammalian genes induced by virus IFN and IRF-1/2 indicating multiple pathways of virus inducibility of the IFNα/β genes (MacDonald *et al.*, 1990). IRFs actively participate in cellular process like defence against viruses and pathogens, regulation of cell growth and differentiation, antioncogenesis, regulation of immune response and apoptosis (Kroger *et al.*, 2001). Therefore, IRFs are important transcription factor for regulation of many genes in mammalian cells and tissues.

IRF-2:

While searching for transcription factors for the regulation of IFN-α/β gene expression, IRF-1 was identified by binding with the virus-responsive DNA sequences from the IFN-α/β promoter (Fujita *et al.*, 1988). IRF-1 was shown to activate positive regulatory sequences in the virus and IFN-stimulated genes (Harada *et al.*, 1990). Subsequently, IRF-1 knock out (IRF-1 $^{-/-}$) mice and murine fibroblasts showed that IFN-α/β genes can still be induced by virus in the absence IRF-1 but expression of certain genes like inducible Nitric Oxide Synthase (iNOS) in the

Macrophages (Kajimo *et al.*,1994) and the level of thymic $CD8^+$ cytosolic T cells were severely affected in the absence of IRF-1. Later, IRF-1 was shown to be more related to the IFNγ-inducible and dsRNA-activated pathways (Harada *et al.*, 1998). IRF family is one of the major group transcription factors for the cytokines network in mammalian cells. IRF-2 is a negative regulator of IRF-1 mediated gene expression. While there is extensive literature on IRF-1, information about IRF-2 is relatively limited. IRF-2 knock out (IRF-$2^{-/-}$) mice showed suppression of hematopoiesis and B-cell lymphopoiesis, they died following Lymphocytic Choriomenengitis Virus (LCMV) infection (Mastsuyama *et al.*,1993). Although, IRF-2 suppresses the activity of IRF-1 (Taniguchi *et al.*, 2001). IRF-2 also activates the transcription of certain genes such as histone H_4 and vascular cell adhesion molecule-1 (VCAM-1)(Jesse *et al.*, 1998). The H_4 gene plays important role in IRF-2 mediated oncogenesis. Mouse and human IRF-2 recognise similar DNA sequences (GAAAGT)n, (GAAAGC)n in the promoter genes.

Functions of IRF-2:

IRF-2 has dual function: activation as well as repression (Yamamoto *et al.*, 1994). The IRF-2 controls the cell cycle dependent expression of H_4 genes. The role of IRF-2 in the control of cell proliferation has been investigated in FDCP1 cell line (F2), (Xie *et al.*, 2001) IRF-2 was experimentally induced by (doxycline) DOX and cells were synchronised in G1 phase Isoleucine deprivation. Flow cytometric analysis indicated that forced expression of IRF-2 had limited effect on cell cycle progression before the first mitosis, however, further elevation of IRF-2 levels resulted in polyploidy, genomic instability and cell death. Western blot analysis revealed that the levels of cell cycle regulatory proteins cyclin B_1 and CDK inhibitory protein, p27 were selectively increased following this IRF-2 expression. It was found that the levels of Fas/FasL were also elevated and also the ratio of Bax (apoptosis promoting protein) and Bcl_2 (antiapoptotic protein) was altered during the process of cell death. Finally, it was concluded that in FDC1 (F2) cell line IRF-2 controlled cell progression through G2 and mitosis in hematopoietic progenitor cells (Xie *et al.*,, 2001).

IRF-2 is the transcription factor of the IRF family that represses interferon mediated gene expression. It has been observed that human monocytic U937 cells express truncated forms of IRF-2 containing DNA binding domain lacking C-terminal regulatory domain. U937 cells are shown to respond to phorbol ester 12-o-

tetradecanoylphorbol-13-acetate (TPA) to induce expression of histone acetylase p300 and p300/CBP-associated factors (PCAF). In addition, TPA treatment led to the appearance of full length IRF-2 along with reduction of truncated protein. Surprisingly, full length IRF-2 in TPA treated U937 cell occurred as a complex with p300 as well as PCAF and was it self acetylated. Consistent with these results recombinant IRF-2 was acetylated by p300 and to a lesser degree by PCAF *in vitro*. Another IRF member, IRF-1, an activator of interferon-mediated transcription, was also acetylated *in vitro* by these acetylases. Finally demonstrated that the addition of IRF-2 but not IRF-1 inhibits core histone acetylation by p300 *in vitro*. The addition of IRF-2 also inhibited acetylation of nucleosomal histones in TPA-treated U937 cells. Acetylated IRF-2 may affect local chromatin structure *in vivo* by inhibiting core histone acetylation and may serve as a mechanism by which IRF-2 negatively regulates interferon-inducible transcription (Masumi *et al.*, 2001).

DNA Binding Domain of IRF-2:

The crystal structure of the DNA binding domain of IRF-1 bound to DNA has been determined (Escalante *et al.*, 1998). The DNA binding region of IRF-1 has an α/β architecture containing three α helices, four stranded antiparallel β sheets, and three long loops. This structure is similar to the helix-turn-helix (HTH) containing DNA binding domain. However, its mode of DNA interaction is distinct from those of other HTH containing proteins and revealed a new HTH motif. The third α-helix contacts the major groove of the GAAA sequence and contacts its surrounding sequence. The crystal structure of the DNA binding domain of IRF-2 was also determined (Fujii *et al.*, 1999). The structure of the DNA binding domain bound to DNA indicates its recognition sequence, AANNGAAA (recognised bases are underlined) and shows co-operative binding to a tandem repeat of the GAAA core sequence induced by DNA structural distortions (Fujii *et al.*, 1999).

Post translation modification of IRF-2:

The carboxyl terminus of IRF-2 contains a repression domain, the deletion of which converts IRF-2 to a transcriptional activator (Taniguchi et. al., *1994*). With regard to the regulatory modifications of IRF-2 proteins, it undergoes inducible proteolytic processing. IRF-2 is cleaved in carboxyl terminal region following viral infection or double stranded RNA treatment, resulting in its conversion to either an activator or a strong repressor (Maniatias *et al.*, 1992; Cohen *et al.*, 1992). IRF-2 is

phosphorylated by Protein kinase A (PKA), Protein kinase C (PKC) and Casein Kinase (CKII) at serine residues (Birnbaum *et. al.*, 1997). IRF-1 also undergoes posttranslational modification such as phosphorylation. IRF-1, is reported to be phosphorylated at serine residues by Protein kinase A (PKA), Protein kinase C (PKC), and Casein Kinase II (CKII) at two cluster sites (residues 138-150 and residues 219-231) (Lin *et al.*, 1999). The mutation of these tyrosine residues inhibited the transactivation of IRF-1, suggesting possible role of phosphorylation by these kinases in the regulation of IRF-1 transcriptional activity (Brinbaum *et al.*, 1997).

IRF-2 was originally described as an antagonist of the IRF-1 mediated transcriptional regulation of IFN and IFN-inducible genes (Fujita *et al.*, 1986). Since Th1 cell development and NK cell development are impaired in IRF-$1^{-/-}$ mice, one could have expected that IRF-$2^{-/-}$ mice might manifest opposite phenotypes. However, rather IL-12 production was suppressed in IRF-$2^{-/-}$ macrophages (Lohoff *et al.*, 2000) and IRF-$2^{-/-}$ mice were susceptible to *Leishmania major* infection due to a defect in the Th1 cell differentiation (Lohoff, *et al.*, 2000). Thus, rather than functioning as a negative regulator, IRF-2 may positively contribute to IL -12 gene expression.

Unlike IRF-1, IRF-2 functions as a transcriptional attenuator, critical in balancing IFN action *in vivo* (Taniguchi *et al.*, 2000). In NIH 3T3 cells, the overexpression of IRF-2 causes oncogenic expression of IRF-1 causes these cells to revert to non transformed phenotype (Tanaka *et al.*, 1993). Although the exact mechanism underlying this cell transformation is still unknown, it is possible that IRF-2 exerts its oncogenic function through mediation of IRF-1 and /or other IRF family members. This possibility is supported by the finding that NIH 3T3 cells expressing only the DBD of IRF-2 were the other hand, IRF-2 activated genes (s) involved in oncogenesis such as Histone 4 (Vaughan *et al.* 1998).

Role IRF-2 in diseases:

IRF-2 is found to be associated with several diseases like Atopic Dermatitis (AD), Acute Myelogenous Leukemia (AML), Breast cancer, Pancreatic cancer, etc. In AD patients IRF-2 gene was found to be mutated and three mutations have been reported, one from promoter region, one silent mutation in exon 9 and a 10 bp deletion in the 3' untranslated region (Nishio *et al.*, 2001). Among these, the -467G allele and haplotype of the -467G, 921 A and 1739 $(ATCCC)_8$ alleles were transmitted preferentially to AD affected children. Lower expression of IRF-1 and higher expression of IRF-2 has been reported in AML thus ratio of IRF-1/2

expression was found to be altered in AML cells compared to normal bone marrow cells. Interestingly, administration of IL-4 could results in normalisation of the ratio of IRF-1/2 expression (Nishio *et. al.*, 2001). Alteration of IRF-1/2 expression may occur in breast cancer tissues. Normal breast tissue and cancerous breast tissue were analysed by using polyclonal antibodies against IRF-1/2. Alteration in the ratio of IRF-1/2 level was observed (Doherty *et al.*, 2001). This indicates that IRF-1/2 is involved breast cancer. Normal level of IRF-1 was reported from the normal breast tissue but in case of cancerous tissue, the situation is reversed. This supports the conclusion that IRF-1 is antioncogenic where as the IRF-2 has oncogenic property. (Doherty *et al.*,2001).

IRF-2 is involved in the repression of Cyclooxogenase-2 (Cox 2) gene. Cox-2 is a rate-limiting enzyme that initiates the conversion arachidonic acid to protanoids. Cox-2 is the inducible isoforms that is upregulated by proinflammatory agents, initiating protanoid mediated pathological aspects of inflammation. Induction of Cox-2 was abrogated in the absence of IRF-1 in mouse peritoneal macrophages. Conversely, the absence of IRF-2 in macrophages resulted in a significantly increase in Cox-2. Here IRF-2 was identified as a negative regulator of Cox-2 gene. (Blanco *et al.*, 2000).

Transcription factors, Histone Nuclear Factor-D, Histone Nuclear Factor-P, Histone 4 Transcription Factor -2 (HiNF-D, HiNF-P/H4TF-2) and IRF-2 complex were analyzed at H_4-site II (histone 4 gene promoter) to study regulation of the human H_4 gene transcription during G1/S phase transition. However, HiNF-D is itself made up of the proteins CDP-cut/CDC2/CyclinA/PARP, yet its role in H4 gene remains to be established. It is believed that at later stage of the cell cycle, IRF-2 attenuates the H_4 gene transcription. Experimentally, HeLa S3 cells in which a mutated H_4sII promoter linked to CAT (CarboxylAcetyl Transferase) reporter gene was introduced. Temporal regulation of the CAT mRNA accumulation under the control of the mutated H_4 promoter was analysed by RNA protection assay. Mutation of the HiNF-D /CDP-cut binding site altered the timing of histone gene expression during cell cycle. Conclusively, HiNF-D influences the timing of IRF-2 dependent cell cycle activation of human histone H4 gene transcription at the G1/S phase transition (Aziz *et al.*, 1998).

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE:

3.1. Discovery of IRF-2:

The induction of the IFN-β gene by a virus is due primarily to transriptional activation that requires virus inducible enhancer-like elements of its gene promoter (Weber *et al.*, 1986;Taniguchi *et al.*, 1988). During the study on the regulation of the IFNβ gene, a factor was found to bind to these elements and was tentatively termed IRF-1 (Taniguchi *et al.*, 1988). Subsequently, cDNA encoding mouse IRF-1 was cloned and its structure elucidated (Taniguchi *et al.*, 1988). Subsequently, a cDNA encoding a molecule structurally related to IRF-1 was isolated by cross hybridisation with IRF-1 cDNA and the molecule was termed IRF-2 (Taniguchi *et al.*, 1989). Infact, the deduced primary structure of IRF-1 and IRF-2 showed 62% homology in the amino terminal region, spanning the 1st 154 residues, where as the rest of the molecules showed only 25% homology (Taniguchi *et al.*, 1989). DNA binding site selection studies revealed the these two factors bind to the same DNA element, termed IRF-E (consensus sequence: G(A)AAA G/C T/C GAAA G/C T/C)(Taniguchi *et al.*,. 1993) which is almost indistinguishable from the interferon stimulated response element (ISRE;consensus sequence:A/G NGAAA NNGAAACT) (Stark *et al.*, 1994) activated by IFN signalling .

3.2 The IRF family of transcription factors

IFNs activate a group of transcription factors called Interferon Regulatory Factors (IRFs) (Kroger *et al.*, 2002). IRFs have helix-turn-helix (HTH) motif and tryptophan (w) repeats in their DNA binding Domain (DBD). They regulate transcription of many mammalian genes induced by cytokines and other agents (Taniguchi *et al.*, 1999). So, far ten members of the IRF family have been reported, they are IRF-1, IRF-2, IRF-3, IRF-4, IRF-5, IRF-6, IRF-7, ICSBP (IRF-8), IRF-9/p48/ISGF3γ and IRF-10 and vIRF (coded by human herpes virus) (Taniguchi *et al.*, 2001). Some properties of IRF members are summarised in Table A. IRFs members structure sketch are depicted in figure 6.

IRF-3 and IRF-7:

IRF-3 and IRF-7 are closely related to each other in terms of their primary structures (Pitha *et al.*, 1997;Hiscott *et al.*, 1999) and Takaoka *et al.*, 2000). IRF-3 was identified through a search of an EST database for IRF-1 and IRF-2 homologs (Pitha

Table A. IRF family members: summary of the properties and functions of IRFs.

IRF members	Expression Pattern	Inducers of expression	Transcriptional role	Physiological role
IRF-1	Most cell types Low-level constitutive	Type I IFN Type II IFN Viral infection ds RNA	Activator	Tumor suppressor
IRF-2	Most cell types constitutive	Type I IFN Viral infection.	Repressor/ Activator	Oncogene Antiviral defence Immune regulation
IRF-3	Most cell types constitutive	unknown	unclear	unknown
IRF-4/ Pip/ LSIRF/ ICSAT	Activated T-Cells	PMA HTLV-1 Tax	Activator/ Repressor	Immune regulation
IRF-5	inducible	Type I IFN(Taniguchi *et al.*, 2001)	unknown	Immune system
IRF-6	Tissue specific pattern	unknown	unknown	unknown
IRF-7	B-cells and lymphoid lineages	Type II IFN	Activator	Transcriptional activator of viral promoter
IRF-8/ICSBP	Cells of Macrophages and lymphoid lineages Low level Constitutive inducible	Type II IFN	Repressor	Antiviral defence Immune regulation
IRF-9/ISGF3γ	Most cell types constitutive	Type II IFN	Activator	Antiviral defence
IRF-10	Tissue specific	Induced by v-Rel protooncogene c-Rel IRF-1	Activator	Later stage of antiviral defence Upregulates IFNγ related genes

(Ref. Adapted from Taniguchi *et. al.* 2001)

et al., 1995). IRF-3 was also identified as the component of the virus inducible dsRNA-activated factor (DRAF1) Complex (Reich *et al.*, 1998). IRF-3 is found to be expressed consitutively involved in the inducible expression of typel IFN gene upon viral infection (Reich 2002). Subsequently, IRF-7 cDNA was cloned as a factor bind to the Epstein-barr virus QP promoter region using the yeast one hybrid system (Pagano *et al.*, 1997). The expression of IRF-7 is ubiquitous (Pagano *et al.*, 1997) and However, it is totally dependent on type 1 signalling. IRF-3 contains an activation domain that includes the NES and IRF association domain (IAD) (Fujita *et al.*, 1998). Evidence has been provided that this domain is flanked by two autoinhibitory domain that interact with each other; in virally infected cells, this interaction relieved by virus induce phosphorylation, thereby unmasking both IAD and DBD (Hiscott *et al.*, 1999).

IRF-4: IRF-4 has been characterised in several contexts with different terminology:

(i) Pip, a binding factor to the murine Ig light chains enhancer (Storb *et al.*, 1995).

(ii) LSIRF, a new IRF family member expresses only in lymphoid cells (Mak *et al.*, 1995).

(iii) ICSAT, a factor that bind to the promoter region of the IL-15 gene. (Hirari *et al.*, 1996). The expression of IRF-4 is restricted to lymphoid lineage. (Storb *et al.*, 1995).

IRF-5 and IRF-6: These factors are structurally related to each other, but information has been scarce regarding their functions. Interestingly, the expression of IRF-5 is induced by type IFN (Tanaka, Taniguchi unpublished observations) suggesting its participation in IFN system. A Xenopus gene reported to encode a protein highly similar to mouse IRF-6, termed x-IRF-6, is expressed in the posterior mesoderm during the early development of *Xenopus laevis* (Asashima *et al.*, 1997).

IRF-8/ICSBP: IFN consensus sequence binding protein ICSBP/IRF-8 was originally identified as a protein that binds to the ISRE motif in the posterior region of MHC class I gene (Ozato *et al.*, 1990). The expression of IRF 8 is restricted to myeloid and lymphoid lineage and is induced by IFN-γ but not by IFN-α/β (Ozato *et al.*, 1997). The DNA binding activity of IRF-8 per se is very weak but is dramatically increased by interaction with IRF-1 and IRF-2 (Ozato *et al.*, 1994). The interaction is mediated by IAD, which is conserved among several IRF members (Levi *et al.*, 1995).

IRF-9 (P48/ISGF3γ): IRF-`9 was originally discovered as a DNA binding subunit of the transcription factor ISGF3γ (IFN stimulated gene factor 3) and termed P^{48}/ISGF3γ (Darnell *et al.*, 1989). In fact, specifically induced in IFN-α-stimulated cells and was subsequently found to consist of three components; Signal transducer and activator of transcription (STAT-1), STAT-2 and IRF-9 (Fu *et al.*, 1992). Like IRF-1 and IRF-2, IRF-9 is expressed in a variety of tissues and shown to be essential for antiviral response by IFN-α/β (stark *et al.*, 1994).

IRF-10: IRF-10 is most closely related to IRF-4 but differs in both its constitutive and inducible expression. The expression of IRF-10 is inducible by Interferons (IFNs) and by concanavalin A. In contrast to that of other IRFs, the inducible expression of IRF-10 is characterized by delayed kinetics and requires protein synthesis, suggesting a unique role in the later stages of an antiviral defense. (Henry *et al.*, 2002).

vIRFs: The viral analogues of these genes (vIRFs) are encoded by HHV-8 (Kaposi's sarcoma herpes virus) genome (Pitha. *et al.*, 1999).Viral IRFs and have shown that they inhibit production of inflammatory cytokines which allow the virus avoid the innate immune response. The kaposi's sarcoma associated virus encodes three IRF homologous (Chang *et al.*, 1996). vIRF unique protein shows 13.4% amino acid identified with IRF-8, including IRF-1, and inhibits IRF mediating transcriptional activation (Moore *et al.*, 1997) suggesting its role in escaping IFN action. vIRF-2 interacts with IRF-1 , IRF-2 and ICSBP *in vitro* (Pitha *et al.*, 1999) and vIRF-3 may inhibit both IRF-3 and IRF-3 activities resulting in the inhibition of the virus-mediated synthesis of IFN α/β (Pitha *et al.*, 2000).

3.3 Structure of IRF-2:

Mutational analysis of IRF-2 revealed that DNA binding activity resides in the amino-terminal region with strictly conserved amino acids, particularly the 5 times tryptophan (W) repeats (fig.1-5) (Taniguchi *et al.*, 1989) the c-terminal IRF-2 of basic in nature where as IRF-2 is acidic. Suggesting the distinct function of these factors. IRF-1 and IRF-2 mRNA are both expressed in a variety of cell types, and their expression levels, particularly IRF-1 mRNA are dramatically unregulated upon viral infection or IFN stimulation (Taniguchi *et al.*, 1989). A series of cDNA transfection experiment revealed that IRF-1 can activate IFN α/β promoters (Taniguchi *et al.*, 1989). In fact, a high -level expression of IRF-1 cDNA resulted in the induction of endogenous IFN-α/β genes in a variety of cell lines, albeit at low efficiency

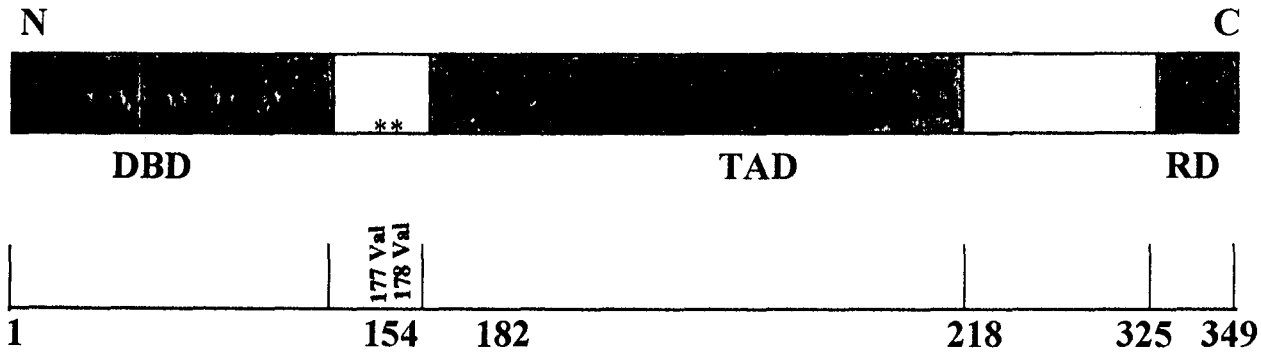


Fig.1.Structure of IRF-2. (Modified from Child *et. al.*, 2003; Taniguchi *et. al.*, 2001 and Mamane *et. al.*, 1997) DBD: DNA Binding Domain, TAD: Transactivation Domain, RD: Repression Domain, WWWWW: Tryptophan repeat.

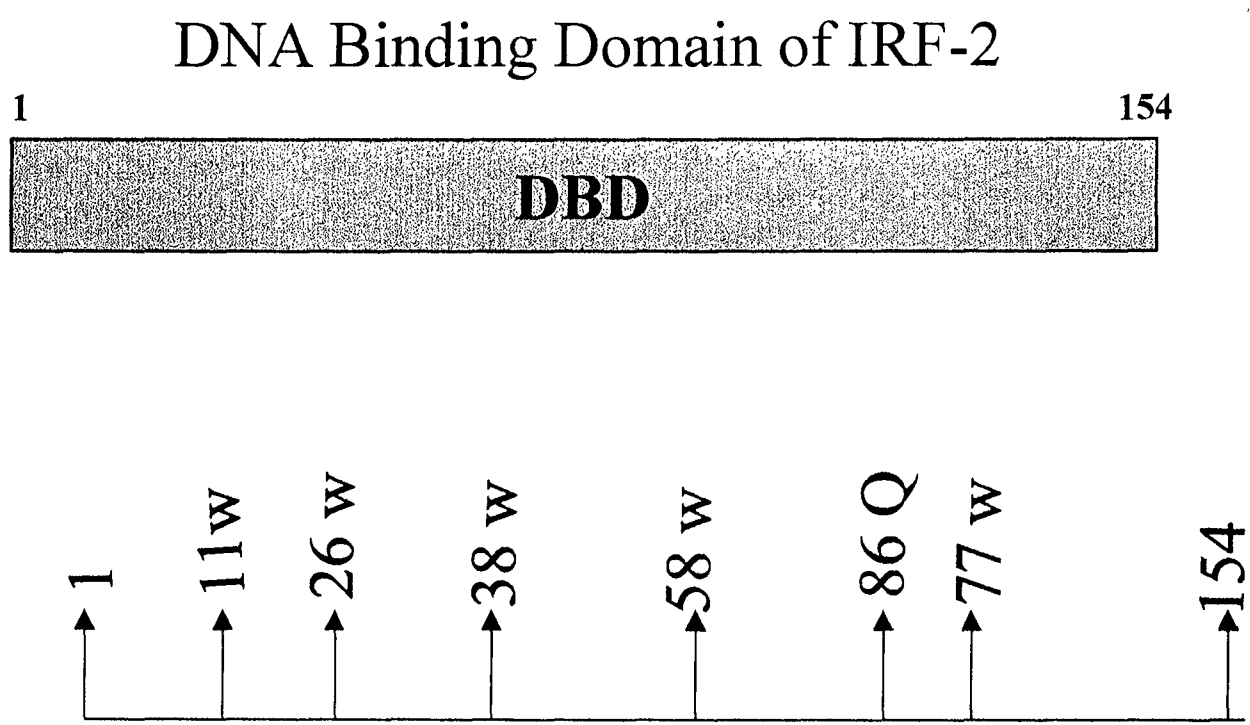
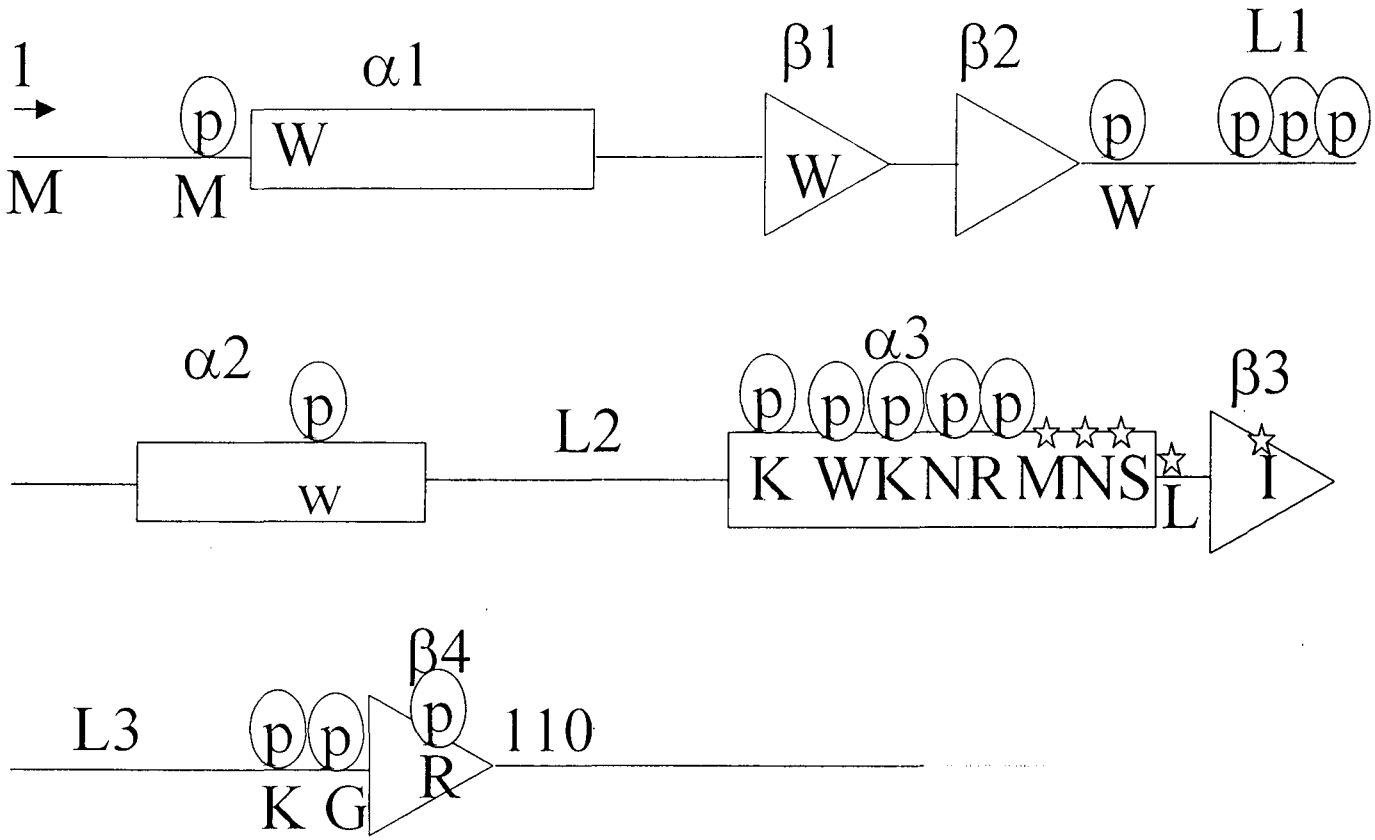


Fig.2.Important amino acids of DBD of IRF-2 (Fuji *et al.*,1999).



☆ K^+-binding sites, (p) -Phosphorylation site.

Fig.3. Detail structure of IRF-2 DBD(Modified from Escalante *et al.*,1998;Fuji *et.al.*,1999).

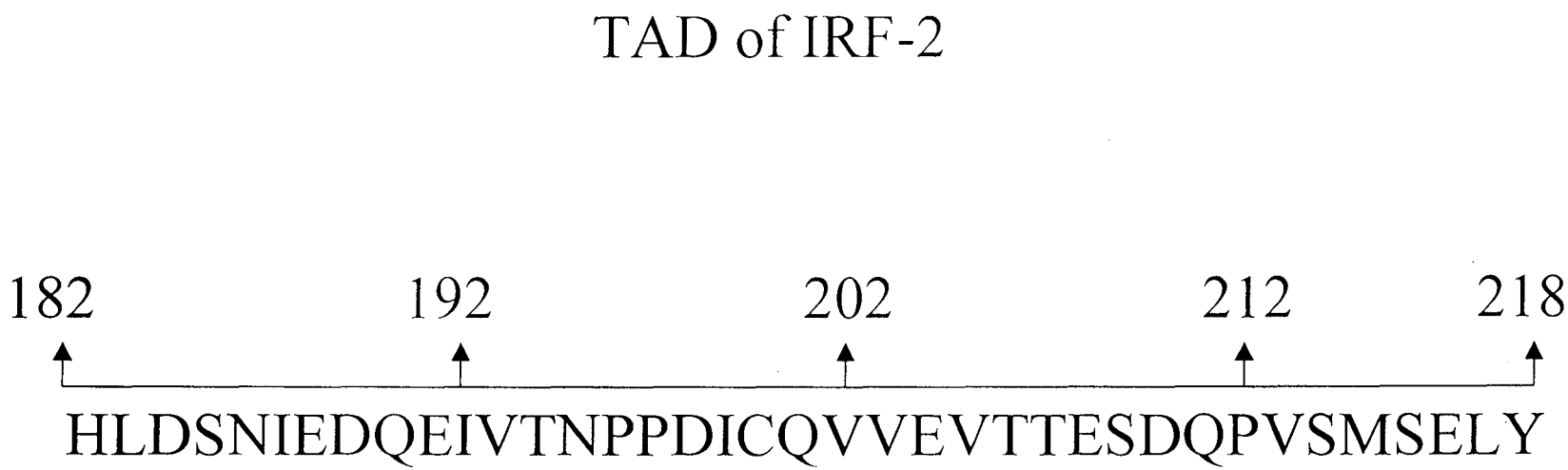


Fig.4. Amino acids sequence of Tranactivation domain (TAD) of IRF-2 (Fuji *et al.*,1999).

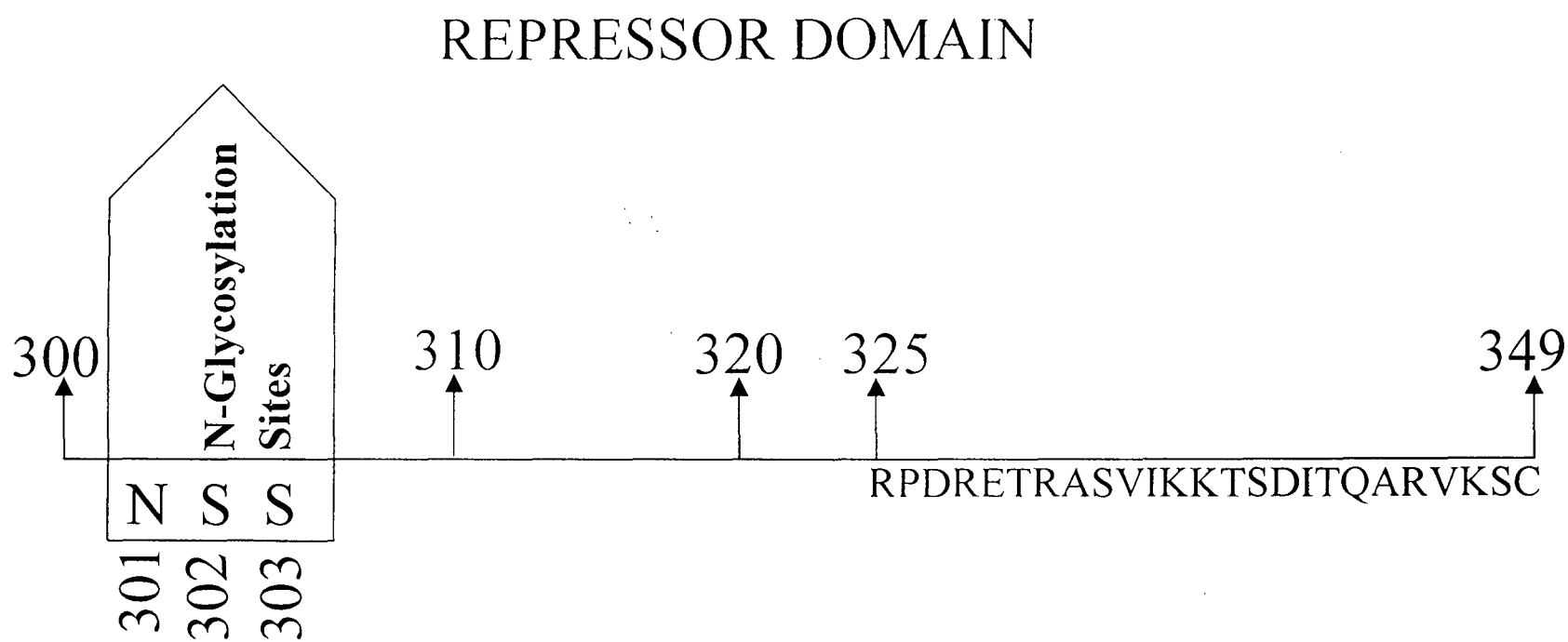


Fig.5. Amino acid sequence of Repression Domain (325-349) and additional N-glycolsylation sequence at 302-303(Harada *et al.*,1989).

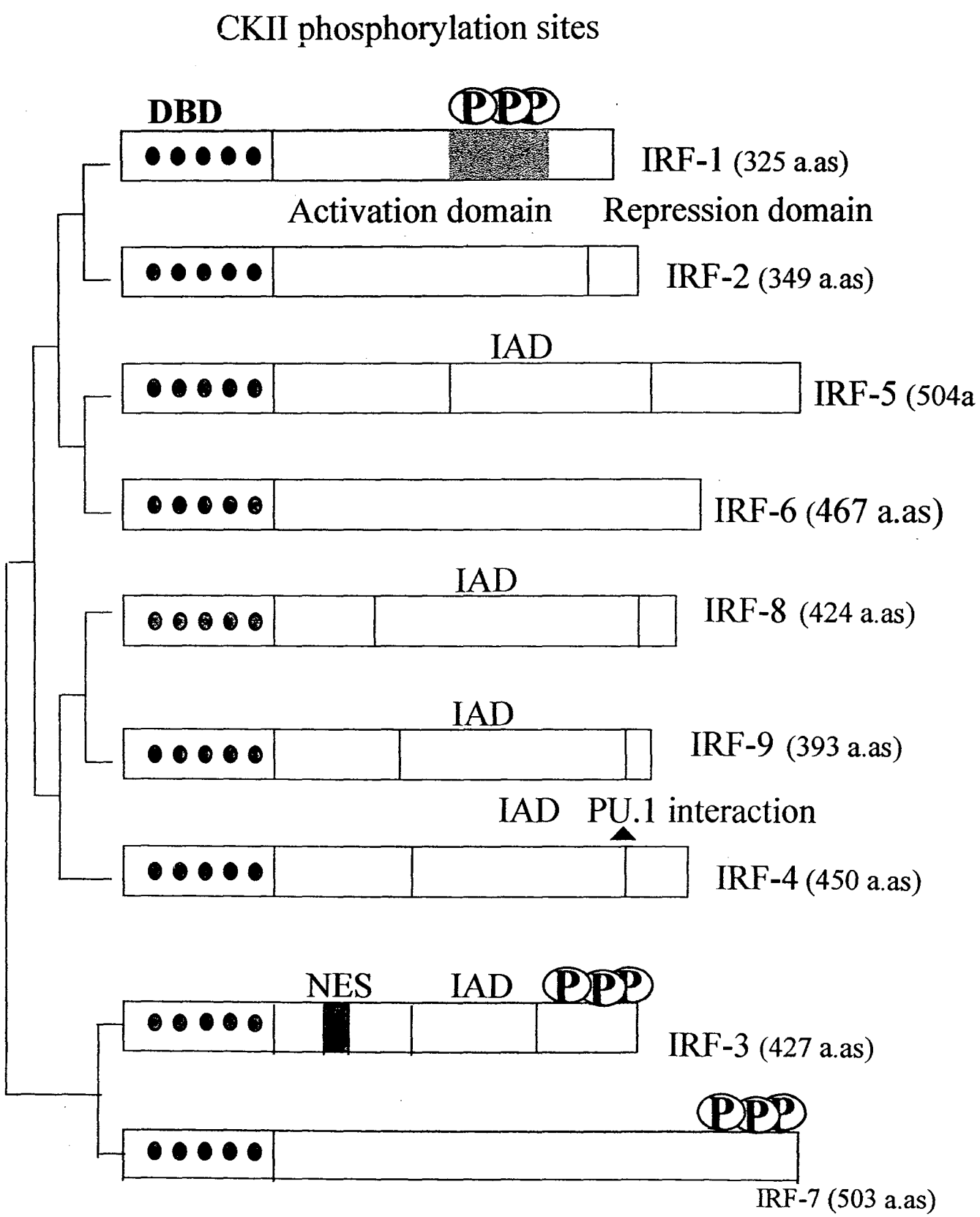


Fig.6. Members of the IRF family ; phylogenetic relationships and their functional domains .members of IRF family show significant homology of the 115 a.as in the amino terminal domain which comprises the DNA Binding Domain. ● Five times repeated tryptophans (W) are conserved among the these family members.different domains are also shown. It was determined that 177 amino acids in the carboxyl-terminal region of IRF-8 comprise the association domain with IRF-1or IRF-2.Phosphorylation sites were also determined. NES :Nuclear Export sequence, IAD :Interferon Association Domain. (Taniguchi *et.al.*,2001).

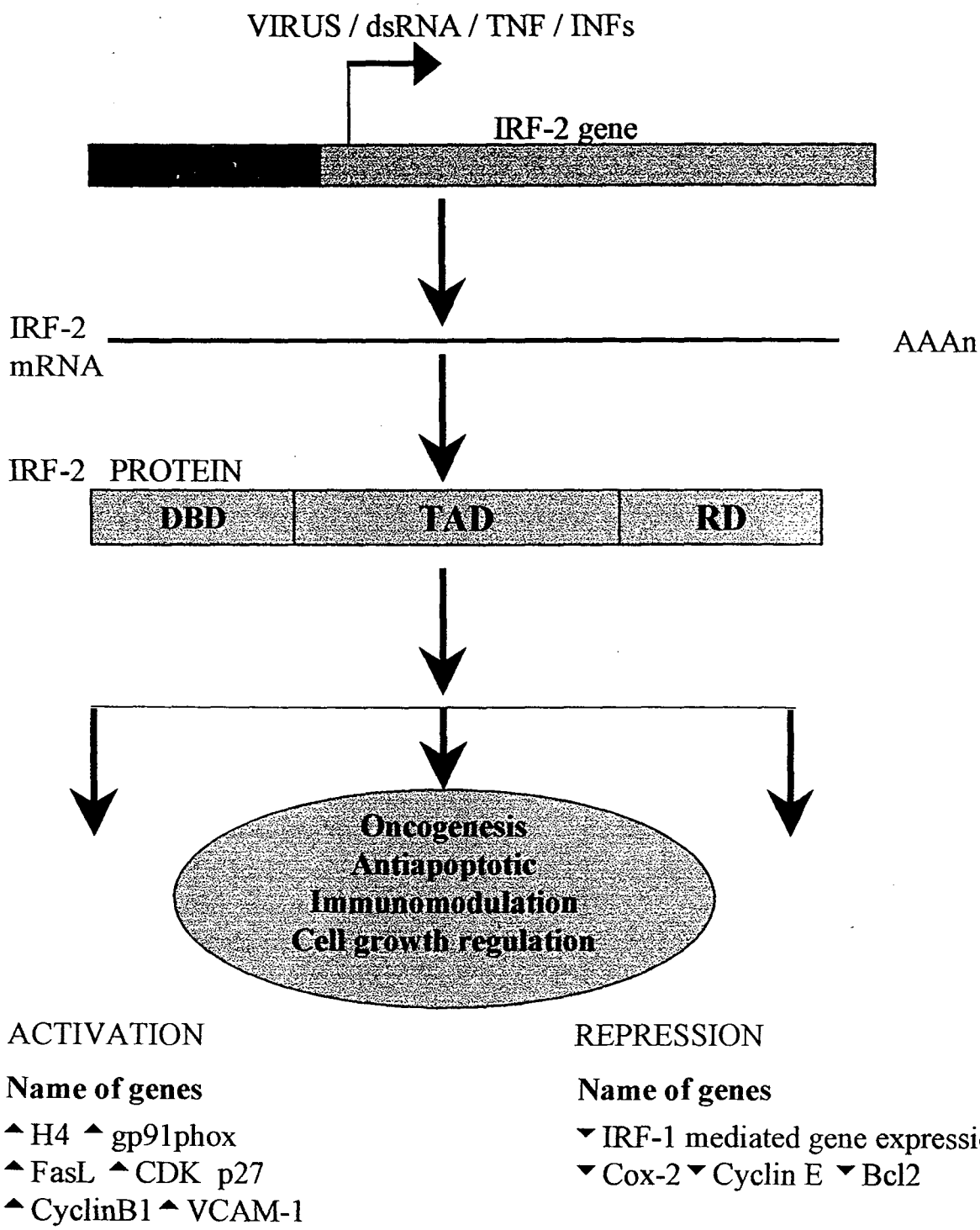


Fig.7. Different cellular responses IRf-2 and some important IRF-2 downstream genes.(Taniguchi *et. al.* 2001; Xie *et.al.*,2003).

(Taniguchi *et al.*, 1990). Unlike IRF-1, IRF-2 had no such effect; rather, it repressed IRF-1 induced transcriptional activation (Taniguchi *et al.*, 2000). IRF-1 protein is very unstable (half-life ~30minutes) where as IRF-2 is apparently stable (half life~8 hrs.) (Fujita *et al.*, 1991). These initial observations were suggestive that IRF-1 and IRF-2 function as transcriptional activator and repressor, respectively, for the IFN-α/β genes. Intrestingly, evidence has also been provided that IRF-2 functions as a trans cription activator for vascular adhesion molecule -1(VCAM). (Dean *et al.*, 1998) and cell cycle regulated Histone H4 genes (Stein *et al.*, 1995).

3.4 Functions of IRF-2:

IRFs play diverse of physiological roles in vertebrates can be categorized as Cell proliferation through regulation of cell growth genes via Interferons, Immunomodulation via regulating immunological cells development, Cell cycle regulation via regulating CCE genes, Host defense etc.

3.4.1.Transcriptional repressor: IRF-2 is a potential transcriptional repressor. It represses the activity of IRF-1 (Fig.7).Transcriptional repression by IRF-2 is localized to the C-terminal. Fusion of this region to the C-terminal end of IRF-1 inhibits transactivation by IRF-1. A latent activation domain exists in the central region of IRF-2, which is considered to be silenced by C- terminal repression domain. These result indicate that IRF-2 is a "mosaic" transcription factor possessing both activation and repression activity (Yamamoto *et al.*, 1994). The exact mechanism by which it causes repression is not known. One mechanism of repression by IRF-2 involves inhibition of IRF-1 transactivation by occupying ISRE sequence and subsequently preventing DNA binding by IRF-1. (Harada *et al.*, 1990;Nguyen *et al.*, 1995).

3.4.2.Transcriptional activator: There is also report of transcription activation by IRF-2. Transcriptional activation domain is resided in between DNA Binding Domain and Repression domain (154-324 amino acids residue). There are atleast three reports that IRF-2 acts as a transcriptional activator (Fig.7). Firstly, human H4 gene found to be directly activated by IRF-2 through binding to cell cycle element (CCE) present in the H4 gene promoter (Vaughan et. al., 1995). This histone gene may play a role in IRF-2 mediated oncogenesis, since it is functionally coupled to DNA replication and cell cycle progression at G1 to S transition. Secondly, QP promoter region of the Epstein Bar Virus encodes EBNA-1 gene has been shown to be activated by IRF-2 and IRF-1 (Nonkwelo *et al.*, 1997). Moreover, IRF-2 found to be involved in the

activation of Vascular Cell Adhesion Molecule -1(VCAM-1) (Jesse *et al.*, 1998). Thus, IRF-2 is a dual transcription factor possess both activation as well as repression property (Yamamoto *et al.*, 1994).

3.4.3. Oncogenic potential of IRF-2: Unlike IRF-1, IRF-2 functions as a transcriptional attenuator, critical in balancing IFN action *in vivo* (Hida *et al.*, 2000). In NIH3T3 cells, the overexpression of IRF-2 causes oncogenic transformation and concomitant constitutive expression of IRF-1 causes these cells to revert to the non-transformed phenotype (Harada *et al.*, 1993). Although the exact mechanism underlying this cell transformation is still unknown, it is possible that IRF-2 exerts its oncogenic function via the mediation of IRF-1 and other IRF family members that bind to the same IRF-E. This possibility is supported by the finding that NIH 3T3 cells expressing only the DNA-binding domain of IRF-2 were also transformed (Nguyen *et al.*, 1995). On the other hand, IRF 2 activates gene (s) involved in oncogenesis, such as Histone 4 (Vughan *et.al.*,1995 ; Vughan, *et .al* .,1998).

3.5.Physiological Role of IRF-2

3.5.1.Role of IRF-2 transcription factor in signaling:

Ligand induced activation of type I and type II receptors, IFNGR, results in the activation or induction of similar transcription factors the IRFs and STAT families. IFNAR stimulation results the activation of Janus family protein tyrosine kinases, Tyk2 and Jak1, which are associated with sub components of IFNAR1 and IFNAR2, respectively. This activation of followed by the site specific tyrosine phosphorylations of STAT1 (residue 701 for mouse STAT1 and STAT-2 (residue 688 for mouse STAT-2) (Darnell *et.al.*,1994 ; Ihle *et al.*, 1995; Taniguchi *et al.*, 1995 and Stark *et al.*, 1998). These two phosphorylated STATs, in combination with IRF-9, form a heterotrimeric complex, ISGF3, which translocates into nucleus and binds to ISRE to activate IFN inducible genes (Darnell *et al.*, 1994). Additionally, the phosphorylated STAT-1 also undergoes homodimerisation and is converted into its transcriptionally active form, termed IFN-γ activated factor/IFN-α-activated factor (GAF/AAF), which binds to the IFN-γ activated site (GAS; consensus sequence TTCCNNGAA) and activates its target genes (Darnell *et al.*, 1994 and Bluyssen *et al.*, 1996). In the case of IFNGR signaling, IFNGR1, the Ligand binding subunit that interact with Jak1, IFNG2, which interacts with Jak2, associate upon of the dimeric form the ligand,

resulting into the activation of these Jak PTKs (Ihle *et al.*, 1995). Like IFNAR stimulation IFNGR stimulation also results in the efficient activation STAT-1 (Darnell *et.al.*, 1994). Furthermore, STAT-2 is also tyrosine phosphorylated by IFN-γ stimulation, albeit at much lower levels than IFN-α/β stimulation, leading to the formation of ISGF3 (Matsumoto *et al.*, 1999). The formation of trimeric complex formed STAT1-p48; which consists of STAT1 dimer and IRF-9, was also reported in a monkey kidney cell line, vero (Bluyssen *et al.*, 1995).

The activation of the STATs during IFN signaling requires their recruitment to the receptors (Greenlund *et al.*, 1995;Yan *et al.*, 1997 and Li *et al.*, 1997) recent study offers a mechanisms by which IFNγ stimulation results INF the activation of ISGF3. In fact, a novel form of cross talk occurs between IFN α/β signaling is dependent on a weak IFNAR stimulation by spontaneously produced IFN-α/β (Takoka *et al.*, 2000). Further evidence was provided for the physical association between IFNAR1 and IFNGR2 subunits. In view of the report demonstrating the STAT-2 -docking site with in the intracellular domain IFNAR1 (Yan *et al.*, 1996) which is physically associated with IFNGR2 at the caveolar membrane domain (Takaoka *et al.*, 2000) this docking site may be utilized for IFN-γ induced activation of the ISGF3 complex, IRF-1 is expressed at low levels in unstimulated cells but is induce by many cytokines such as IFNs (-α, -β, -γ), TNF-α, IL-1, IL-6 and by viral infection (Miyamoto *et al.*, 1988; Abdollahi *et al.*, 1991; Fujita *et al.*, 1989). The analysis of the IRF-1 promoter region revealed that this induction is mediated by STAT and NFκB transcription factors, the binding sites for which are found within this region (Pine *et al.*, 1994 and Harada *et al.*, 1994). Infact, IFN induced expression of IRF1 mRNA was completely abolished in STAT-1 deficient (STAT-$1^{-/-}$) cells (Meraz *et.al.*, 1996). As described above, the recognition sequence of IRF-1 (IRF-E) overlaps with that of ISRE, which binds ISGF3. These observations suggest that IRF- 1 function as a regulator of cellular response to IFNs by affecting a set of IFN-inducible genes. In contrast, IRF 2 is inducible by IFN α/β, albeit with slower kinetics of induction than IRF-1 (Harada *et.al.*, 1989).This induction is mediated by ISGF3,the binding site for which found with in the IFF-2 promoter region (Harada *et al.*, 1994).

3.5.2. IRF-2 in regulation of Immune Responses:

(a) NK *cell development:* Role of IRF 2 has been investigated in NK cell devlopment. IRF-$2^{-/-}$ mice were shown to carry defects in NK cell development (Lohoff *et al.*, 2000). IRF-$2^{-/-}$ splenocytes displayed a larger decrease in NK cell cytotoxicity than did IRF $2^{+/-}$splenocytes stimulated with poly (I): (C) *in vivo*. A notable defect in NK cell activity was also noted *in vivo* in a tumor rejection model using the NK sensitive cell line. Furthermore, the number of NK cells (NK1.1+TCRα/β-) was dramatically decreased in IRF-$2^{-/-}$ mice (Lohoff *et al.*, 2000)

(b) Macrophage function: Neutrophils and macrophages play an important role in restricting bacterial replication (e.g. *Listeria monocytogenes* infection) in the early phase of primary infection in mice, and cytokines IFN-γ and TNF-α are essential for protection. IRF-$2^{-/-}$ and IRF-$8^{-/-}$ mice are highly susceptible to *Listeria* infection compared with IRF-$1^{-/-}$ mice. Therefore, IRF-8 and IRF-2 are critical for IFN-γ production of reactive oxygen intermediates (ROIs) and possibly others in macrophages. (Fehr *et al.*, 1997).

(c) Cytotoxic T cell function: CD8+T cells have cytotoxic effector functions. Cytotoxic T Lymphocyte (CTL) response to LCMV (lymphocytic choriomeningitis virus)-infected target cell was significantly reduced in IRF-$1^{-/-}$ mice. In contrast, IRF-$2^{-/-}$ mice showed normal CTL activity against LCMV-infected target cells, although some nonspecific cytotoxicity was detected (Matsuyama *et al.*, 1993).

(d) Regulation of $CD8^+$ *T cell response:* cytokines are not always beneficial to the host because many cytokines are multifunctional and often invoke antigen non-specific response of the cell. In this context, regulatory mechanisms have been reported for other cytokine systems, downregulating their signaling events (Ihle 1995;Naka.1999; Yasukawa *et al.*, 2000 and Miyaazono, 2000). Recently, IRF $2^{-/-}$ mice in C57BL/6 background spontaneously were found to have developed an inflammatory skin disease resembling psoriasis (Hida *et al.*, 2000 and Matsuyama *et al.*, 1993). The pathogenic development is suppressed upon the selective depletion of these T cells. $CD8^+$ T cells exhibit *in vitro* hyper-responsiveness to antigen stimulation, accompanied with a notable upregulation of the expression of genes induced by IFN-α/β (Hida *et al.*, 2000). Furthermore, both disease development and $CD8^+$T cell abnormality are suppressed by the introduction of nullizygosity to the genes that positively regulate the IFN- α/β signaling pathway. (Hida *et al.*, 2000). Thus, IRF-2 may represent a unique negative regulator, attenuating IFN-α/β induced

gene transcription, which is necessary for balancing the beneficial and harmful effects of IFN-α/β signaling in the immune system.

(e) Regulation of Th1/Th2 differentiation: IRF-2 was originally described as an antagonist of the IRF-1 mediated transcriptional regulation of IFN-inducible genes (Harada *et al.,* 1990). Since Th1 cell development and NK cell development are impaired in IRF1$^{-/-}$ mice, one would have expected that IRF-2$^{-/-}$ macrophages (Lohoff *et al.,* 2000), and IRF-2$^{-/-}$ mice are susceptible to *Leishmania major* infection due to defect in Th1 cell differention (Lohoff *et al.,* 2000). Thus, rather than functioning as negative regulator, IRF 2 may to IL-2 gene expression in cooperation with other factors, such as IRF-8, in activated macrophage.

STATEMENT OF PROBLEM

4. STATEMENT OF PROBLEM:

Interferon Regulatory Factor-2 belongs to the IRF family of transcription factors involved in the regulation of many genes, the expression of which are induced by type I IFN, IRF-1, viruses, poly I:C, dsRNA etc. IRF-2 occupies important position in regulation of the cytokine network. Most of the studies highlighting functions of IRF-2 have been carried out in murine and human cell lines as well as IRF-2 knock out mice using various inducers. However, the information regarding physiological role is limited.

A variety of agents like Type I IFN, IRF-1, viruses, poly I:C, dsRNA and other agents stimulate mRNA expression of IRF-2. IRF-2 participates in regulation of IFN signaling by binding on ISRE sequence. IRF-2 controls the overexpression of IRF-1 mediated gene expression by competitively binding on ISRE sequence of that gene. Moreover, IRF-2 regulates cell cycle progression by causing expression of H4 gene. Thus IRF-2 is a very important transcription factor, having both repression as well as activation function.

IRF-2 DNA Binding Domain is almost similar to rest of the members of IRFs.Strikingly, IRF-2 via its DNA Binding Domain doing repression as well as activation. Thus binding pattern of IRF-2 is crucial for its functions.

A few properties still not clearly understood about IRF-2 are given as follows:

1. *How IRF-2 is involved in different responses? The function of IRF-2 is different in different cell types; presumably due to presence or absence of other cell type specific factors that may associate with it.*

2. *Different levels of both IRF-1 and IRF-2 expression seems to dictate differential gene expressions, how such functions are brought about and regulated by IRF-2?*

3. *Post-translational modification of IRF-2 may influence its function in terms of DNA binding as well as transcription.*

With this background, the present work has been proposed and carried out, aimed at generation of murine IRF-2 clones and expresssion of recombinant IRF-2 protein in *vitro.*

Objectives:

- Generation of recombinant pBS/IRF-2 clones.
- Expression of recombinant IRF-2 as a GST fusion protein in pGEX2TK plasmid.

MATERIALS & METHODS

5. MATERIALS AND METHODS:

5.1.MATERIALS:

Table.1

	Name	Features	Reference
1	*E.coli* cell: XL-1 Blue	E.coli XL-1 Blue genotype: endA1,hsdR 17 (rk-,mk^+) supE44, thi-1,λ, rec.A1 gyr A96, relA1,(lac) [F',pro AB,lac^qZ Δ M15,Tn10(tet^R)]	Stratagene, U.S.A
2	Plasmids: pIRFL pIRF2.5 pCDM8 pBluescript pGEX2TK	Containing 2082 bp murine IRF-1cDNA in pCDM8 vector. Containing 2435 bp murine IRF-2 cDNA in pCDM8 vector. 4.4kbmammalian expression vector. 2961 bp plasmid with origin, col E1, with T3 andT7 promoter. 4950bp pGEX based GST expression system containing strong tac promoter.	Prof.T.Taniguchi (Tokyo) Prof.T.Taniguchi (Tokyo) Invitrogen,U.S.A Stratagene Amersham biopharmacia.
3	Primers: IRF-2: P1 P2 IRF-1: GST 5' 3' NC	5'AAGGATCCATGCCGGTGGAACG GATGCGA 3' 5'AAGGATCCTTAACAGCTCTTGA CACGGGC 3' 5'AAGGATCCACCATGCCAATC 3' 5'AAGGATCCAAGAACGGGTCAGA GA 3'	Sigma-aldrich resources.
5.	GST-IRF-1	GST-IRF-1fusion protein expressed in *E.coli*.XL-Blue 1 cells, ~67kd.	Meenakshi upreti J.N.U.,N.D.-67

5.2. REAGENTS:

Acetic acid	Acetic acid glacial (*Merck*-GR, Qualigens-AR). Concentration 99.7%. Stored at RT.
30% Acrylamide	29.2 g. Acrylamide (Sigma, A-9909) + 0.8 g. N,N'-methelyene bis-acrylamide (Sigma, M-7256) dissolved in 60 ml. Warmed on the magnetic stirrer to dissolve and made final volume to 100 ml, added 1 g. activated charcoal/100ml. acrylamide, stirred for an hour, filtered through Whatman 1 MM filter paper, stored at 4°C in dark brown bottles.
Antibiotics	Ampicillin: Ampicillin-sodium salt injection - Biocillin 500mg. Injection (Biochem Pharmaceuticals Industires) was dissolved in the 5ml. sterile H_2O provided with the antibiotic. Kept frozen as 500 ul aliquots at -20^0C. Working concentration: 100 µg/ml. Tetracycline: Tetracycline hydrochloride (Sigma, T-8032). Stock concentration was 10 mg/ml in 50% ethanol. Stored in aliquots at -20^0C. Working concentration: 15µg/ml.
APS 10%	0.10 g. Ammonium per sulphate (Sigma, A-9164) was dissolved in 1 ml. H_2O to get a 10% solution. Prepared fresh just before use.
β-Mercaptoethanol	β-Mercaptoethanol (14.4 M *Merck*-GR.) Stored at 4°C.
BSA, 10 mg/ml	Dissolved 10 mg. Albumin, bovine (Fraction V, Sigma, A9647) in H_2O and stored as 1 ml. aliquots at –20°C.
$CaCl_2$, 1 M	Dissolved 14.7 g. of $CaCl_2.2H_2O$ (Sigma, C-3306) in 100 ml. H_2O and sterile filtered through a 0.22-micron

filter. Stored at –20°C.

CIAP — Calf intestinal alkaline phosphatase. (New England Biolabs, 290S). Concentration: 10 U/µl. Stored at –20^{0}C in the supplier's buffer.

CIAP buffer (10X) — 1 M NaCl, 500 mM Tris.Cl, pH 8.2, 100 mM $MgCl_2$ and 10 mM DTT, pH 7.9. Stored at –20^{0}C.

Chloroform. — *E.Merck* (Germany, Cat. No. 2445). Stored at RT

Destaining solution — MeOH:H_2O:AcOH::40:50:10. The destaining solution changed several times until the background was satisfactorily removed.

DTT, 1M — 154.5 mg $_{D\text{-}L}$-Dithiothreitol (Sigma, D-9779) dissolved in deionised 800 µl H_2O and the volume made to 1 ml.Kept frozen as 100 µl. aliquots at –20^{0}C.

Ethanol — *Merck*-GR 1.00983.0511., Bengal Chemicals and Pharmaceuticals, Ltd.

EDTA, 0.5M — Suspendeded 93.05g of di-sodium ethylene-diaminetetra- acetate.$2H_2O$(Qualigens-ExcelaR; Sigma, E-5134) to 400 ml. H_2O and adjusted pH to 8.0 with 40 g. NaOH pellets and 5 M NaOH, autoclaved and stored at RT.

Ethidium bromide, 10 mg/ml — Dissolved 100 g. ethidium bromide (Sigma, E-8751) to 10 ml. sterile H_2O, stored as 1ml. aliquots at 4^{0}C in dark coloured Eppendorf tubes. Working concentration being 0.5 µg/ml.

Formaldehyde — Formaldehyde solution (37%), Qualigens-ExcelaR.

	Stored at RT.
Glycerol	Sigma, G-5150; Qualigens AR
Hydrochloric Acid	Qualigens-AR
Isopropanol	Isopropanol (Spectrochem, HPLC grade). Stored RT.
IPTG	Isopropyl-β-D-thiogalactopyranoside (MBI Fermentas, #R0392). 100 mM stock solution was made in sterile H_2O and was stored as 1 ml. aliquots at -20^0C.
Kinase	*T_4 Polynucleotide Kinase* New England Biolabs, 10U/μl, # M0201L Stored at -20^0C and incubated at 37^0C. 1X NEBuffer: 70 mM Tris.Cl (pH 7.6), 10 mM $MgCl_2$, 5mM DTT
Ligase	*T4 DNA Ligase* (Gibco BRL, 15224-025), concentration: 1 Weiss unit/μl. Stored at -20^0C in supplier's buffer.
Ligation Buffer	50 mM Tris.Cl (pH 7.6), 10 mM $MgCl_2$, 1mM DTT, 1 mM ATP, 5% PEG-8000. Supplied by Gibco BRL. Stored at -20^0C in supplier's buffer
Lysis Buffer (E.coli)	(10 mM HEPES, pH 7.9, 2 mM EDTA, pH 8.0, 1 mM EGTA, pH 7.0, 400 mM KCl, 0.1% Triton X-100, 10% Glycerol, 1 mM DTT, 1 mM PMSF and 5 μg/ml each of the protease inhibitors- aprotinin, leupeptin and benzamidine)
$MnCl_2$	Manganese chloride (Qualigens-AR)
Medium	LB medium: 10 gm Bacto-tryptone (Hi-media, RM-

014), 5 g. Bacto Yeast Extract (Hi-media RM-027), 10 gm NaCl (Qualigens-ExcelaR) in 1 L H_2O. and adjusted the pH to 7.0 with 5 M NaOH. Autoclaved at 15 lb/sq.in. pressure for 15 minutes and stored at 4^0C.

MOPS, 1 M (3-[N-Morpholino]propanesulphonic acid), MOPS free acid (Sigma, M-8899). Dissolved 20.93 g. in 100 ml. H_2O, adjusted pH to 7 with NaOH. Filter sterilised and stored at 4^0C.

Normal saline 0.98% NaCl solution in sterile H_2O. Generally used chilled.

Phenol (Ranbaxy-AR) Double-distilled, added hydroxyquinoline to a final concentration of 1%, equilibrated with 0.5 M Tris.Cl pH 8.0, followed by equilibration in Tris.Cl pH 8.0 and stored in 200 ml. aliquots at -20^0C. Working solution stored at 4^0C.

PBST 1 x PBS, pH 7.6 containing 0.1% Tween-20

PAGE Polyacrylamide gel electrophoresis:
Stacking gel: 5% Polyacrylamide in Tris.Cl pH 6.8.
Resolving gel: 8 to 10% Polyacrylamide Tris.Cl pH 8.8

30% PEG Polyethylene glycol-8000 (Sigma, P-5413). 30% made in H_2O, autoclaved and stored at RT.

Polymerases *Taq DNA polymerase, recombinant*: Gibco BRL, Cat. No. 10342-020, 5U/µl, stored at -20^0C.
10X buffer: 200 mM Tris.Cl (pH 8.4), 500 mM KCl. Stored at -20^0C.
GeneTAQ, Recombinant Taq DNA polymerase:MBI Fermentas, # EPO407, Fermentas, stored at -20^0C.

10X buffer: 100 mM Tris.Cl (pH 8.8), 500 mM KCl, 15 mM $MgCl_2$, 0.1% Gelatin, 0.05% Tween 20 and 0.05% NP-40.

Cloned Pfu DNA polymerase: Stratagene, # 600153, 2.5U/µl, stored at -20^0C.

10X buffer: 200 mM Tris.Cl (pH 8.75), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1000 µg/ml BSA. Stored at -20^0C.

Potassium acetate, 5M — 49.07 g of Potassium acetate (Qualigens-SQ) was dissolved in 60 ml distilled H_2O and volume made up to a final of 100 ml autoclaved and was stored at RT.

Potassium Chloride, 2M — Dissolved 14.91 g of KCl in 60 ml of distilled H_2O and the volume made to 100 ml. Autoclaved and stored at 4^0C.

Restriction enzymes — *(a) Banglore Genei*

BamHI, 10U/µl, storesd at -20°c

EcoRI, 20U/l stored at -20°c

10X buffer for BamHI and E.coRI (100mM Tris-Cl pH 8,100mM NaCl,100mM $MgCl_2$,100mM B.M.E.), stored at -20°c.

RNase A, 10 mg/ml — Pancreatic RNase A, Type 1-A (Sigma, R-4875) 10 mg/ml dissolved in 1X TNE, incubated at 85^0C for 10 min to inactivate any contaminating Dnase. Stored as 100 µl. aliquots at -20^0C.

Ribonuclease inhibitor (Rnasin) — *Promega*, 20-40 U/µl , Cat.# N2111

SDS, 10% — Lauryl sulfate-Sodium salt (Sigma, L-4390) 10% (w/v) SDS dissolved in 50 mM Tris.Cl pH 8.0. Stored at RT.

Sephadex G-50	Sephadex G-50 (Sigma,G-50-80), 10 g. was soaked and washed several times in excess H_2O to remove all traces of dextran, re-suspended in 100 ml 1 X TNE, autoclaved and was stored at 4^0C.
Sodium acetate, 3 M pH 5.2	Dissolved 40.8 g. sodium acetate.$3H_2O$. (Qualigens-ExcelaR), in 80 ml. H_2O and adjust the pH to 5.2 with glacial acetic acid, autoclave and store at RT.
Sodium acetate, 3 M pH 4.6	Dissolved 40.8 g. sodium acetate.$3H_2O$ (Qualigens-ExcelaR), in 80 ml. H_2O and adjust the pH to 4.6 with glacial acetic acid, make 20 ml. aliquots, autoclave and store at RT.
Sodium Chloride, 5 M	Dissolved 29.2 g. Sodium chloride (Qualigens-ExcelaR) in 100 ml. H_2O, autoclaved, stored at RT.
TAE, 50 X	Mixed 242 g. Tris base (Qualigens-ExcelaR), 57.1 ml. glacial acetic acid (Qualigens-ExcelaR), 100 ml. 0.5 M EDTA pH 8.0 (Qualigens-ExcelaR) and made to 1 L with H_2O, autoclaved and stored at RT.
TBE, 5 X	54 g. Tris base, 27.5 g. Boric acid (Qualigens-ExcelaR) and 20 ml. 0.5 M EDTA pH 8.0, autoclaved and stored at RT. If on long storage a precipitate was formed the solution was discarded.
Tris-Glycine-SDS, 5 X	15.1 g. Tris base, 75.07 g. Glycine and 5 g Lauryl Sulphate Sodium salt was dissolved in 800 ml. H_2O and final vol. made to 1 L. [Final concentration: 0.025 M Tris.Cl, 0.192 M Glycine, 0.1% (w/v) SDS, pH 8.3.
TE buffer	10 mM Tris.Cl pH 7.5 or 8.0 and 1 mM EDTA in H_20,

	autoclaved and stored at RT.
TEMED	N,N,N',N', Tetra methyl ethylene diamine. (Sigma, T-7024) stored at 4^0C.
Transformation Buffer II	To sterile H_2O added 15% glycerol and autoclaved. Added to a final concentration 10 mM MOPS pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, and sterile filtered through 0.22μm membrane and stored at 4^0C. For every 100 ml. of starting culture required 4 ml. of transformation buffer II.
Tris.Cl, 1.5M	(Qualigens-ExcelaR) 18.2 g was dissolved in 80 ml distilled H_2O, pH adjusted to 8.8, volume made to 100 ml. Autoclaved and stored at 4^0C
Tris.Cl, 0.5M	(Qualigens-ExcelaR) 6.1 g was dissolved in 80 ml distilled H_2O, pH adjusted to 6.8, volume made to 100 ml. Autoclaved and stored at 4^0C
Tween-20	*Merck*-GR
Water	Milli Q Water (Millipore deionizer) or quartz double-distilled, autoclaved H_2O.
Whatman	Whatman 3 MM Paper (3030917), stored in a clean dry place.
X- gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside (Sigma, B-4252). Stock solution of 20 mg/ml in dimethylformamide made in glass or polypropylene tubes. The aliquots were stored at -20^0C, in Eppendorf tubes that were wrapped in Al-foil to circumvent damage by light.

X-Gal solution	0.2% X-Gal (from 2% stock in DMF), 2mM $MgCl_2$, 5 mM $K_4Fe(CN)_6.3H_2O$, 5 mM $K_4Fe(CN)_6$.

Plasmids:

pBluescript II SK (+/-) phagemid: 2961 bp from Stratagene, USA

Important sites are *lacZ* gene (*lac* promoter 816-938 bp), MCS (657-759 bp) and Ampicillin-resistance gene (Amp^r) (1975-2832 bp).

pGEX-2TK: ~ 4950 bp from Amersham Pharmecia Biotech

Important sites are MCS (951-966 bp), GST-region: *tac* promoter-10 (205-211 bp), *tac* promoter-35 (183-188 bp), thrombin cleavage region (918-935 bp), Kinase recognition site (936-950 bp), β-lactamase (Amp^r) gene region (1377-2235 bp), $LacI^q$ gene region (3318-4398 bp).p

5.3. METHODS:

5.3.1. Maintanence of *E.coli strains:* Glycerol stocks

(A) ***Bacterial cultures growing in liquid media***

0.85 ml of bacterial culture was aliquoted to a labeled eppendorf tube and to it was added 0.15 ml of sterile glycerol. The culture was vortexed to ensure that the glycerol was evenly dispersed. The tube was then stored at –70 ^{0}C.

(B) Bacterial cultures on agar plates

A colony of bacteria scraped from the surface of an agar plate incubated overnight was inoculated into 2ml LB with the appropriate antibiotic. To it was added an equal volume of LB medium containig 30% sterile glycerol. The mixture was vortexed to ensure the complete dispersal of glycerol. Aliquots of the glycerinated culture were dispensed into sterile tubes and frozen at –70 ^{0}C.

(C) Stab cultures

2-3 ml glass vials with screw caps were used for this purpose. Molten LB agar was added until the vials were two thirds full and autoclaved and stored at room temperature until needed. A single well-isolated bacterial colony was picked and stabbed several times through the agar to the bottom of the vial. The cap was replaced and the vial labeled before being stored in the dark at room temperature.

5.3.2. Preparation of competent cells.

Day 1: The *E.coli* cells was made competent by a modification of the method of Hanahan (1985) streaked on LB agar plate with the appropriate antibiotic and incubated overnight at 37^{0}C.

Day 2: A single colony was picked and inoculated in 5 ml of LB with required antibiotic and incubated overnight at 200 rpm and 37 ^{0}C.

Day 3: 1 ml of the overnight culture was used to inoculate 100 ml of LB with the antibiotic. The culture was incubated for about 2 hrs. at 37 ^{0}C with shaking at 200 rpm till

an $O.D._{550}$ between 0.5-0.6 is reached. The cells were immediately put into pre-chilled 250 ml. GSA bottles and spun at 2.5K rpm for 5 minutes at 4°C in Sorval RC-5B refrigerated centrifuge. The supernatant was discarded and 40ml. Transformation Buffer I per 100ml. starting culture was added. (Added about 10ml. Buffer initially and resuspended the cell pellet with a sterile Pasteur pipette. When no cell clumps were visible rest of the buffer was added). After the cell pellet had been completely resuspended the cells were allowed to incubate on ice for 20 minutes. This was followed by centrifugation of the cells at 2.5K rpm for 5 minutes at 4°C. The supernatant was discarded and ice-cold 4ml. Transformation Buffer II per 100ml. starting culture was added. The cell pellet was resuspended with a sterile Pasteur pipette. 200µl. aliquots in pre-chilled Eppendorf tubes were made and stored at -70°C.

5.3.3. Transformation using competent cells

The competent *E. coli* cells were thawed on ice for 10 minutes. 1µl of plasmid DNA (0.1ng/µl concentration) or 4µl. ligation mixture was added to 200 µl. competent cells and allowed to incubate for 20 minutes on ice. A heat-shock at 42°C for 90 seconds was given followed by incubation on ice for 5 minutes. To it was added 800 µl. LB medium and incubated at 37°C for 1 hour with shaking at 200 rpm. 200 µl. of the transformation mixture was plated on a LB agar plate containing the appropriate antibiotics. The plate(s) were allowed to dry in the hood for 5 minutes and incubated overnight at 37°C.

On the following day total number of colonies were counted and the transformation efficiency measured by the formula given below:

Transformation efficiency = Total no. of colonies/µg DNA X100.

5.3.4. Plasmid isolation

(A) Plasmid isolation from 50 ml. bacterial culture

Midipreparation of plasmid DNA was carried out according to Sambrook et. al., (1989). 50 ml of LB + antibiotic was inoculated with a single transformed colony, allowed to grow overnight at 37° C, 200 rpm. Cells were pelleted down by spinning at 5000 rpm for 5 min. at 4° C in a Sorvall RC 5B centrifuge. The cell pellet was

resuspended well by vortexing in 1.5 ml lysis buffer I (15% sucrose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA, pH 8.0). A final concentration of 2 mg/ml freshly prepared Lysozyme (40mg/ml in 50 mM Tris-HCl pH 8.0) was added to the cell suspension and left on ice for 10 min. The lysed cell suspension was denatured by adding 3.0 ml of freshly prepared lysis buffer II (0.2 N NaOH + 1% SDS) and mixed by inverting the tube. After 10 min. on ice, 1.6 ml of 3 M sodium acetate (pH 4.6) was added, mixed by inverting and left on ice for 20 min. The tubes were then centrifuged at 12000 rpm for 15 min at 4 ^{0}C and to the supernatant collected in a fresh tube, 5 µl of DNase free RNase (10 mg/ml) was added, incubated for one hour at 37°C. The plasmid DNA solution was extracted twice with equal volume of phenol : chloroform (1:1) and once with chloroform. The plasmid DNA was precipitated by adding 0.6 volume of isopropanol in presence of 0.3 M sodium acetate and kept on ice for 15 min. DNA was pelleted by spinning at 15000 rpm for 20 min. at 4°C and the pellet was washed with 80% ethanol to remove excess salt. The pellet was then air dried for 30 min. at RT and dissolved in 100 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 1µl DNA was loaded on 1% agarose-TAE gel to check the quality and quantity of plasmid DNA.

PEG precipitation: The pellet was then resuspended in 400 µl of sterile double distilled water, to which was added 120 µl of 4 M Sodium Chloride. Mixed thoroughly and transferred to an Eppendorf tube. To it was added 500 µl of 13% PEG8000 and mixed well by vortexing. The sample was then incubated on ice for 1 hr. followed by centrifugation at 12K rpm, 4^{0}C for 10 min. After discarding the supernatant the pellet was washed with 1 ml. of prechilled 70% ethanol, centrifuged at 12K rpm, 4^{0}C for 10 min. The supernatant discarded, pellet dried and resuspended in 100 µl of 10 mM Tris. HCl.

Estimation of DNA concentration: A 1:10 and 1:20 dilution of the DNA was made to estimate its concentration. O.D. was measured at 260 nm and 280 nm and the spectrum taken from 200 nm to 300 nm. In a 1-cm-wide cuvette 50µg/ml dsDNA has an absorbance at 260 nm of 1.00. The concentration of the DNA can be estimated from the formula:

$$\text{Concentration of DNA} = A_{260} \times {}^{50\mu g/ml}/_{1\ \text{absorbance unit}} \times \text{dilution factor}$$

The O.D. $_{260/280}$ ratio of pure DNA (free of proteins) is 1.7 - 2.0. Alternatively the concentration of plasmid DNA can be estimated from the intensity of the bands on an ethidium bromide stained agarose gel.

5.3.5. Restriction digestion of plasmid DNA

The plasmid DNA was dissolved in the appropriate 1X Restriction Enzyme Buffer to get a final concentration of 50ng DNA/µl in the reaction mixture. If required BSA was added to a final concentration of 0.1mg/ml. The restriction enzyme was added to a final concentration of 1U/µg DNA and the Eppendorf tube left at the appropriate incubation temperature overnight.

5.3.6. Purification of DNA fragments through low melt agarose

DNA fragments purified from agarose gels by as per the method of Ausubel *et. al* (1995). The digested DNA fragments were electrophoresed through the required percentage of high melting temperature agarose in 1X TAE buffer containing 0.5µg/ml ethidium bromide at 100mA. When the fragment had resolved sufficiently a window was cut below the target fragment and 1% low melt agarose was poured into it. The agarose was polymerised at 4^{O}C, the gel was then brought to room temperature by submerging it in the buffer tank for 15 min. and electrophoresed again till the target fragment completely entered the window. The fragment was cut and any extra pieces of agarose trimmed off. The minced piece of agarose containing the target fragment was kept at 65°C to melt the agarose and ascertain the volume, mixed with TE buffer, pH 8.0. to a final concentration of ≤ 0.4%, vortexed and kept at 37°C for 10 minutes. To remove the agarose, added an equal volume of buffered phenol, vortexed for 15 min, centrifuged for 10 min at 12K rpm at RT. Collected the aqueous phase and set aside. Re-extracted the phenol phase and the interphase with an equal volume of TE buffer, pH 8.0 (if required). Centrifuged at 12K rpm, for 10 min. and at RT. The phenol extraction step was repeated again. This was followed by adding equal volume of Chloroform mixed to get an emulsion and subsequent centrifugation at 12K rpm, 4 ^{0}C for 10 min.

The aqueous phase was collected and the DNA was ethanol precipitated in the presence of 0.1 vol. 3 M sodium acetate, pH 5.2. The pellet was dissolved in 20µl. Tris.Cl, pH 8.0. The DNA concentration was ascertained after visualising an ethidium bromide stained agarose gel containing the gel purified DNA fragment.

5.3.7. Subcloning of murine IRF-2 PCR DNA

- **Vector preparation:** pBluescript was used as a cloning vehicle. pBSIISK plasmid DNA was digested with *Eco RI* (5'G↓AATTC3') in 20 µl reaction at 30°c for 4hrs. After complete digestion of the plasmid the enzyme was heat inactivated. Then purification was done by chloroform:phenol extraction method. it was subjected to *Eco RI* Restriction Digestiong as follows:

Table: 2.Restriction Digestion of pBSIIKS:

Reagents	**Volume taken**	**Final con^{c}.**
pBluescript SKII(3µg/µl)	6µl	18µg/µl
Eco RI(10u/µl)	2µl	20u/µl
Buffer(10x)	2µl	1x
Milli Q water	10µl	----
Total vol.	20µl	----

Reaction was placed at 30°c in water bath for overnight. Next day gel was run to confirm digestion. Vector was subsequently purified by chloroform: phenol extraction method quantified by running 1% agarose gel (12µg).

- **Klenow filling of pBSIISK/Eco RI product:** *Eco RI* is a cohesive end cutter (5'G AATTC 3'). It produces overhang that should be filled for blunt end ligation. Vector DNA was made blunt at their 5' and 3' ends by treatment with 4µl Klenow DNA polymerase (5U/µl) in 20µl reaction at 37°c for 30 minutes 1x buffer of all dNTP's

(0.1 mM each). Klenow polymerase was heat inactivated at 65°c for 20 minutes and the reaction was stores at -20°c.

Calf intestinal alkaline phosphates (CIP) treatment of vector: To avoid self-ligation of vectors, 1U CIP in 50µl reaction, 1x CIP buffer at 37°c for 1 hour treatment was done. Dephosphorytion reaction was performed as per following reaction:

Table: 3. Dephosphorylation of pBSIISK:

Reagents	**Volume taken**	**Final con^c.**
Vector (1µg/µl)	6µl	6µg/µl
CIP (10u/µl)	1µl	0.2u/µl
Buffer(10x)	5µl	1x
Milli Q water	38µl	---
Total	50µl	----

CIP inactivated by heating at 60°c for 10 minutes. DNA was once extracted with equal volume of phenol: chloroform (1:1) mixture, percipitated by adding 2.5 volume ethanol (Merck) in presence of Na-accetate (pH 6.0) at -70°c for 15 minutes, collected by centrifugation at 12k rpm for 15 minutes at room temperature washed once with 80% ethanol (Merck), dried and dissolved in TE buffer.1µl DNA was checked on 1% agarose TAE gel to assess the DNA concentration (150ng/µl) Then vector was used for ligation.

5.3.8. Insert preparation (PCR amplified murine IRF-2 cDNA):

- pIRF-2.5 possesses cDNA of IRF-2 was subjected to PCR reaction in order to get amplification. The reaction was settled as follows:

Table: 4. PCR of murine IRF-2 cDNA:

S.No.	Reagents	Stock conc.	Vol. taken	Final conc.
1.	Template DNA (pIRF2.5)	1 ng/μl	1μl	0.04ng/μl
2.	$Mgcl_2$	25mM	2μl	2mM
3.	dNTP's	10mM	0.75μl	0.3mM
4.	Taq. DNA Pol.buffer	10x	2.5μl	1x
5.	Taq. DNA Pol. Enzyme	3u/μl	0.3μl	0.05u/μl
6.	Forward primer P1	25pmol/μl	0.5μl	1pmol/μl
7.	Reverse primer P2	25pmol/μl	0.5μl	1pmol/μl
8.	Milli Q water (PCR grade)	----	17.45μl	----
	Total volume	----	25μl	----

Cycle parameters for PCR reaction:

1. 94°C ----5 minutes
2. 94°C ----30 seconds
3. 62°C ----1 minute
4. 72°C ----1.5 minutes
5. Go to step 2, 30 times
6. 72°C ----10 minutes
7. 4°C ----1 hr.
8. END

PCR IRF-2 was then purified by chloroform: phenol extraction method.

- **Insert preparation (PCR amplified murine cDNA IRF-1):**

$pIRF_L$ possesses cDNA of IRF-1 was subjected to PCR reaction in order to get amplification. The reaction was settled as follows:

Table: 5. PCR of cDNA of murine IRF-1:

S.No.	Reagents	Stock conc.	Vol. taken	Final conc.
1.	Template DNA ($pIRF_L$)	1 ng/μl	1μl	0.04ng/μl
2.	$MgCl_2$	25mM	2μl	2mM
3.	dNTP's	10mM	0.75μl	0.3mM
4.	Tap DNA Pol. Buffer	10x	2.5μl	1x
5.	Taq DNA Pol. Enzyme	3u/μl	0.3μl	0.05u/μl
6.	3'NC	25pmol/μl	0.5μl	1pmol/μl
7.	GST5'C	25pmol/μl	0.5μl	1pmol/μl
8.	Milli Q water (PCR grade)	----	17.45μl	----
	Total volume	----	25μl	----

Cycle parameters:

1.94°c ----5 minutes
2.94°c ----30 seconds
3.42°c ----1minute
4.72°c ----1.5minutes
5.Go to step 2, 30 times
6.72°c ----10 minutes
7.4°c ----1hr.
8.END

- **Klenow filling of PCR amplfied product:** Taq DNA polymerase adds A nucleotide at 3' end of amplified product as overhang. 300ng of PCR DNA was made blunt at their 5' and 3' ends by treatment with 1U klenow DNA polymerase (5U/μl) in 20μl reaction at 37°c for 30 minutes 1x buffer of all dNTP's (0.1 mM each). Klenow polymerase was heat inactivated at 65°c for 20 minutes and the reaction was stores at -20°c.
- **Kinasing of PCR amplified IRF-2 gene:** polynucleotide kinase was used for adding phosphate group at 5'duplex DNA of PCR amplified IRF-2.20μl reaction was settled containing 10x buffer, ATP (SRL)(0.1M) final concentration 20pmol/μl and polynucleotide kinase enzyme (500U) at 37°c for 30 minutes. Then Polynucleotide

kinase enzyme was heat inactivated. Finally, chloroform : phenol extraction was done for purification.DNA was quntified by runing on 1% agarose gel(150ng).

- **Ligation**: Ligation was done as per following reaction. Firstly, vector: insert ratio was calculated by following formulae:

  ng of vector × length of insert (in Kb) / size of vector in Kb×molar ration of insert/vector = ng of insert.

Table: 6. Control reaction:

Reagents	**Volume taken**	**Final con$^{c.}$**
Vector (pBSIISK/*E.coRI*) 150ng/µl	1µl	15ng/µl
T4 ligase buffer(10x)	2µl	1x
T4 DNA Ligase (5u/µl)	1µl	0.25u/µl
Milli Q water	16µl	----
Total	20µl	----

Table: 7. IRF-1/vector (1:1) ratio:

Reagents	**Volume taken**	**Final conc.**
Vector (pBSIISK/E.coRI) 150ng/µl	1µl	15ng/µl
PCR amplifiedIRF-1(81ng/µl)	1µl	4.2ng/µl
T4 ligase buffer(10x)	2µl	1x
T4 DNA Ligase (5u/µl)	1µl	0.25u/µl
Milli Q water	16µL	----
Total	20µl	----

Table: 8.IRF-1/vector (1:3) ratio:

Reagents	Volume taken	Final con[c.]
Vector (pBSIISK/*E.coRI*) 150ng/µl	1µl	15ng/µl
PCR amplified IRF-1(27ng/µl)	1µl	1.4ng/µl
T4 ligase buffer (10x)	2µl	1x
T4 DNA Ligase (5U/µl)	1µl	0.25u/µl
Milli Q water	15µL	----
Total	20µl	----

Table: 9. IRF-2/vector (1:1) ratio:

Reagents	Volume taken	Final con[c.]
Vector (pBSIISK/*E.coRI*) 150ng/µl	1µl	15ng/µl
PCR amplified IRF-2 (81ng/µl)	1µl	4.2ng/µl
T4 ligase buffer(10x)	2µl	1x
T4 DNA Ligase (5u/µl)	1µl	0.25u/µl
Milli Q water	15µL	----
Total	20µl	----

Table: 10.IRF-2/vector (1:3):

Reagents	Volume taken	Final con$^{c.}$
Vector (pBSIISK/*Eco RI*) 150ng/µl	1µl	15ng/µl
PCR amplified IRF-2 (28ng/µl)	1µl	4.2ng/µl
T4 ligase buffer(10x)	2µl	1x
T4 DNA Ligase (5u/µl)	1µl	0.25u/µl
Milli Q water	15µl	----
Total	20µl	----

Ligation reaction was then placed in water bath at16°c for 18-hrs. Next day transformation was performed.

Transformation of ligation reactions: In order to sub-clone lgation reactions were transformed in XL1-blue *E.coli* competent cells.

Table: 11.

Vector transformation mix:

Competent cell (*XL-1Blue*)	200µl
Lgation mix (vector)	1µl
TCM	2µl

IRF-1/vector (1:1):

Competent cell (*XL-1Blue*)	200µl
Lgation mix (vector/IRF-1)	1µl
TCM	2µl

IRF-1/vector (1:3)

Competent cell (*XL-1Blue*)	200µl
Lgation mix (vector/IRF-1)	1µl
TCM	2µl

IRF-2/vector (1:1)

Competent cell (*XL-1Blue*)	200µl
Lgation mix (vector/IRF-2)	1µl
TCM	2µl

IRF-2/vector (1:3):

Competent cell (*XL-1Blue*)	200µl
Lgation mix (vector/IRF-2)	1µl
TCM	2µl

Then heat shock was given at 42°c for 90 seconds, immediately placed on ice for 5 minutes. Then 800µl LB was added and transfomation mix were put in the incubator shaker 37°c for 1 hour. Finally, all cells plated on the X-gal/IPTG plate.For X-gal IPTG plate preparation, 20µl to each plate (stock 20mg/ml in DMF) was mixed with 8µl IPTG (1M) to each plate. Subsequently, 47µl X-gal/IPTG mix was spreaded on LB ampicillin plate,allowed the plate to dry to 45 minutes. Transfomant cells were plated on plate and incubated at 37°c in incubator.

Next day, blue and white colonies were counted.(fig.10).

Table: 12. Colony counting:

S.No.	Samples	Ligation /transformation	Total no. of colonies	No.of white colonies	No.of blue colonies
1.	pBSIISK / *Eco RI* vector		25	----	25
2.	pBSIISK / *Eco RI* /IRF-1(1:1)	1/10 th	70	10	60
3.	pBSIISK / *Eco RI* /IRF-1 (1:3)	1/10 th	105	12	117
4.	pBSIISK / *Eco RI* /IRF-2(1:1)	1/10 th	82	08	74
5.	pBSIISK / *Eco RI* /IRF-2 (1:3)	1/10 th	132	16	116

5.3.9.Construction of GST-IRF-2 expression clones

(A) pGEX-2TK -IRF-2 construct

Mouse IRF-2 complete coding sequence (1051 bp) was obtained by PCR from pIRF-2-5 plasmid (accession no. J03168, ref. NCBI). PCR primers were as follows:

P1: 5'AAGGATCCATGCCGGTGGAACGGATGCGA 3'

P2: 5'AAGGATCCTTAACAGCTCTTGACACGGGC 3' with Bam HI overhangs at both ends. *pfu* DNA polymerase was used to avoid any error during amplification. The gel purified IRF-2 cDNA fragment was ligated into pBluescript vector at *Eco RI* gap filled site, blue/white selection in presence of X-Gal and IPTG was carried out to pick white colonies. The Bam HI insert from the Bluescript-IRF-2clone was then subcloned into Bam HI site of pGEX-2TK expression vector (Pharmacia) in *E.coli.* XL-1 Blue cells, to obtain the pGEX-IRF-2 plasmid for expressing GST-IRF-2.

5.3.10.Expression of GST-IRF-2

(A) Induction of recombinant murine IRF-2

Expression of GST-IRF-1 fusion protein was carried out by standard method (Sambrook *et al.*, 1989) with certain modifications. 5 ml LB medium containing ampicillin (100 μg/ml) was inoculated with a single colony of pGEX-IRF-2 /*E.coli* XL-1 and grown overnight at 37 ^{0}C. 100 μl of the overnight grown culture was used to inoculate another 10 ml LB with appropriate ampicillin and grown for 3-4 hrs at 37^{0}C until $O.D_{550\ nm}$ reached between 0.6/ml and 0.8/ml. IPTG (0.5 mM) induction was carried out at 37^{0}C for 3 hrs along with the control (*E.coli* XL-1 Blue cells containing pGEX-2TK vector). Extracts from equal number of cells (~1.0 $O.D_{550\ nm}$) was prepared and used to assess GST-IRF-2 expression. Cell pellet from 1.0 O.D. volume of each culture was resuspended in 150 μl of water and 50 μl of 4x loading dye (0.06M Tris.Cl, pH.8.0, 2% SDS, 10% Glycerol, 0.025% Bromophenol blue) and subjected to boiling in a water bath at 95^{0}C for 10 min. The samples were given a spin at 10K rpm, RT for 30 sec and resolved in 10% SDS-PAGE at 100V for 6 hrs. The gel was stained with Coomassie Brilliant Blue R250. Several colonies were checked to finally selected one (2.3) pGEX-IRF-2 clones, which expressed GST-IRF-2.

(B) Sodium dodecyl sulphate polyacrylamide gel electrophoresis. (SDS-PAGE)

SDS-PAGE gels were prepared as per Laemeli's method (Laemali, 1970). 30% stock solution of degassed acrylamide was mixed with 1.5 M Tris.Cl pH 8.8, water and 10% SDS. The solution was swirled gently to avoid the formation of bubbles and quickly added freshly prepared 10% APS and TEMED. All ingredients were added as per the requirements of a 10% acrylamide gel. The solution was poured into sealed glass plates. A layer of Isopropanol was poured on the resolving gel and the gel allowed polymerizing at room temperature for one hour. The isopropanol layer was removed completely and the stacking gel which should be atleast 1 cm below the bottom of the comb was poured. The stacking gel was allowed to polymerize for an hour. The wells were washed carefully after removing the comb to remove any traces of unpolymerised acrylamide. The gel was placed in the vertical gel apparatus, ensuring that there were no air-bubbles trapped between the agarose and the buffer at the bottom of the gel. 60 µl of the each protein sample was loaded into one well and electrophoresed in 1 X Tris-glycine SDS Buffer at 100V for 6 hrs. or 50 V overnight.

(C) Fixing and staining of SDS-PAGE gels using Coomassie Brilliant Blue R-250

Polypeptides resolved in SDS-PAGE gels can be simultaneously fixed with 50% (v/v) methanol, 10% (v/v) acetic acid and stained with Coomassie Brilliant Blue R-250[1](Merril *et al.*, 1979; Switzer *et al.*, 1979). The gels was immersed overnight in 5 times its vol. of Coomassie Brilliant Blue R-250 with slow agitation. The stain was removed and kept for future use. Soaking and shaking it in 50% (v/v) methanol, 10% (v/v) acetic acid, changing the solution till the background became clear, destained the gel. The next day the gel was stored in 20% glycerol.

RESULTS

6. RESULTS:

- ***PCR amplification of IRF-2:***

To obtain sufficient amount of pIRF2-5 plasmid DNA for cloning purpose *E.coli* competent cells (MC1061/p3) were transformed with above plasmid. Transformants were selected on antibiotic plates, isolated single colony was picked up and inoculated in LB broth plasmid isolation was done and quantity was estimated on 0.8 % agarose gel in presence of Ethidium bromide.

To facilitate subcloning in expression plasmid pGEX2TK (at Bam HI site), Bam HI was introduced on the either end of the IRF-2 gene by PCR. The oligonucleotide primers used for this purpose are mentioned in table 1. The PCR done with the above mentioned primed pair generated sufficient amount of IRF-2 amplicon (with Bam HI sequence at end) was 1063 bp (fig.9).

- ***Cloning of PCR amplified IRF-2:***

PCR amplified IRF-2 was gap filled by Klenow fragment as overhang may be generated by Taq polymerase enzyme. The IRF-2 amplicon was then phosphorylated at both 5' terminus using T4 Polynucleotide kinase. Vector pBSIISK was cut with *EcoRI, gap* filled and dephosphorylated with Calf Intestinal alkaline Phosphatase (CIAP). The vector and insert DNA was estimated on 1% agarose gel electrophoresis along with molecular weight standards of known concentration. IRF-2 fragment was ligated in CIP treated pBSIISK vector in 1:1 and 1:3 molar ratio. 20 µl ligation reactions were carried out the conditions and constituents are given in Table 6,7,8,9 and 10.

- ***Selection of IRF-2 transformed clones:***

After transformation of ligation mixture of different ratio 1:1 and 1:3 were plated on X-gal/IPTG plates. Cells were allowed to grow overnight at 37°C. Next day, plates were placed at 4°C in order to develop colour (fig.10). I got 8 colonies out of 82 colony .The white colonies were selected for mini-preparation. The colonies were assigned as 2.1,2.2,2.3 and 2.5. Plasmid isolation was done and checked on 0.8% agarose gel (fig.11). To confirm colonies possessing recombinant plasmid, restriction digestion of isolated plasmid with Bam HI was performed and digestion mixture was checked on 8% agarose gel (fig.12).

- ***Nucleotide and protein sequences of IRF-2:***

LOCUS IRF-2 2435 bp mRNA linear

DEFINITION Mus musculus interferon regulatory factor 2 (Irf2), mRNA.

ACCESSION NM_008391

VERSION NM_008391.1 GI:6680468

SOURCE Mus musculus (house mouse)

ORGANISM Mus musculus

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;

Mammalia; Eutheria; Rodentia; Sciurognathi; Muridae; Murinae; Mus.

REFERENCE 1 (bases 1 to 2435)

AUTHORS Harada,H., Fujita,T., Miyamoto,M., Kimura,Y., Maruyama,M., Furia,A., Miyata,T. and Taniguchi,T.

TITLE Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes

JOURNAL Cell 58 (4), 729-739 (1989)

NCBI review. The reference sequence was derived from J03168.1.

Protein sequences

"MPVERMRMRPWLEEQINSNTIPGLKWLNKEKKIFQIPWMHAARHGWDVEK
DAPLFRNWAIHTGKHQPGIDKPDPKTWKANFRCAMNSLPDIEEVKDRSIKKG
NNAFRVYRMLPLSERPSKKGKKPKTEKEERVKHIKQEPVESSLGLSNGVSGFS
PEYAVLTSAIKNEVDSTVNIIVVGQSHLDSNIEDQEIVTNPPDICQVVEVTTESD
DQPVSMSELYPLQISPVSSYAESETTDSVASDEENAEGRPHWRKRSIEGKQYL
SNMGTRNTYLLPSMATFVTSNKPDLQVTIKEDSCPMPYNSSWPPFTDLPLPAP
VTPTPSSSRPDRETRASVIKKTSDITQARVKSC"

IRF; Region: Interferon regulatory factor transcription factor. This family of transcription factors is important in the regulation of Interferons in response to infection by virus and in the regulation of interferon-inducible genes. Three of the five conserved tryptophan residues bind to DNA"

BASE COUNT 681 a 630 c 508 g 616 t

```
   1 tctcaggcaa gccggggact aacttttagt tttgctcctg cgattattca actgacgggc
 161 tttcatttcc attttacaca ccctaacaac actcacacct tgcgggattg tattggtagc
 121 gtggaaaaaa aaaaagcaca ttgagagggt accATGccgg tggaacggat gcgaatgcgc
 181 ccgtggctgg aggagcagat aaattccaat acgataccag ggctaaagtg gctgaacaag
 241 gagaagaaga ttttccagat cccctggatg catgcggctc ggcacggatg ggacgtggaa
 301 aaggatgctc cgctcttcag aaactgggcg atccatacag gaaagcatca accaggaata
 361 gataaaccag atccaaaaac atggaaagca aattttcgat gtgccatgaa ttccctgccc
 421 gacattgagg aagtgaagga cagaagcata aagaaaggaa acaacgcctt cagagtctac
 481 cggatgctgc ccttatccga acgaccttcc aagaaaggaa agaaaccaaa gacagaaaaa
 541 gaagagagag ttaagcacat caagcaagaa ccagttgagt catctttggg gcttagtaat
 601 ggagtaagtg gcttttctcc tgagtatgcg gtcctgactt cagctataaa aaatgaagtg
 661 gatagtacgg tgaacatcat agttgtagga cagtcccatc tggacagcaa cattgaagat
 721 caagagatcg tcactaaccc gccagacatc tgccaggttg tagaagtgac cactgagagt
 781 gatgaccagc cagtcagcat gagtgagctc taccctctac agatttctcc tgtgtcttcc
 841 tacgcagaaa gcgaaactac cgacagtgtg gccagtgatg aagagaacgc agaggggaga
 901 ccacactgga ggaagaggag catcgaaggc aagcagtacc tcagcaacat ggggacacgg
 961 aacacctatc tgctgcccag catggcgacc tttgtcacct ccaacaagcc agatctgcag
1021 gtcaccatca aagaggatag cTGTccgatg ccttacaaca gctcctggcc cccatttaca
1081 gaccttcccc ttcctgcccc agtgaccccc acgcccagca gcagtcggcc agaccgggag
1141 acccgggcca gtgtcatcaa gaagacatct gatatcaccc aggcccgtgt caagagctgt
1201 taagcctttg actctccctg gtggttgttg ggatttctta gctttgtgtt gttctttgtt
1261 tgtattatat tatttttttt ctctatgata cctatcttag acacatctaa gggagaaagc
1321 cttgacgata gattattgat tgctgtgtcc aactccagag ctggagcttc ttcttaactc
1381 aggactccag ccccccccc ccctcggtag atgcgtatct ctagaacctg ctggatctgc
1441 cagggctact ccctcaagtt caaggaccaa cagccacacg ggcagtggag gtgctgcgtt
1501 gcctacggtc aaggccagca tggtggagtg gatgcctcag aacggaggag aaaatgtgaa
1561 ctagctggaa ttttttttatt cttgtgaata tgtacatagg cagtacgagc aatgtcgcgg
1621 gctgcttctg caccttatct tgaagcactt acaataggcc ttcttgtaat cttgctctcc
1681 ttcacagcac actcggcgac cccttctgtg tccactaccc cactacccac ccctccctcc
1741 tcaacccctc catcccggtc ctctatgcgc cccttccccc caaccaatcc catcacaacc
1801 tcttacctat cctttccctc ccaacccctt ctatcccagc ccaccaccta ccccactcct
1861 ccccaactcc tccattctag cccattaccc acgcctctct cctcagccca gcctacccca
1921 tcccaccctg ttcctttcct ccagtttcct ctcctcaaag gcaaggctct acatcttgga
1981 ggaggaggag gagaagaaaa tgagtttctt caccgctgtc ccattttaag actgcttgaa
2041 taataaaaaa aaatctttct aatctgctat gcttgaatgg cacgcggtac aaaggaaaac
2101 tgtcatggaa atattatgca aattcccaga tctgaagacg gaaaatactc taattctaac
2161 cagagcaagc ttttttattt ttttatacaa ggggaatatt ttattcaagg taaaaaaatt
2221 ctaaataaaa tataattgtt ttttatcttt tctacagcaa atttataatt ttaagattcc
2281 ttttcctgtt catcagcagt tgttattaca tcccttgtgg cacatttttt ttttaatttt
2341 gtaaaggtga aaaaaaaact tttatgagct catgtagcaa tcaaattatc ctgtggattg
2401 ataataaatg aatatggtat atagttaaag atttt.
```

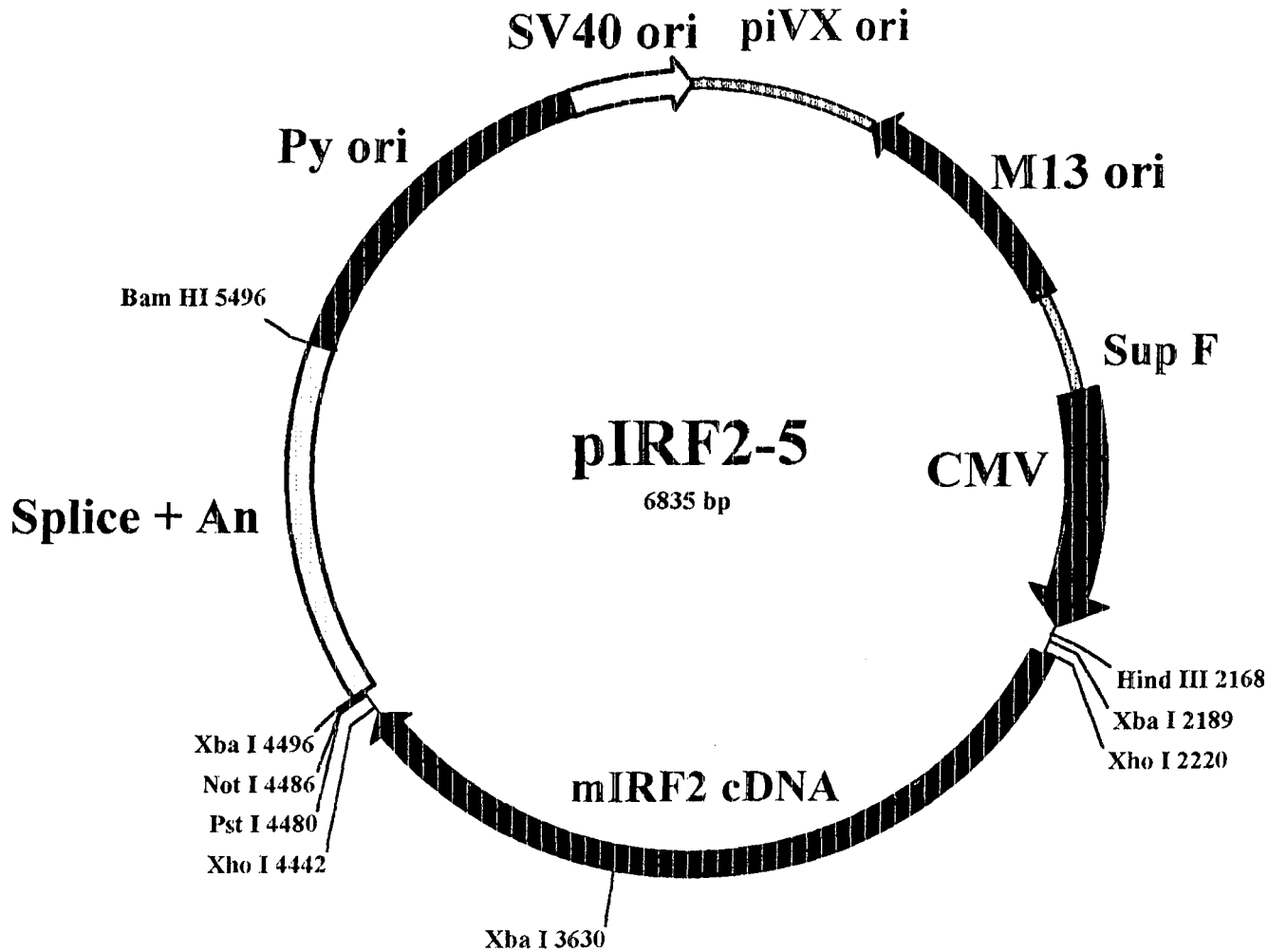


Fig. 8. Map of pIRF 2-5 plasmid

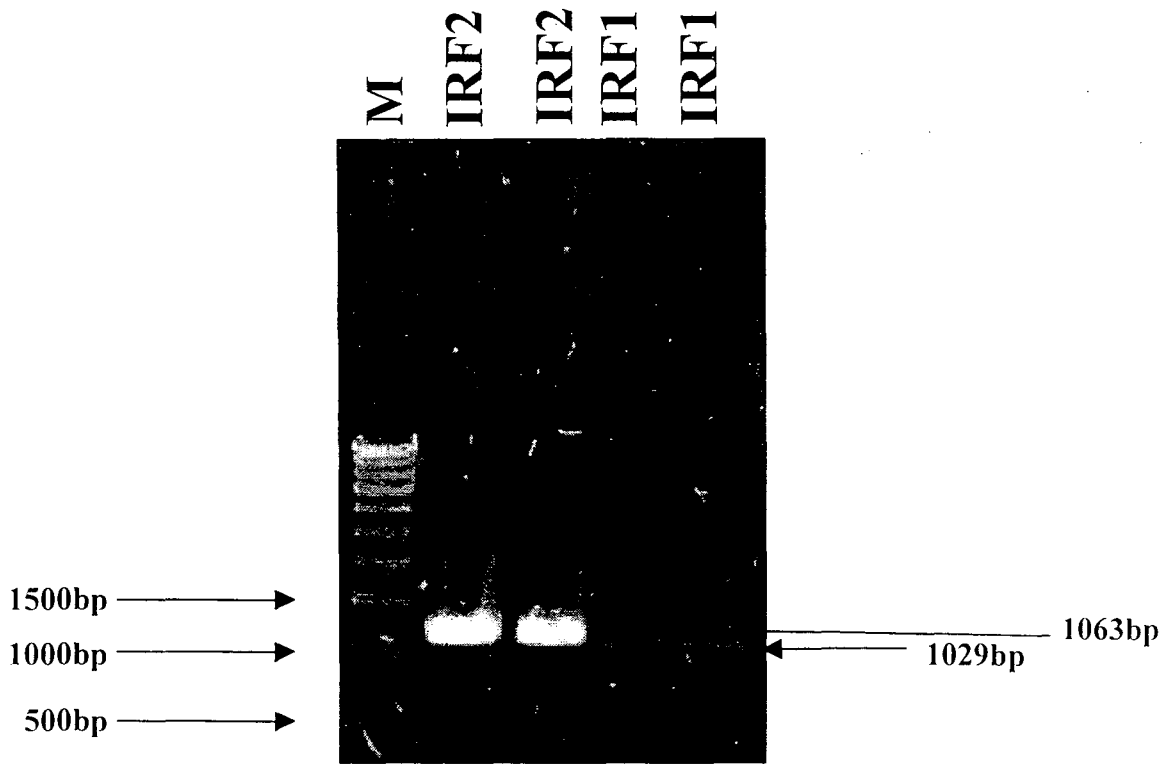
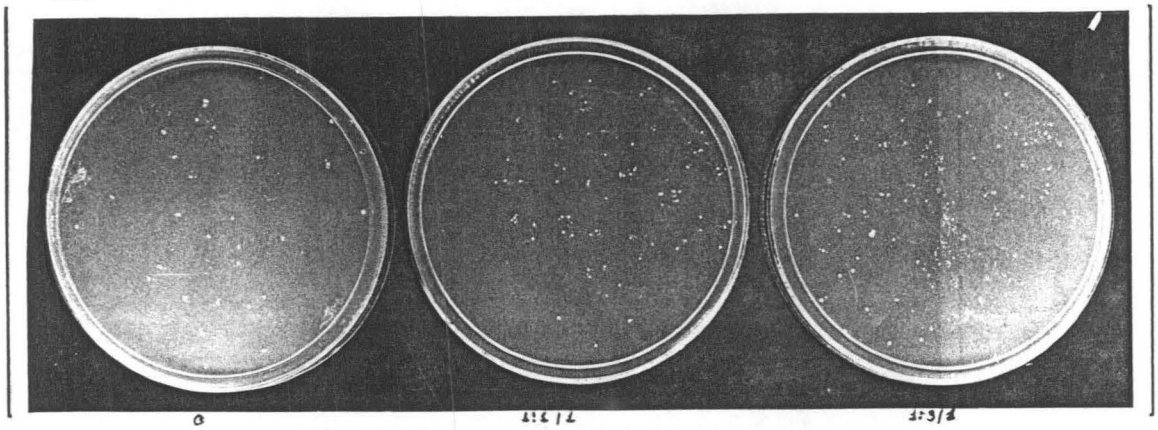


Fig. 9. PCR amplification of IRF-2 and IRF-1 amplicons from IRF-2 and IRF-1 cDNAs.

A.



B.

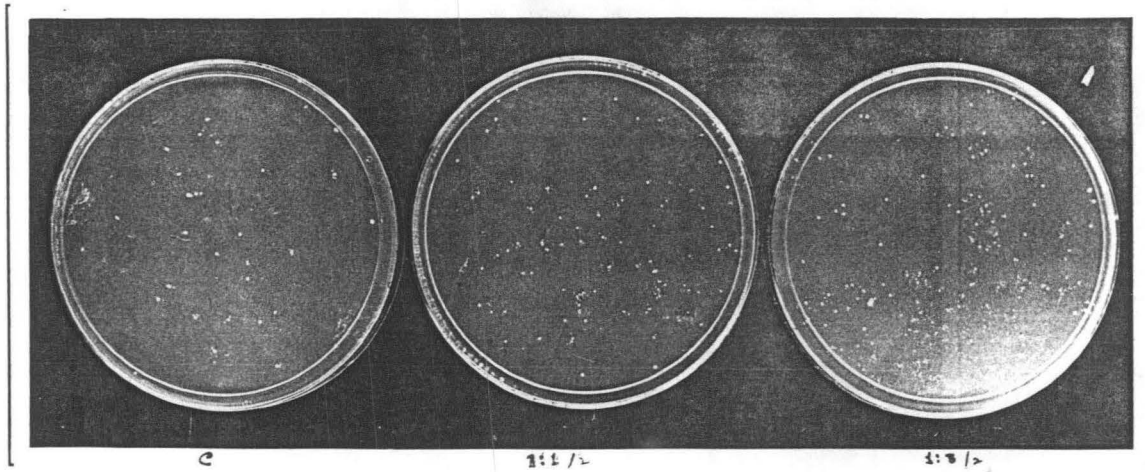


Fig. 10. Agar plates showing transformed colonies with ligation mixture from pBSIISK/*EcoRI*:IRF-1 (A) and pBSIISK/ *EcoRI* :IRF-2 (B).

Ligation reactions:

A. 1. Control plate: pBSIISK/*Eco RI* vector.
2. pBSIISK/*Eco RI* : IRF-1 fragment :: 1:1
3. pBSIISK/*Eco RI* : IRF-1 fragment :: 1:3

B. 1. Control plate: pBSIISK/*Eco RI* vector.
2. pBSIISK/*Eco RI* : IRF-2 fragment :: 1:1
3. pBSIISK/*Eco RI* : IRF-2 fragment :: 1:3

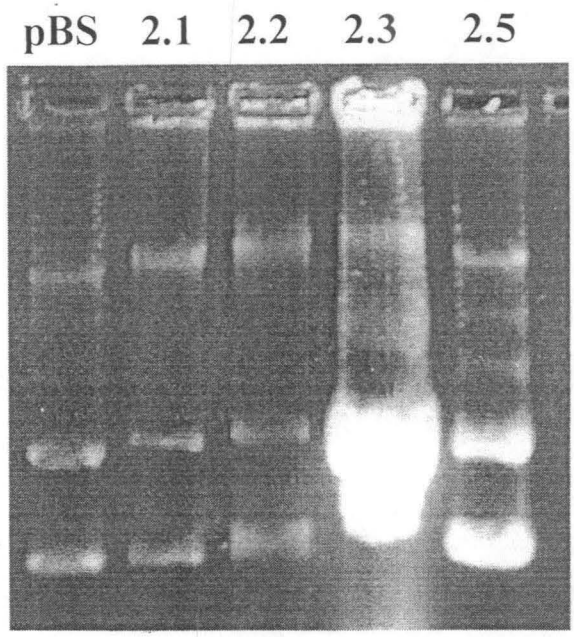


Fig. 11. Ethidium Bromide stained gel showing plasmids picked from plates transformation of the mixture (figure 10).
pBS from control plate.
2.1 form pBS/IRF-2 (1:1) ratio plate.
2.2 from pBS/IRF-2 (1:1) ratio plate.
2.3 from pBS/IRF-2 (1:3) ratio plate.
2.5 from pBS/IRF-2 (1:3) ratio plate.

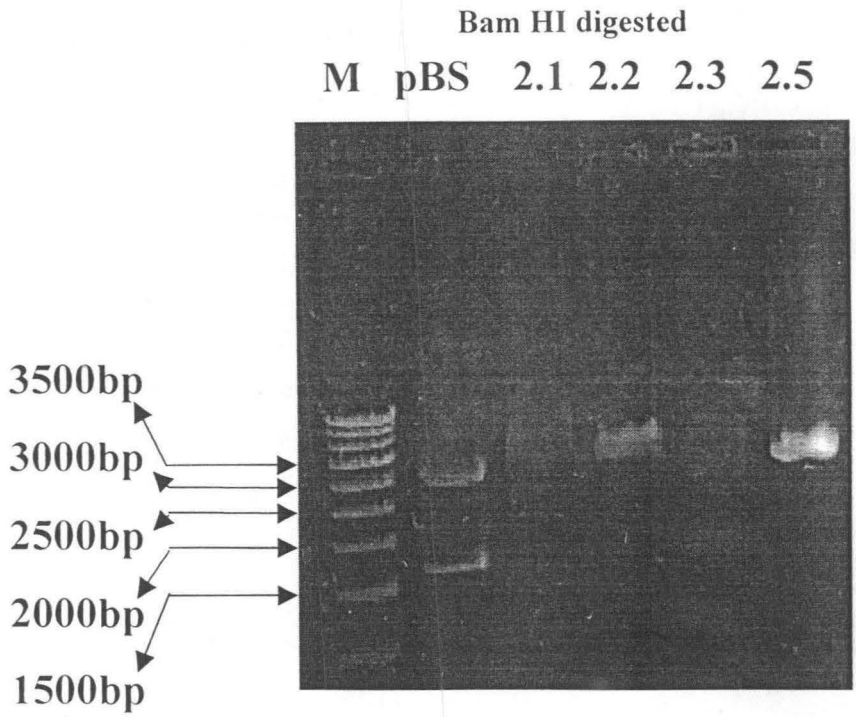


Fig.12.Restriction digestion (Bam HI) of the plasmid DNA of probable clones(2.1,2.2,2.3,2.5).

DISCUSSION

7. DISCUSSION:

A cDNA encoding a molecule structurally related to IRF-1 was isolated by cross hybridization with cDNA of IRF-1, and the molecule termed IRF-2. (Harada *et al.*, 1989). By further study IRF-2 structure showed 62% homology at N-terminal with IRF-1, spanning the first 154 amino acids residues, where as rest of the molecule showed 25% homology. DNA binding site selection studies revealed that IRF-2 bind to the DNA element, termed IRF-E (consensus sequence: G(A)AAA^{G}/c^{T}/cGAAA^{G}/c^{T}/c) (Tanaka *et al.*,1993).

Some of the genes that have been identified to be transcriptionally repressed by IRF-2 are: IRF-1 mediated gene expression like Interferon-β, Cyclooxygenase-2 (COX-2), Interleukin (IL)-15, IL-12, MHC class II transactivator (CIITA), p21$^{WAF1/CIP1}$ and Caspase-1.It has been reported that IRF-2 also activates the gene expression some of them are namely Vascular Cell Adhesion Molecule-1 (VCAM-1) (Jesse *et.al.*,1998),H_4 gene of cell cycle (Vaughan *et al.*,1995),gp^{91phox}gene (Luo *et.al.*1996),FasL,Cyclin B,CDK inhibitor protein (Xie *et al.*, 2002*)*.The activity of IRF-2 is modulated by Phosphorylation,acetylation,interaction with coregulatory factors (Birnbaum *et al.*,1997; Masumi *et al.*,2001; Staal *et al* 2000).

Biologicaly, IRF-2 plays an important role in cell growth regulation, and has been shown potential oncogene, as over expression of IRF-2 causes anchorage-dependent growth in NIH 3T3 cell and tumor formation in mice (Harada *et.al.*, 1993). Recently, two novel co-repressor molecules have been recognized namely IRF-2BP1 and IRF-2BP2, nuclear protein and have the properties of IRF-2 dependent transcriptional co-repressor that can inhibit basal transcription in manner that is not dependent upon hasten deacetylation. (Childs *et a.l*, 2003). IRF-2 mRNA levels accumulate in response to virus, double stranded RNA or poly (I:C), IFN-γ, IFN-α, IFN-β, cytokines like TNF-α, IL-1, and other agents in tissues specific manner. An interesting question is how a single transcription factor can be involved in all of these different responses? Function of IRF-2 apparently differs with cell type, presumably due to the presence or absence of cell-type-specific factors that direct IRF-2 to particular set of promoters or that require IRF-2 for their own function (Taniguchi *et al.*, 2000; Bovolenta *et al.*, 1994; Sharf *et al.*, 1995). Secondly, different levels of IRF-2 expression seem to dictate its effect on target gene expression. Posttranslational modification (Sharf *et al.*, 1997; Lin and Hiscott, 1999) and chromatin remodelling by

interacting with Histone acetylases like PCAF (Masumi *et al.*, 1999) or p300/CREB binding protein (CBP) (Yoneyama M *et al.*, 1998) may also influence its function. Despite extensive information regarding its involvement in diverse cellular processes, very little is known about the mechanism (s) by which its structural domains function. IRF-2 is a typical modular protein comprising of individual domains.

The present study has been to express recombinant IRF-2 in *E. Coli.*

- Expression of recombinant mouse IRF-2 as GST-IRF-2 fusion Protein.

We tried to express IRF-2 from the pGEX-2TK vector, a GST-gene fusion vector for inducible, high-level intracellular expression of IRF-2 as a fusion with the 26 kd glutathione S-transferase (GST) from *Schistosoma japonicum*. The pGEX-2TK vector has a *tac* promoter for inducible, high level expression by IPTG; an internal *lac* I^q gene for use in any *E. coli* host; a translational terminator in the reading frame; thrombin recognition site for cleaving the protein from the fusion product and it contains the recognition sequence for the catalytic subunit of cAMP-dependent protein kinase from heart muscle at a site located between the GST domain and the multiple cloning site. The expressed recombinant IRF-2 protein can be directly studied.

CONCLUSION

8. CONCLUSIONS:

The present study on cloning and *in vivo* expression of oncogenic protein IRF-2 comprises of following aspects:

1.PCR amplification of murine IRF-2 cDNA as a 1063bp DNA.

2. Recombinant construction of pBS/IRF-2 for cloning the 1063bp IRF-2 cDNA.

3.Construction of pGEX2TK-IRF-2 for expression of GST-IRF-2 fusion protein in *E.coli.*

Major works done pertaining to above study may be concluded as follows:

- Murine cDNA of IRF-2 (349 a.a.) was amplified by PCR reaction as a 1063bp DNA.
- Murine cDNA of IRF-1 (325 a.a.) was amplified by PCR reaction as a 1029bp DNA.
- Recombinant pBS/IRF-2 (4023 bp) was not obtained.
- Recombinant IRF-2/pGEX2TK (~66 Kd) expression plasmid has to be constructed.

REFERENCES

9. REFERENCES:

1. Au, WC, Yeow, WS, Pitha, PM.2001.Analysis of functional domains of interferon regulatory factor 7 and its association with IRF-3 .*Virology* **280** (2) :273-287
2. Aziz, F., van Wijnen, A. J., Stein, J. L. and Stein, G. S. (1998). HiNF-D (CDP-cut/CDC2/cyclin A/pRB-complex) influences the timing of IRF-2-dependent cell cycle activation of human histone H4 gene transcription at the G1/S phase transition. *J Cell Physiol.* **177**(3): 453-464.
3. Aziz, F., van Wijnen, A. J., Vaughan, P. S., Wu, S., Shakoori, A. R., Lian, J. B., Soprano, K. J., Stein, J. L. and Stein, G. S. (1998). The integrated activities of IRF-2 (HiNF-M), CDP/cut (HiNF-D) and H4TF-2 (HiNF-P) regulate transcription of a cell cycle controlled human histone H4 gene: mechanistic differences between distinct H4 genes. *Mol Biol Rep.* **25**(1):1-12.
4. Behera AK, Kumar M, Lockey RF, Mohapatra SS. (2002). 2'-5' Oligoadenylate synthetase plays a critical role in interferon-gamma inhibition of respiratory syncytial virus infection of human epithelial cells. *J. Biol. Chem.* **277**(28): 25601-8.
5. Birnbaum, M. J., Zundert, B., Vaughan, P. S., Whitmarsh, A. J., van, Wijnen, A. J., Davis, R. J., Stein, G. S. and Stein, J. L. (1997). Phosphorylation of the oncogenic transcription factor interferon regulatory factor 2 (IRF2) in vitro and in vivo. *J Cell Biochem.* **66**(2):175-183.
6. Blanco JC, Contursi C, Salkowski CA, DeWitt DL, Ozato K, Vogel SN. (2000). Jun.Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon gamma-dependent cyclooxygenase 2 expression. *J. Exp. Med.* **191**(12):2131-44.
7. Blanco, C., Salkowski, C. A., DeWitt, D. L., Ozato. and Vogel, K. (2000). Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon gamma-dependent cyclooxygenase 2 expressions. *J. Exp. Med.* **191**(12): 2131-2144.
8. Bovolenta, C., Driggers, P, H., Marks, M, S., Medin, J, A., Politis, A,D., Vogel, S, N., Levy, D, E., Sakaguchi, K.,Appella, E. and Coligan, J, E.(1994) Molecular interactions between interferon consensus sequence binding protein and members of the interferon regulatory factor family. *Proc. Natl.Acad. Sci. U S A.* **91**(11): 5046-5050.

9. Burysek L, Pitha PM. (2001) Mar.Latently expressed human herpesvirus 8-encoded interferon regulatory factor 2 inhibits double-stranded RNA-activated protein kinase. *J. Virol.*; 75(5):2345-52.

10. Burysek L, Yeow WS, Lubyova B, Kellum M, Schafer SL, Huang YQ, Pitha PM. (1999). Sep. Functional analysis of human herpesvirus 8-encoded viral interferon regulatory factor 1 and its association with cellular interferon regulatory factors and p300. *J. Virol.* 73(9): 7334-42.

11. Burysek L, Yeow WS, Pitha PM.(1999). Unique properties of a second human herpesvirus 8-encoded interferon regulatory factor (vIRF-2). *J. Hum. Virol.*;2(1):19-32.

12. Childs KS, Goodbourn S. (2003). Identification of novel co-repressor molecules for Interferon Regulatory Factor-2. *Nucleic Acids Res.* 31(12):3016-26.

13. Choi Y, Johnson GA, Burghardt RC, Berghman LR, Joyce MM, Taylor KM, StewartMD, Bazer FW, Spencer TE. (2001). Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. *Biol Reprod.*; 65(4):1038-49.

14. Chow WA, Fang JJ, Yee JK. (2000). The IFN regulatory factor family participates in regulation of Fas ligand gene expression in T cells. *J. Immunol.* 164(7): 3512-8.

15. Coccia EM, Del Russo N, Stellacci E, Orsatti R, Benedetti E, Marziali G, Hiscott J, Battistini A.(1999). Activation and repression of the 2-5A synthetase and p21 gene promoters by IRF-1 and IRF-2.*Oncogene.* 18(12): 2129-37.

16. Coccia EM, Stellacci E, Valtieri M, Masella B, Feccia T, Marziali G, Hiscott J, Testa U, Peschle C, Battistini A.(2001). Ectopic expression of interferon regulatory factor-1 potentiates granulocytic differentiation. *Biochem J.* 360(2): 285-94.

17. Cuesta N, Salkowski CA, Thomas KE, Vogel SN. (2003). Regulation of lipopolysaccharide sensitivity by IFN regulatory factor-2. *J. Immunol.*170 (11): 5739-47.

18. Darnell, J. E., Kerr, I. M. and Stark G. R. (1994). JAK-STAT pathways and transcriptional activation in response to IFNs and other extracellular singling proteins. *Science* 264:1415-1421.

19. Doherty GM, Boucher L, Sorenson K, Lowney J. (2001). Interferon regulatory factor expression in human breast cancer. *Ann Surg.* 233(5): 623-9.

20. Doherty, G. M., Boucher, L., Sorenson, K.and Lowney, J. (2001) Interferon regulatory factor expression in human breast cancer. *Ann Surg.* **233**(5): 623-9.

21. Du MX, Sotero-Esteva WD, Taylor MW. (2000). Analysis of transcription factors regulating induction of indoleamine 2,3-dioxygenase by IFN-gamma. *J Interferon Cytokine Res.* **20**(2): 133-42.

22. Elser B, Lohoff M, Kock S, Giaisi M, Kirchhoff S, Krammer PH, Li-Weber M. (2002). IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. *Immunity.* **17**(6): 703-12.

23. Escalante, C. R., Yie, j., Aggarwal, A. K. and Thonos, D. (1998). Structure of IRF-1 with bound DNA reveals determinants of IFN regulation. *Nature.* **391**:103-106.

24. Escalante, C. R.1998 Structure of IRF-1 with Bound DNA Reveals Determinants of Interferon Regulation, *Nature*, **391**, pp.103.

25. Falvo JV, Parekh BS, Lin CH, Fraenkel E, Maniatis T. (2000). Assembly of a functional beta interferon enhanceosome is dependent on ATF-2-c-jun heterodimer orientation. *Mol Cell Biol.* **20**(13): 4814-25.

26. Fan, C, M., and Maniatis, T. (1989) Two different virus inducible elements are required for human IFN gene regulation. *EMBO J.* **8**:101-110.

27. Feng X, Petraglia AL, Chen M, Byskosh PV, Boos MD, Reder AT. (2002). Low expression of interferon-stimulated genes in active multiple sclerosis islinked to subnormal phosphorylation of STAT1. *J Neuroimmunol.* **129**(1-2): 205-15.

28. Fuji, Y.1999.Crystal Structure of an IRF-DNA Complex Reveals Novel DNA Recognition and Cooperative Binding to a Tandem Repeat of Core Sequence, *EMBO J.*, **18**, pp.5028.

29. Fujii J, Uegaki K, Yamazaki T, Shirakawa M, Swindells MB, Harada H, Taniguchi T, Kyogoku Y. (1998) Solution structure of the IRF-2 DNA-binding domain: a novel subgroup of the winged helix-turn-helix family. Structure. 6(4): 491-500.

30. Fujii Y, Shimizu T, Kusumoto M, Kyogoku Y, Taniguchi T, Hakoshima T. (1999). Crystal structure of an IRF-DNA complex reveals novel DNA recognition and cooperative binding to a tandem repeat of core sequences. *EMBO J.* **18**(18): 5028-41.

31. Fujii, Y., Shimizu, T., Kusumoto, M., Kyogoku, Y., Taniguchi, T. and Hakoshima, T. (1999) Crystal structure of an IRF-DNA complex reveals novel

DNA recognition and cooperative binding to a tandem repeat of core sequences. *EMBO J* **18**(18): 5028-5041.

32. Fujita, T. Kimura, Y., Miyamoto, M. and Taniguchi, T.(1989) Induction of endogenous IFNα and IFNβ genes by a regulatory transcription factors IRF-1 .*Nature* **337**:270-272.
33. Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, Furia A, Miyata T and Taniguchi T. (1989) Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell.* **58**(4): 729-739.
34. Harada H, Kitagawa M, Tanaka N, Yamamoto H, Harada K, Ishihara M, Taniguchi T. (1993) Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1and -2.*Science*. **259**(5097): 971-974.
35. Hida S, Ogasawara K, Sato K, Abe M, Takayanagi H, Yokochi T, Sato T, Hirose S, Shirai T, Taki S, Taniguchi T. (2000). CD8(+) T cell-mediated skin disease in mice lacking IRF-2, the transcriptional attenuator of interferon-alpha/beta signaling.*Immunity*. **13**(5): 643-55.
36. Hiscott J, Pitha P, Genin P, Nguyen H, Heylbroeck C, Mamane Y, Algarte M, Lin R. 1999.Triggering the interferon response: the role of IRF-3 transcription factor. *J. Interferon Cytokine Res.* **19**: 1-14.
37. Itoh, S., Harada, H., Fujita, T., Mimura, T. and Taniguchi, T. (1989) Nucleotide, Protein Sequence of a cDNA coding for human IRF-2. *Nucleic Acids Res.* **17**(20): 8372.
38. Iwase S, Furukawa Y, Kikuchi J, Saito S, Nakamura M, Nakayama R, Horiguchi-Yamada J, Yamada H. (1999). Defective binding of IRFs to the initiator element of interleukin-1beta-converting enzyme (ICE) promoter in an interferon-resistant Daudi subline. *FEBS Lett.* **450**(3): 263-7.
39. Jesse, T. L., Lachance, R. and Ladermann, M. F. (1998) IRF-2 is a transcriptional activator inmuscle where it regulates expression of Vascular and Cell Adhesion Molecule-1 (VCAM-1). *J.Cell.Biol* **140**:1265-76.
40. Juang YT, Lowther W, Kellum M, Au WC, Lin R, Hiscott J, Pitha PM.1998.Primary activation of IFNa and IFN§ gene expression by IRF-3. *Proc. Nat. Acad. Sci.* **95**: 9837-9842.

41. Juang, Y. T., Lowther, W., Kellum, M., Au, W. C., Lin, R., Hiscott, J., and Pitha, P.M. 1998.Primary activation of interferon A and interferon B gene transcription by interferon regulatory factor 3. *Proc. Natl. Acad. Sci. USA* **95**, 9837.

42. Kamijo, R., Harada, H. and Matamyame, T. (1994) Requirement for transcription factor IRF-1 in Nitric oxide synthetase induction in Macrophages. *Science* **263**:1612-1615.

43. Kim, T., Kim, T.Y., Song, Y.-H., Min, I.M., Yim, J., and Kim, T.K.1999.Activation of IRF3 in response to DNA damaging agents. *J. Biol. Chem.* **274**: 30686.

44. Kirchhoff S, Oumard A, Nourbakhsh M, Levi BZ, Hauser H. (2000). Interplay between repressing and activating domains defines the transcriptional activity of IRF-1. *Eur J Biochem.* **267**(23): 6753-61.

45. Ko J, Gendron-Fitzpatrick A, Splitter GA. (2002). Susceptibility of IFN regulatory factor-1 and IFN consensus sequence binding protein-deficient mice to brucellosis. *J Immunol.* **168**(5): 2433-40.

46. Koenig Merediz SA, Schmidt M, Hoppe GJ, Alfken J, Meraro D, Levi BZ, Neubauer A, Wittig B. (2000). Cloning of an interferon regulatory factor 2 isoform with different regulatory ability. *Nucleic Acids Res.* **28**(21): 4219-24.

47. Kroger, A., Koster, M., Schroeder, K., and Mueller, P.P.(2002) Activities of IRF-1 . *J.I.C.R.* **22**:5-14.

48. Lee PT, Holt PG, McWilliam AS. (2001). Failure of MHC class II expression in neonatal alveolar macrophages: potential role of class II transactivator. *Eur J Immunol.* **31**(8): 2347-56.

49. Li W, Nagineni CN, Efiok B, Chepelinsky AB, Egwuagu CE. (1999). Interferon regulatory transcription factors are constitutively expressed and spatially regulated in the mouse lens. *Dev Biol.* **210**(1): 44-55.

50. Lian RH and Kumar V. (2002). Murine natural killer cell progenitors and their requirements for development. *Semin. Immunol.* **14**(6): 453-60. Review.

51. Lin R and Hiscott J. (1999). A role for casein kinase II phosphorylation in the regulation of IRF-1transcriptional activity. *Mol Cell Biochem.* **191**(1-2): 169-80.

52. Lin R, Heylbroeck C, Genin P, Pitha PM, Hiscott J.1999.Essential role of IRF-3 in direct activation of RANTES gene transcription. *Mol Cell Biol.* **19**: 959-966.

53. Lin RT, Mamane Y, Hiscott J Structural and functional analysis of interferonregulatory factor 3: localization of the transactivation and autoinhibitory domains Molecular and cellular biology **19**(4): 2465-2474 1999

54. Lin, R. and Hiscott, J. (1999) A role of casein kinase II phosphorylation in the regulation of IRF-1 transcriptional activity. *Mol.Cell.Biochem.* **191**: 169-180.

55. Lohoff M, Duncan GS, Ferrick D, Mittrucker HW, Bischof S, Prechtl S, Rollinghoff M, Schmitt E, Pahl A, Mak TW. (2000). Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1cells. *J. Exp Med.* **192**(3): 325-36.

56. Lohoff, M., Duncan, G. S., Ferrick, D., Mittrucker, H, W., Bischof, S., Prechtl, S., Rollinghoff, M., Schmitt, E., Pahl, A. and Mak, T. W. (2000). Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells. *J Exp Med.* **192**(3): 325-336

57. Lohoff, M., Duncan, G. S., Ferrick, D., Mittrucker, H. W., Bischof, S., Prechtl, S., Rollinghoff, M., Schmitt, E., Pahl, A., and Mak, T. W. (2000) *J. Exp. Med.* **192**, 325-336.

58. Lowney JK, Boucher LD, Swanson PE, Doherty GM. (1999). Interferon regulatory factor-1 and -2 expression in human melanoma specimens. *Ann Surg Oncol.* **6**(6): 604-8.

59. Lu, R., and Pitha, P.M. 2001.Monocyte differentiation to macrophase requires interferon regulatory factor 7, *J. Biol. Chem.*, **276**.

60. Malakhova O, Malakhov M, Hetherington C, Zhang DE. (2002). Lipopolysaccharide activates the expression of ISG15-specific protease UBP43 via interferon regulatory factor 3. *J Biol Chem.* **277**(17): 14703-11. Epub.

61. Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, LePage C, DeLuca C, Kwon H, Lin R, Hiscott J. (1999). Interferon regulatory factors: the next generation. *Gene.* **237**(1): 1-14. Review.

62. Marecki S, Riendeau CJ, Liang MD, Fenton MJ. (2001). PU.1 and multiple IFN regulatory factor proteins synergize to mediate transcriptional activation of the human IL-1 beta gene. *J Immunol.* **166**(11): 6829-38.

63. Masumi A, Ozato K. (2001). Coactivator p300 acetylates the interferon regulatory factor-2 in U937 cells following phorbol ester treatment. *J Biol Chem.* **276**(24): 20973-80. Epub.

64. Masumi A, Yamakawa Y, Fukazawa H, Ozato K, Komuro K. (2003). Interferon regulatory factor-2 regulates cell growth through its acetylation.. *J. Biol Chem.* [Epub ahead of print]

65. Masumi, A. and Ozato, K. (2001) Coactivator p300 acetylates the interferon regulatory factor-2 in U937 cells following phorbol ester treatment. *J Biol Chem.* **276**(24): 20973-20980.

66. Meraro D, Hashmueli S, Koren B, Azriel A, Oumard A, Kirchhoff S, Hauser H, Nagulapalli S, Atchison ML, Levi BZ. (1999). Protein-protein and DNA-protein interactions affect the activity of lymphoid-specific IFN regulatory factors. *J. Immunol.* **163**(12): 6468-78. .

67. Nakao F, Ihara K, Kusuhara K, Sasaki Y, Kinukawa N, Takabayashi A, Nishima S, Hara T. (2001). Association of IFN-gamma and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. *J Allergy Clin Immunol.* **107**(3): 499-504.

68. Nguyen H, Teskey L, Lin R, Hiscott J. (1999). Identification of the secretory leukocyte protease inhibitor (SLPI) as a target.of IRF-1 regulation. *Oncogene.* **18**(39): 5455-63.

69. Nguyen, H., Hiscott, J. and Pitha, P.M. (1997) The growing family of interferon regulatory factors. *Cytokine Growth Factor Rev.* **8**(4): 293-312. Review.

70. Nguyen, H., Hiscott, J., and Pitha, P. M. (1997) *Cytokine Growth Factor Rev.* 8, 293-312.

71. Nishio Y, Noguchi E, Ito S, Ichikawa E, Umebayashi Y, Otsuka F, Arinami T. Genet. (2001). Mutation and association analysis of the interferon regulatory factor 2 gene (IRF2) with atopic dermatitis. *J Hum.* **46**(11): 664-7.

72. Nonkwelo, C., Ruf, I. K. and Sample, J. (1997) Interferon-independent and induced regulation of Epstein-Barr virus EBNA-1 gene transcription in Burkitt lymphoma. *J Virol.***71** (9): 6887-97.

73. Oberg K. (2000). Interferon in the management of neuroendocrine GEP-tumors: a review. *Digestion.*; **62** Suppl 1:92-7. Review.

74. Rubinstein YR, Driggers PH, Ogryzko VV, Thornton AM, Ozato K, Pontzer CH. (2000). Mar.An IFN regulatory factor-2 DNA-binding domain dominant negative mutant exhibits altered cell growth and gene expression. *Oncogene.* **19**(11): 1411-8.

75. Saito H, Morita Y, Fujimoto M, Narazaki M, Naka T, Kishimoto T. (2000). IFN regulatory factor-1-mediated transcriptional activation of mouse STAT-induced STAT inhibitor-1 gene promoter by IFN-gamma. *J Immunol.* **164**(11): 5833-43.

76. Salkowski CA, Kopydlowski K, Blanco J, Cody MJ, McNally R, Vogel SN. (1999). Aug.IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice. *J Immunol.* **163**(3): 1529-36.

77. Schafer SL, Lin RT, Moore PA, Hiscott J, Pitha PM 1998 Regulation of type I interferon gene expression by interferon regulatory factor-3. *Journal of biological chemistry* **273** (5): 2714-2720

78. Sherry B. (2002). The role of interferon regulatory factors in the cardiac response to viral infection. *Viral Immunol.* **15**(1): 17-28. Review.

79. Staal A, Enserink JM, Stein JL, Stein GS, van Wijnen AJ. (2000). Molecular characterization of celtix-1, a bromodomain protein interacting with the transcription factor interferon regulatory factor 2. *J Cell Physiol.* **185**(2): 269-79.

80. Stark, G, R., Kerr, I. M., Williams, B.R.G., Silvermann, R. H. and Schreiber, R, D. (1998) How cells respond to IFNs. *Ann.Rev.Biochem.* **67**:227--264.

81. Taki S. (2002). Type I interferons and autoimmunity: lessons from the clinic and from IRF-2-deficient mice. *Cytokine Growth Factor Rev.* **13**(4-5): 379-91.

82. Tanaka, K., Miyamoto, H., Harada, H., Ishihara, M. and Taniguchi, T. (1993) Recognition DNA sequence of IFN Regulatory Factor -1(IRF-1) and IRF-2, regulatore of cell growth and the IFN system. Mol. *Cell.Biol.***13**: 4531-4538.

83. Taniguchi T, Ogasawara K, Takaoka A, Tanaka N. (2001). IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol.* **19**:623-55. Review.

84. Taniguchi, T. (1989) Regulation of interferon-beta gene: structure and function of cis-elements and trans-acting factors. *J Interferon Res.* **9**(6): 633-640. Review.

85. Taniguchi, T., Ogasawara, K., Takaoka, A. and Tanaka, N. (2001). *Annu. Rev. Immunol.* **19**:623-655.

86. Taniguchi, T., Ogasawara, K., Takaoka, A., and Tanaka, N. (2001) IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol.* **19**:623-655. Review.

87. Van der Fits L, van der Wel LI, Laman JD, Prens EP and Verschuren MC. (2003). Psoriatic lesional skin exhibits an aberrant expression pattern of interferon regulatory factor-2 (IRF-2). *J Pathol.* **199**(1): 107-14.

88. Vaughan, P. S., Meijden, C. M., Aziz, F., Harada, H., Taniguchi, T., van Wijnen, A. J, Stein, J. L. and Stein, G. S. (1998). Cell cycle regulation of histone H4 gene transcription requires the oncogenic factor IRF-2. *J.Biol Chem.* **273**(1): 194-199.

89. Wang IM, Contursi C, Masumi A, Ma X, Trinchieri G, Ozato K. (2000). An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *J. Immunol.* **165**(1): 271-279.

90. Xi H, Blanck G.(2003). The IRF-2 DNA binding domain facilitates the activation of the class II transactivator (CIITA) type IV promoter by IRF-1. *Mol Immunol.* **39**(11): 677-84.

91. Xi H, Eason DD, Ghosh D, Dovhey S, Wright KL and Blanck G.(1999). Co-occupancy of the interferon regulatory element of the class II transactivator (CIITA) type IV promoter by interferon regulatory factors 1 and 2.*Oncogene.* **18**(43): 5889-903.

92. Xi H, Goodwin B, Shepherd AT and Blanck G. (2001). Impaired class II transactivator expression in mice lacking interferon regulatory factor-2. *Oncogene.* **20**(31): 4219-27.

93. Xie R, van Wijnen AJ, van Der Meijden C, Luong MX, Stein JL and Stein GS. (2001). The cell cycle control element of histone H4 gene transcription is maximally responsive to interferon regulatory factor pairs IRF-1/IRF-3 and IRF-1/IRF-7. *J. Biol. Chem.* **276**(21): 18624-32. Epub.

94. Xie RL, van Wijnen AJ, van der Meijden CM, Stein JL and Stein GS. (2002). Forced expression of the interferon regulatory factor 2 oncoprotein causes polyploidy and cell death in FDC-P1 myeloid hematopoietic progenitor cells. *Cancer Res.* **62**(9): 2510-5.

95. Yamaguchi H, Ikeda Y, Taylor A, Katsumura M, Miyazawa T, Takahashi E, Imakawa K and Sakai S. (1999). Effects of PMA and transcription factors on ovine interferon-tau.transactivation in various cell lines. *Endocr J.* **46**(3): 383-8.

96. Yamomoto, H., Lauphier, M, S., Taniguchi, T. and Harada, H. (1994). The oncogenic transcription factor IRF-2 posses a transcriptional repression and an activation domain. *Oncogene*. **9**: 1423-1428.

97. Yano H, Iemura A, Haramaki M, Ogasawara S, Takayama A, Akiba J and Kojiro M. (1999). Interferon alfa receptor expression and growth inhibition by interferon alfa in human liver cancer cell lines. *Hepatology*. **29**(6): 1708-17.

98. Yim JH, Wu SJ, Lowney JK, Vander Velde TL and Doherty GM. (1999). Enhancing in vivo tumorigenicity of B16 melanoma by overexpressing interferon regulatory factor-2: resistance to endogenous IFN-gamma. *J Interferon Cytokine Res.* **19**(7): 723-9.

99. Zhang S, Thomas K, Blanco JC, Salkowski CA and Vogel SN. (2002). The role of the interferon regulatory factors, IRF-1 and IRF-2, in LPS-induced cyclooxygenase-2 (COX-2) expression in vivo and in vitro. *J Endotoxin Res.* **8**(5): 379-388.

100. Zhang, L. and J.S. Pagano. (2000). Interferon regulatory factor 7 is induced by Epstein- Barr virus latent membrane protein 1. *J. Virol.* **74**: 5748-57.

101. Zhang, LW and Pagano, JS. Structure and function of IRF-7.2002. *Journal of interferon and cytokine research* **22**(1): 95-101.