CDFD

ANNUAL REPORT

April 2008 to March 2009

Center for DNA Fingerprinting and Diagnostics
Hyderabad

CDFD MANTRA

"We dedicate ourselves to achieve world class excellence in basic research and simultaneously endeavour to transfer the benefits of modern biology to every section of society. We view our population not as a problem, but as an advantage, which is unique to India. We believe we can use our extraordinarily large pool of genetic diversity as a "genetic playground" to address a number of questions; questions which will continue to accumulate and will demand answers, as the new millennium is bombarded with increasing arrays of nucleotide sequences as a consequence of global genome projects. Compassion, when coupled with science, can realize its highest ideal, viz., improving the quality of life of the average citizen. This is the lifeline of our activities at CDFD. We are prepared to face the challenging tasks ahead of us with our exceptional human resources. We are confident that we will meet and surpass the expectations and responsibilities reposed in us by the creation of the CDFD".

CONTENTS

From the Director’s Desk

Mandate

MANDATE

The objectives for which the Centre for DNA Fingerprinting and Diagnostics (CDFD) was established as enumerated in the EFC documents are:

- To establish DNA diagnostic methods for detecting genetic disorders and to develop probes for such detection;
- To use DNA fingerprinting techniques for the authentication of plant and animal cell material, cell lines and to develop new probes where necessary for such purposes;
- To provide training in DNA fingerprinting techniques and offer consultancy services to medical institutions, public health agencies and industry in the country;
- To undertake basic, applied and development R & D work;
- To collaborate with foreign research institutions and laboratories and other international organizations; and establish affiliation with recognized universities and institutions;
- To acquire or transfer technical know-how from/to entrepreneurs & industries and, to register patents, designs & technical know-how in the interest of the Centre;
- To carryout DNA profile and related analysis in civil cases like paternity disputes, immigration, and exchange of new-borns in hospitals, for various agencies including private parties, on appropriate payment;
- To provide DNA fingerprinting and related analysis and facilities to crime investigation agencies;
- To assist police personnel, forensic scientists, lawyers and the judiciary in understanding the evidential value of the DNA profile analysis and related techniques in crime investigation and family matters.

Services

Laboratory of DNA Fingerprinting Services

Coordinator	J Nagaraju	Staff Scientist
Scientist In-charge	Madhusudan Reddy Nandineni	Staff Scientist
Other Members	Varsha	Staff Scientist (till 15.03.2009)
	SPR Prasad	Senior Technical Examiner
	V N Sailaja	Technical Officer Gr. II
	Ch V Goud	Technical Examiner
	D S Negi	Technical Examiner
	S Pranitha	Junior Assistant-I (since 31.07.2008)
	Md Mahfooz Alam	Junior Assistant-I (till 27.06.2008)
	G Rajalingam	Junior Assistant-II

Objectives:

1. To provide DNA fingerprinting services in the cases forwarded by law enforcing agencies of State and Federal Governments and Hon'ble Judiciary, relating to maternity/paternity, murder, rape, immigration, child-swapping, body identification, wildlife identity, seed purity testing, strain identification, etc.;
2. To develop human resources skilled in DNA fingerprinting, to cater to the needs of State and Federal Government agencies;
3. To impart periodical training to manpower involved in DNA fingerprinting sponsored by State and Federal Government agencies;
4. To provide advisory services to State and Federal Government agencies in establishing DNA fingerprinting facility; and
5. To create DNA marker databases of different caste populations of India.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

A total number of 148 cases were received for DNA fingerprinting examination during this period. Out of these, 67 cases were related to paternity/ maternity disputes, 65 cases were related to identification of deceased, 10 cases were pertaining to rape/sexual assault, 2 cases were pertaining to murder and 4 cases were related to wildlife poaching. The cases involving paternity/maternity (45%), identification of the deceased (44%), rape/sexual assault cases (7%), wildlife (3%) and murder (1%) constituted the bulk of the cases received.

Eighteen States and Union territories of India have availed DNA fingerprinting services of CDFD during this period. Rajasthan State forwarded the highest number (43) of cases, followed by Karnataka (37), Andhra Pradesh (13), Uttar Pradesh (10), Punjab (8), Haryana (7), Chhattisgarh (6), Maharashtra (5), Kerala (4), Bihar (4), Goa (2), Himachal Pradesh (2), and one case each from Andaman & Nicobar, Assam, Jharkhand, Madhya Pradesh, Manipur and Tamil Nadu. The details of the cases received from different States and Union territories of India are given in the following table and the percentage of cases received is shown in the figure.

An amount of Rs.19,22,936/- (Rupees nineteen lakhs, twenty two thousand, nine hundred and thirty six only) has been received towards DNA fingerprinting analysis charges (which is inclusive of service charge as levied by Government of India) in various cases.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

A total number of 91 cases were received for DNA fingerprinting examination during the above-mentioned period. Out of these, 49 cases were related to paternity/maternity/ biological relationship disputes, 32 cases were related to identification of deceased, 5 cases were pertaining to rape/sexual assault, 2 cases were pertaining to murder and 3 cases were related to wildlife poaching. Similar to previous year, the cases involving paternity/maternity (55%), identification of the deceased (35%), rape/ sexual assault cases (5%), wildlife (3%) and murder (2%) constituted the bulk of the cases received.

Seventeen States and Union Territories of India have availed DNA fingerprinting services of CDFD during

this period. Rajasthan State forwarded the highest number (27) of cases, followed by Karnataka (8), Maharashtra (8), Andhra Pradesh (7), Kerala (6), Uttar Pradesh (6), Andaman & Nicobar (5), Chhattisgarh (5), Madhya Pradesh (5), Punjab (4), Himachal Pradesh (3), Bihar (2), and one case each from Delhi, Haryana, Puducherry, Uttarkhand and Tamil Nadu. The details of the cases received from different States and Union Territories of India are given in the following table and the percentage of cases received is shown in the figure.

An amount of Rs.16,47,038/- (Rupees sixteen lakhs, forty seven thousand and thirty eight only) has been received towards DNA fingerprinting analysis charges (which is inclusive of service charge as levied by Government of India) in various cases.

Deposition of evidence in Hon'ble Courts

During this reporting year, the DNA experts defended their reports in about a dozen cases in various Hon'ble Courts throughout the country.

Training/Lectures/Workshops on DNA fingerprinting examination

Training on DNA fingerprinting examination has been provided to the scientists from the State Forensic Science Laboratory, Rajasthan, Jaipur, State Forensic Science Laboratory, Bengaluru, Karnataka, and State Forensic Science Laboratory, Sagar, Madhya Pradesh.

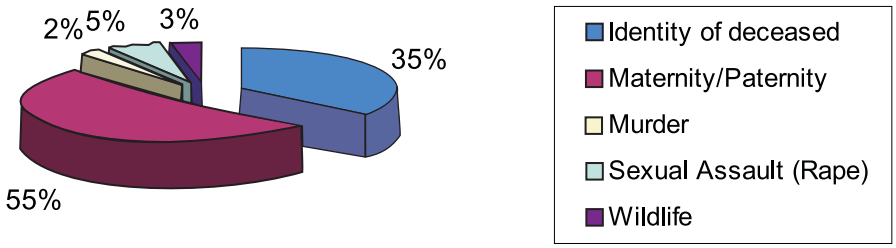
Lectures have been delivered for the benefit of the:

1. Foreign delegates from 22 different countries, coordinated by the National Crime Records Bureau (NCRB), New Delhi.
2. Trainee investigating officers from Central Detective Training School, Hyderabad;
3. IPS and other Senior Police Officers from Sardar Vallabbhai Patel National Police Academy, Hyderabad, and
4. Indian Air Force trainee Officers from the Air Force Intelligence School, Pune.

Summary of the state-wise breakup of DNA Fingerprinting cases:

State-wise case list	Identity of deceased	Maternity/ Paternity	Murder	Rape	Wildlife	Total No. of Cases
Andaman & Nicobar	0	5	0	0	0	5
Andhra Pradesh	0	5	0	0	2	7
Bihar	0	2	0	0	0	2
Chhattisgarh	0	3	0	2	0	5
Delhi	1	0	0	0	0	1
Haryana	0	1	0	0	0	1
Himachal Pradesh	2	1	0	0	0	3
Karnataka	0	6	1	0	1	8
Kerala	2	4	0	0	0	6
Madhya Pradesh	1	4	0	0	0	5
Maharashtra	0	8	0	0	0	8
Puducherry	0	1	0	0	0	1
Punjab	3	0	0	1	0	4
Rajasthan	19	5	1	2	0	27
Tamil Nadu	0	1	0	0	0	1
Uttar Pradesh	4	2	0	0	0	6
Uttarkhand	0	1	0	0	0	1
Total No. of Cases	**32**	**49**	**2**	**5**	**3**	**91**

Cases received from April 2008 to March 2009:



Diagnostics Division

Principal Investigator	Ashwin Dalal	Staff Scientist
Other Members	Padma Priya	DBT PDF
	Angalena R	Technical Officer II
	S.M. Naushad	Technical Officer II
	Usha Rani Dutta	Technical Officer I
	Binod Pradhan	Technical Officer I
	Jamal Md Nurul Jain	Technical Assistant
	C Krishna Prasad	Technician II
	R Sudheer Kumar	Technician II
	V.Murali Mohan	Junior Assistant II
	G Srinivas	Junior Assistant II
	P Rajitha	Project Associate
Collaborators	Muralidharan Bhashyam, Molecular Oncology, CDFD	
	Suman Jain, Thalassemia Society, Hyderabad	

Objectives

- Clinical evaluation and counseling of patients with genetic disorders.
- Research on single gene disorders
- Providing social awareness in early detection and prevention of genetic disorders
- Training the medical and paramedical students in genetic testing

I. Services provided during the year 2008-2009

Clinical Genetics

At total of 1664 patients attended the genetic clinic for genetic evaluation and counseling, during the year 2008-09. These consisted of patients with chromosomal disorders, monogenic disorders, mental retardation, congenital malformations, multiple malformation syndromes, hemolytic anemias, short stature, skeletal dysplasias, myopathies, neurodegenerative disorders, ataxia, hypogonadism (male/female), ambiguous genitalia, inborn errors of metabolism, and familial disorders.

A Memorandum of Understanding was signed between NIMS and CDFD for establishment of a "Medical Genetics Unit" to provide services, training students and conducting research in the field of Medical Genetics. A total of 225 patients were examined and counseled in the unit since August, 2009.

Genetic investigations done during 2008-09

Investigation	Total cases	Positives
Cytogenetics	918	62 (6.7 %)
Proband	845	62 (7.3 %)
Prenatal	73	0 (0%)
Molecular Genetics	426	110 (25.8 %)
Proband		403 107 (26.5 %)
Prenatal	23	3 (13 %)
Biochemical Genetics	565	90(15.9%)
Proband	561	89 (15.8 %)
Prenatal	4	1 (25 %)

Cytogenetics

A total of 918 cases were analyzed for cytogenetic analysis, of which 62 were positive giving a yield of 6.7 %. From this year, we have begun providing services FISH analysis for hematologic malignancies like chronic myeloid leukaemia, microdeletion syndromes and rapid prenatal testing by FISH and Quantitative Fluorescent PCR.

Disease	Abnormality	Number of cases
Down syndrome	Trisomy 21	30
	46, XY, rob (21; 21) +21	2
Edwards syndrome	46, XY,+18	1
Turner syndrome	Monosomy X (45,X)	3
	Iso X (46,X, i(X))	1
	Mosaic Turner syndrome	3
	(46, XY / 45XO)	2
	(45,XO/46,X r(X)	1
Klinefelter syndrome	47,XXY	2
Sex Reversal	Phenotypic male with 46, XX	3
	Phenotypic female with 46, XY	5
True Hermaphrodite	46,XX/46,XY	1
Structural chromosomal abnormalities		11
	46,XY,inv(2)	1
	46,X,t(X;22)(p11.21;q13.3)	1
	46,XY,t(1;16)(p31.1;p11.2)	1
	46,XX,t(1;18)(pter;p11.2)	1
	46,XY,t(5;16)(q33.3;p13.3)	1
	46, XY, 15p+	1
	45,XX,rob(13;14)(p10;q10)	1
	46,XY,t(9;10)(p22;p15)	1
	46,XX,t(7;16)(q31.1;q23)	1
	47,XX+marker	2

Molecular Genetics

NAME OF DISORDER	No of cases	POSITIVE	NEGATIVE		
DMD/BMD	43	20	23		
DMD Carrier Analysis	6	5	1		
Spinal Muscular Atrophy	23	8	15		
		Homozygous	**Heterozygous**	**Compound Heterozygous**	**Normal**
Thalassemia and Sickle cell anemia	95	52	26	10	7
Factor V Leiden	69	-	6	-	63
Factor II mutation	29	-	-	-	29
Cystic Fibrosis	69	6	14	1	48

NAME OF DISORDER	No of cases	POSITIVE	NEGATIVE		
TRIPLET REPEAT DISORDERS		POSITIVE	NEGATIVE		
Fragile X Syndrome	29	1	28		
Friedrichs Ataxia	12	5	7		
Myotonic Dystrophy	2	-	2		
Huntington Disease	8	5	3		
SCA Panel	18	5	13		
PRENATAL DIAGNOSIS		Normal	Homozygous	Heterozygous	Compound
Heterozygous					
Thalassemia	12	5	1	6	-
Cystic Fibrosis	1	-	-	1	-
DMD	1	Negative-1			
Spinal muscular atrophy	9	Positive- 2			

Biochemical Genetics

DISEASE	NUMBER OF POSITIVES (%)
Biochemical (N = 565)	**91**
AMINO ACID DISORDERS (N=173)	**17 (9.8%)**
Maple syrup urine disease	2
Non Ketotic Hyperglycinemia	5
Hyperornithinemia	8
Tyrosinemia	1
Hyperhomocysteinemia	1
Lysosomal storage disorders (N=66)	16 (10.5 %)
Metachromatic Leukodystrophy (19)	5
Gaucher's disease (11)	5
GM1-Gangliosidosis (9)	3
Mucopolysaccharidosis (8)	0
Pompe's disease (1)	0
Tay Sach's disease (4)	1
Sandhoff syndrome (1)	1
Fabry disease (3)	1
Chititriosidase (5)	0
Alpha mannosidosis (1)	0
Krabbe disease (2)	0
Arylsulphatase B (2)	0
METABOLIC SCREENING TESTS	
Newborn screening (N=35)	Biotinidase deficiency- 1
Urine Metabolic screening (128)	5
Urine MPS Screening (41)	13
Oligosaccharide screening (19)	11
HIGH RISK PREGNANCY SCREENING	
Triple Marker screening (N=32)	16
First Trimester screening (N=67)	11
PRENATAL DIAGNOSIS (4)	**1**
MPS I (1)	0
Tay Sach disease (1)	0
Metachromatic Leukodystrophy (1)	1
Pompe disease (1)	0

II. Research

1. Research in single gene disorders

We reported on a case of hypertrichosis, hyperkeratosis and mental retardation which is second such case to be reported in literature of this new syndrome. We have been actively involved with activities of the Thalassemia and Sickle cell society of Hyderabad and detected a novel mutation in beta globin gene in a case of thalassemia i.e. IVS 1-129 A>C mutation. We identified a case of H-syndrome characterized by cutaneous hyperpigmented, hypertrichotic, and indurated patches associated with hearing loss, short stature, hepatosplenomegaly, and hypogonadism, for which the gene has been recently characterized. Our patient showed a novel mutation i.e R133C in exon 4 of the SLC29A3 gene. We also identified mutations in cases of Myotonia congenita and Seckel syndrome which helped in counseling these families.

2. Clinical, biochemical and molecular analysis of treatable lysosomal storage disorders

This is a new activity

Lysosomal storage disorders are a heterogeneous group of disorders associated with specific lysosomal enzyme deficiency. Enzyme replacement therapy (ERT) is available for at least 5 of these disorders, namely Gaucher disease, Fabry disease, Mucopolysaccharidosis type I and VI, and Pompe disease. Phase II trials are underway for Niemann Pick disease. Our study aims to characterize the clinical features, biochemical parameters and molecular defects in these treatable disorders. The results would form the basis for revealing the spectrum of mutations for these disorders in the Indian population which will in turn help in better diagnosis of carriers for these disorders and accurate prenatal diagnosis. At present the diagnosis and prenatal diagnosis of these disorders is primarily based on enzyme assay, which has a number of disadvantages. There is a large amount of overlap in enzyme levels among carriers and normal people; hence it is very difficult to detect carriers by enzyme assay. Therefore mutation detection is imperative for carrier detection. In addition, the data regarding mutations will be helpful in genotype phenotype studies in these disorders. Further, the knowledge regarding mutations in Indian patients will help in establishing testing for these disorders as a service to the patients. The project was submitted to ICMR and ICMR has recommended to resubmit it as a multicentric project with CDFD as nodal centre for the country.

3. Establishment of EBV transformed cell lines from lymphocytes of patients with rare disorders

This is a new activity

One of the important tasks following mapping & sequencing of the Human Genome is to identify disease-causing genes. It is equally important to resolve genetic heterogeneity & establish genotype-phenotype correlations. For both the above objectives, it is essential to have informative clinical material. The informative index cases & their families are rare, and very often not willing to get investigated, particularly if they have to pay for it. It is important to harness this material as much as possible. The Diagnostics Division of CDFD has a very active clinical and laboratory programme in the field of clinical genetics. It sees over 2000 new cases for various genetic disorders each year, many of which are rare or unique. Since most of the genetic disorders are rare, a significant number of families with of one type of disorder need to be accumulated over a period of time. The present project aims to establish EB virus transformed cell lines from patients with genetic disorders of interest. The project is under consideration with DBT.

Publications

1. Naushad SM, Jamal MNJ, Prasad CK and Rama Devi AR (2008) Relationship between methionine synthase, methionine synthase reductase genetic polymorphisms and deep vein thrombosis among South Indians. ***Clinical Chemistry and Laboratory Medicine*** 46(1):73-79.

2. Prabhakara K, Bruno DL, Padman P, Prasad S, Kumar SR, Slater HR and Ramadevi AR (2008) Prenatal detection of deletion-duplication of chromosome 3 arising from meiotic recombination of familial pericentric inversion. ***Prenatal Diagnostics*** 28(5):466-468.

3. Agarwal S, Tamhankar PM, Kumar R and Dalal A (2009) Clinical and haematological features in a compound heterozygote (HBB:c.92+5G>C /HBB:c.93-2A>C) case of thalassemia major. ***International Journal of Laboratory Hematology*** (In press).

4. Angalena R, Chaudhary A, Bashyam MD and Dalal A (2009) Hemoglobin D (Iran) masquerading as Hemoglobin E: An interesting case report. ***Newsletter of Genetics Chapter of Indian Academy of Pediatrics*** (In press).

5. Dalal A and Mehrotra RN (2009). Hypertrichosis, hyperkeratosis and mental retardation syndrome: further delineation of phenotype. ***Clinical Dysmorphology*** (In press).

Laboratory of Molecular Genetics

Centre of Excellence on Genetics and Genomics of Silkmoths

and

Basmati Rice Genetics and Genomics

Principal Investigator	J Nagaraju	Staff Scientist
Ph D Students	S Jayendranath	SRF
	Jyoti Singh	SRF
	K P Arun Kumar	SRF
	Asha Minz	JRF
	Chandrapal Singh	JRF
	S Suresh Kumar	JRF
	Deepa Badrinarayan	JRF
Other Members	V V Satyavathi	Technical Officer IV (CoE)
	A Sobhan Babu	Technical Officer I
	M Muthulakshmi	Technical Officer I
	S Annapurna	Technical Officer I (CoE)
	Archana Tomar	Bioinformatician (CoE)
	V Satish	Research Associate
	E V Subbaiah	Research Associate
	N Mrinal	Research Associate
	A Srividya	Project Associate
	A Kaliappan	Project Assistant
	K Vijayasarathy	Project Assistant
	D Swarna kumari	Project Assistant
	K Adarsh Gupta	Project Assistant
	Santhosh R Jadhav	Project Assistant
	S Venkateswari	Project Assistant
	B Ramesh Babu	Secretary (CoE)
	P Chandra Shekar	Technician II
	A V Bhaskar	Lab Attendant

Project 1

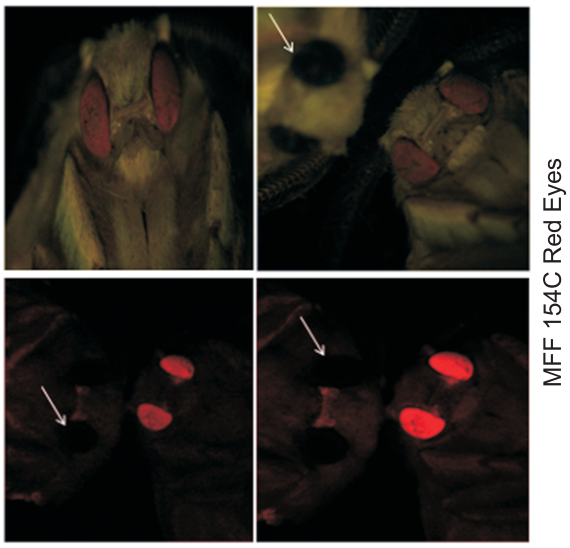
Development of RNAi-based baculovirus resistant transgenic silkmoths

Objectives: Generation of transgenic silkworms resistant to *Bombyx mori* nucleo-polyhedrosis virus (BmNPV) using RNAi strategy and introduction of anti-baculoviral property to commercial silkworm strains by expression of multiple RNAi viral targets.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

The transgenic lines expressing dsRNA for essential baculoviral immediate early gene (*ie-1*) were generated and tested against baculovirus infection. The transgenic lines were characterized for (i) copy number and chromosomal location of the integrated transgenes, (ii) baculoviral abundance as quantified by qPCR of the baculoviral gene, and (iii) western blot analysis using antibody for baculovirus coat protein, GP64. We also generated eight *piggyBac* mediated transgenic lines that expressed dsRNA for four essential baculoviral genes (**Fig. 1**). The gene encoding red fluorescent protein was used as a marker driven by 3xP3 promoter for the identification of transgenic individuals by red fluorescent eyes (**Fig. 2**).

Fig. 1. Multigene "flip-flop" construct of four viral target genes transcribed from a single promoter. DsRed2 is under 3xP3 promoter and acts as a marker gene.



White arrow: Non-Transgenic eyes

Fig. 2. The red fluorescent eyes are seen in the transgenic silkmoths that are absent in non-transgenic moths.

Details of progress made in the current reporting year (April 1, 2008 – March 31, 2009)

During the year under report, the transgenic lines were characterized for site of integration of the transgenes, copy number and their physical location in the silkworm genome. Transposable element display (TED) method was used for identifying the site of insertion and copy number of the insertions (Fig. 3). In all the transgenic lines the genomic integration of the transgene was found to be mediated by piggyBac as evidenced by the presence of signature sequence TTAA characteristic of piggyBac mediated integration. These

sequences were blasted against the silkworm physical map assembly of sequences and the chromosomal location and the relative position of the transgene on the chromosome was determined for each of the transgenic line (Fig. 4).

To commercially exploit these transgenics, we introduced the transgenes from Nistari which is a non-diapausing strain to the diapausing and baculovirus susceptible silkworm strain CSR_2, the most productive silkworm strain that is currently in use in the sericulture states of India, through recurrent back cross breeding coupled with transgene marker selection as outlined in Fig. 5. The hybrids were tested using microsatellite markers specific to Nistari and CSR_2 strains as well as phenotypic fluorescence marker to establish the identity of the genome. Further, the F_1 hybrid males were backcrossed to CSR_2 females to obtain backcross progeny.

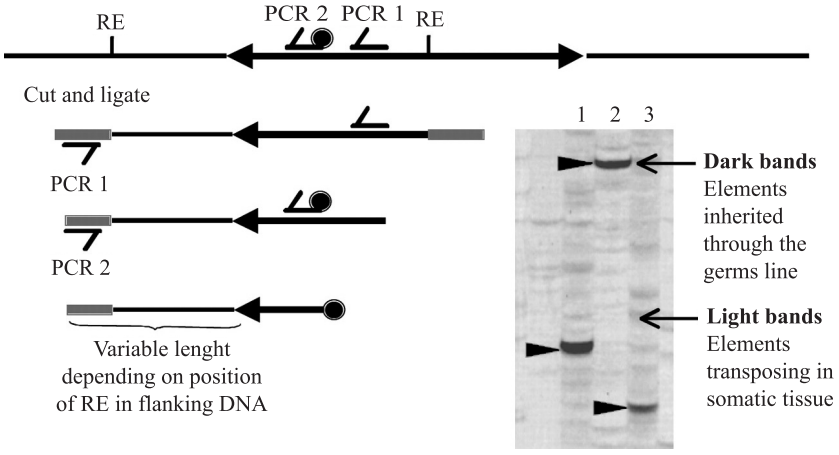


Fig. 3. The Transposable Element Display (TED) method

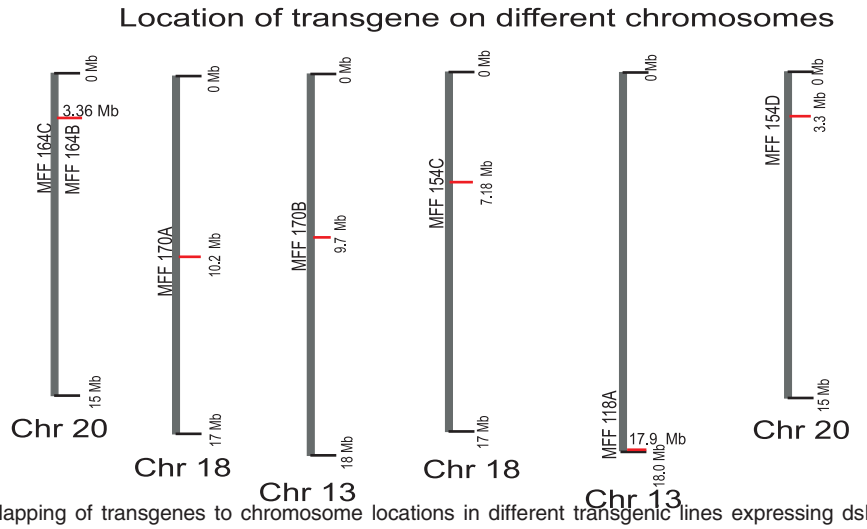


Fig. 4. Mapping of transgenes to chromosome locations in different transgenic lines expressing dsRNA for essential baculoviral genes

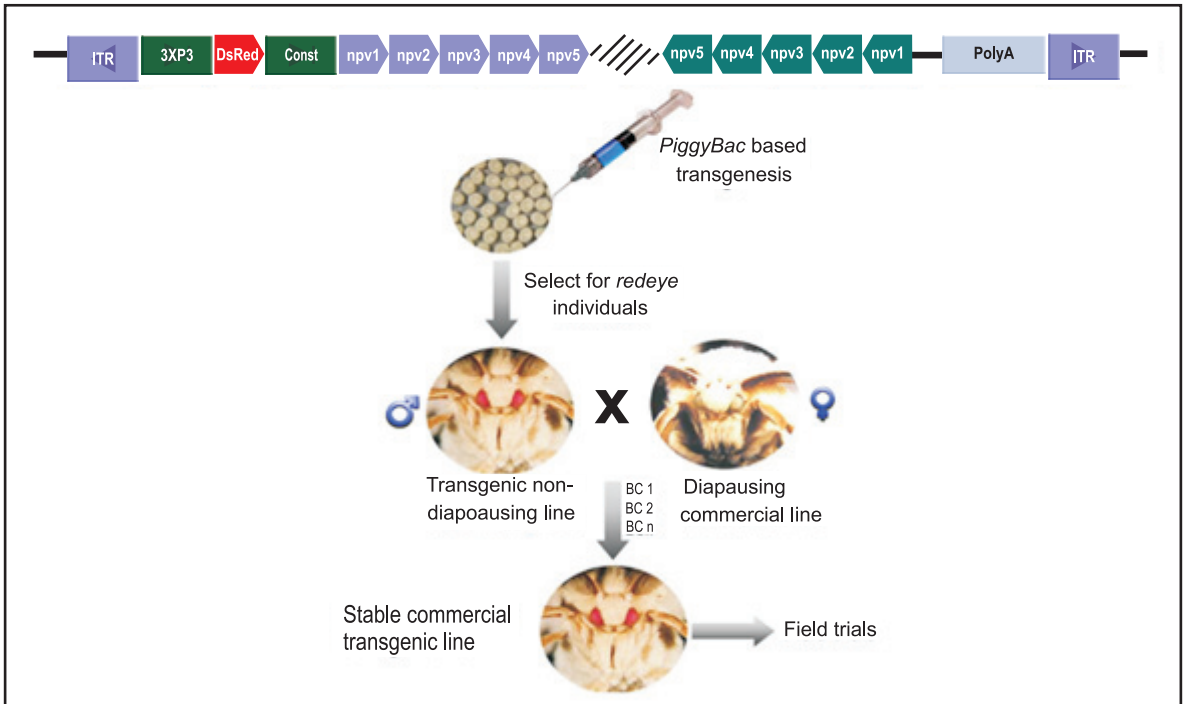


Fig. 5. The transgenic line, Nistari, expressing red fluorescence in the eyes is crossed to high yielding commercial non-diapausing strain, CSR_2 to obtain F_1 offspring. The F_1 males were back-crossed to CSR_2 females to obtain BC_1 progeny.

Project 2: Comparative and functional genomics of silkmoths

Objective: Characterization and functional analysis of various ESTs identified from the silkmoths

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

A large scale sequencing of expressed sequence tags (ESTs) from three wild silkmoth species, *Antheraea assama* (Golden silkmoth of Assam), *Samia cynthia ricini* (Eri silkmoth) and *Antheraea mylitta* (Tasar silkmoth) was carried out. A total of 57,113 ESTs were sequenced and characterized from several tissues at different developmental stages. In order to disseminate this information to the scientific community, a BLAST searchable database of ESTs called 'Wild Silkbase' was developed.

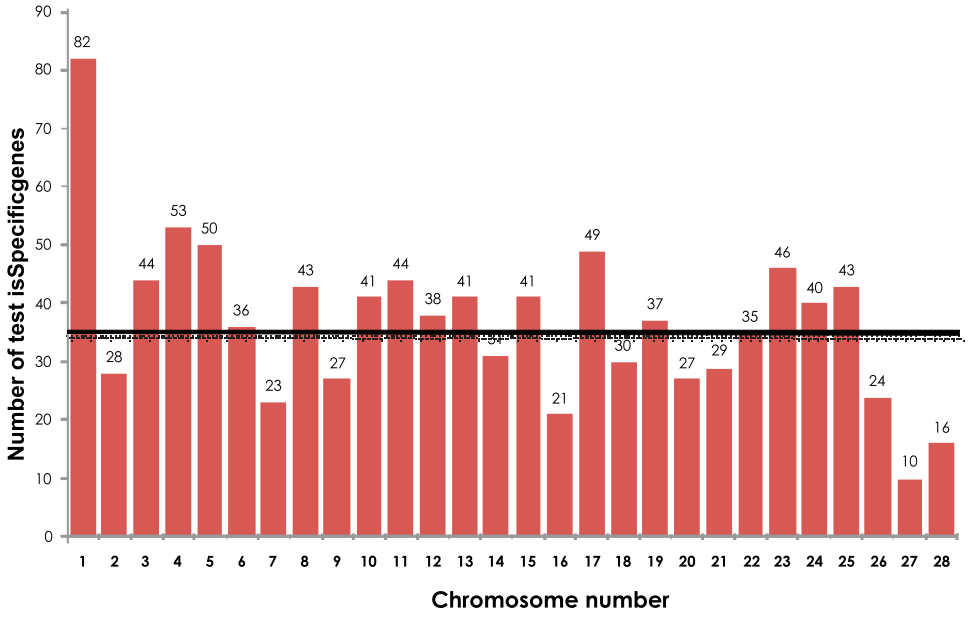
Details of progress made in the current reporting year (April 1, 2008 – March 31, 2009)

In silico analysis of testis derived ESTs from *A. assama* revealed an unusual phenomenon of conservation of several testis-specific genes in diverse species of distant taxa, from insects to mammals. Due to limited availability of genomic resources for *A. assama*, a study was initiated in *Bombyx mori* system that has large EST dataset and a 9X solid coverage of genome sequence. We downloaded 9614 testis ESTs and a total of 95,051 ESTs derived from tissues other than testis and carried out *in silico* differential display to identify genes expressed specifically in the testis. This study identified several families of testis-specific genes like *tektins*, *dyneins*, *kinases* and *tubulins*, involved in spermatogenesis of the silkworm.

Testis-specific genes validated in this manner by microarray results were assigned chromosomal positions on the *B. mori* genome to examine their distribution on different chromosomes. We assayed two datasets: one set comprised of a total of 1104 microarray validated testis-specific genes and the second included 1984 testis-specific genes derived from fl-cDNAs and ESTs. We were able to successfully map 1029 and 1857

genes in the first and second datasets, respectively, onto their respective chromosomal location. We also mapped 465 genes from the other tissues onto chromosomes to compare their distribution with that of testis-specific genes. Z showed significantly higher frequency of testis-specific genes with 11.45 genes per Mb of chromosome compared to autosomes (Student t-test, P<0.001). Z chromosome harboured highest number (82 and 151) in the first and second datasets of testis-specific genes, respectively, as against the average number of testis-specific genes of 35+10 and 63 + 21 on autosomes (**Fig. 6**). Physical mapping of these genes revealed that there is apparent bias in their distribution on the chromosomes. In order to identify if there are any clusters of testes-specific genes, we constructed a physical map. The map showed the spatial distribution of genes on *B. mori* chromosomes (**Fig. 7**). It was evident from the map that genes are quite evenly and densely distributed on Z chromosome. The analysis confirmed the presence of several clusters of testis- specific genes on different chromosomes.

Our results show that in comparison with the autosomes, the Z chromosome (Linkage Group 1) harbours a significantly higher number of testis-specific genes. In *B. mori*, four muscle protein genes, *Bmkettin, Bmtitin1, Bmtitin2, Bmprojectin,* and another gene involved in locomotor behaviour, *Bmhig* located on the Z chromosome expressed more transcripts in males than in females suggesting that these genes are not dosage compensated. These genes are functionally conserved between *B. mori* and *Drosophila*. In silkworm, although adult moths have lost flight during the course of domestication, male moths flap their wings more vigorously to approach sedentary females. In this context, the location of muscle proteins encoding genes on the Z chromosome ensures higher quantity of these proteins in males. It is assumed that the Z chromosome has possibly evolved through a process of genome shuffling to accumulate genes whose products are required at higher levels in males. We speculate that lack of dosage compensation and sexual antagonism has possibly led to the accumulation of male specific genes on Z chromosome. In the course of evolution, proteins which are required in higher amounts in males, than in females would have been favored on Z chromosome. Also, initial analysis of testis-specific paralogs gave evidence for possible translocation of male advantageous genes onto Z chromosome.



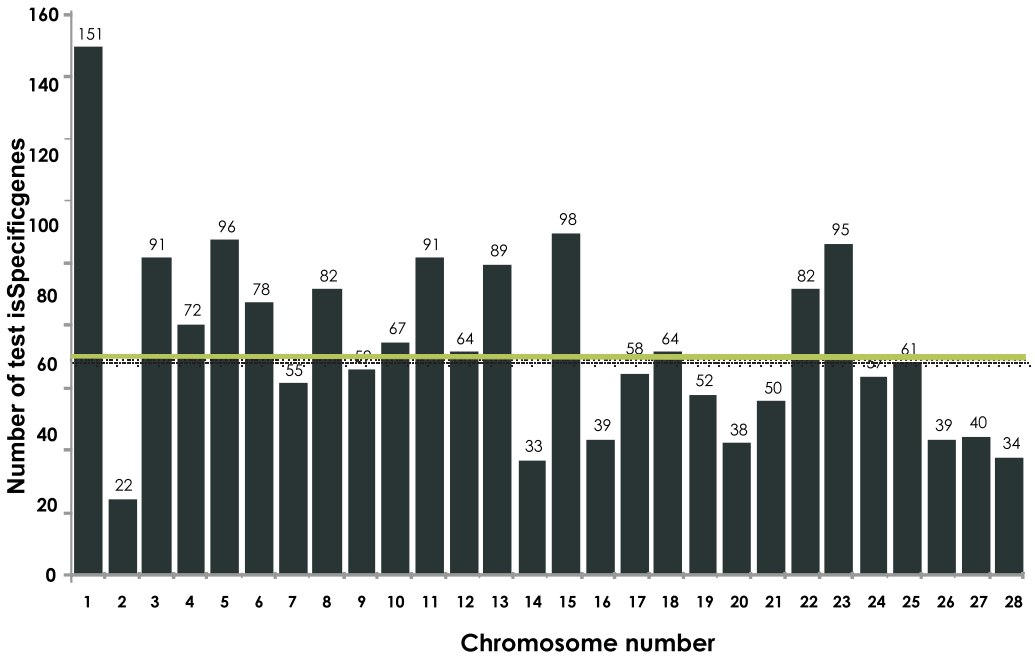


Fig. 6. Distribution of testis-specific genes as identified through microarray analysis (Upper panel) and through EST and fl-DNA sequence analysis (Lower panel) on different chromosomes of *B. mori*. There is a significant difference (P<0.001) between number of testis-specific genes present on Z chromosome and autosomes in both the cases.

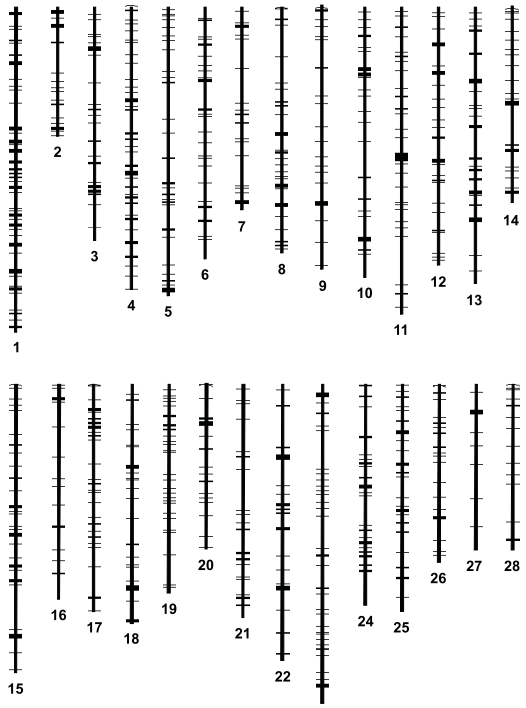


Fig.7. Physical map of *B. mori* chromosomes showing the distribution of testis-specific genes on different chromosomes

Basmati rice genetics and genomics

Project 3: Fine-mapping and positional cloning of candidate genes in a promising region on chromosome 5 for grain quality traits of Basmati rice.

Objective: To fine-map and characterize the candidate genes of important grain quality traits of Basmati rice on chromosome 5.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

QTLs were mapped in an F_2 mapping population of 181 plants, derived from a cross between a traditional Basmati variety, Basmati370 and a semi-dwarf variety Jaya. Phenotypic data for 18 agronomic and quality traits of the parents, F_1 hybrids and F_2 mapping population was recorded. 502 microsatellite primers were screened for polymorphism between parental varieties and 203 markers were identified as polymorphic. These markers are distributed on all the 12 rice chromosomes. The F_2 mapping population was screened with these 203 polymorphic markers and a Basmati rice linkage map was constructed. We identified 47 QTLs for 16 different agronomic and quality traits. Interestingly, a single region on chromosome 5 at the marker interval of M12-M18 was found to be controlling some important grain quality traits.

Details of progress made in the current reporting year (April 1, 2008 – March 31, 2009)

Further fine-mapping of the region on chromosome 5 between markers M12-M18 using SSRs, InDels and ESTs as molecular markers led to the identification of four QTLs relating to Basmati grain quality traits of grain length, grain breadth, length-breadth ratio and grain elongation ratio (**Fig. 8**). Based on japonica and indica rice genomic information we have identified three candidate genes for grain quality trait in this region. Currently, we are sequencing these genes from Basmati varieties.

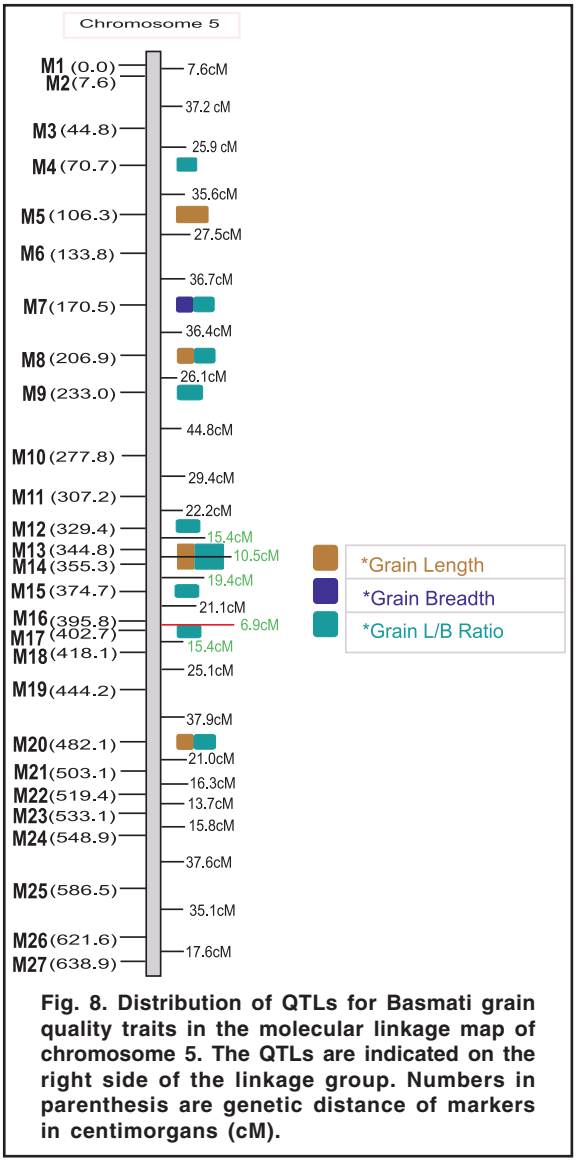


Fig. 8. Distribution of QTLs for Basmati grain quality traits in the molecular linkage map of chromosome 5. The QTLs are indicated on the right side of the linkage group. Numbers in parenthesis are genetic distance of markers in centimorgans (cM).

Publications:

1. Arunkumar KP, Kifayathullah L and Nagaraju J (2008) Microsatellite markers for the Indian golden silkmoth, *Antheraea assama* (Saturniidae: Lepidoptera). ***Molecular Ecology Resources*** 9: 268-270.

2. Arunkumar KP, Tomar A, Daimon T, Shimada T and Nagaraju J (2008) WildSilkbase: An EST database of wild silkmoths. ***BMC Genomics*** 9: 338.

3. Kanginakudru S, Metta M, Jakati RD and Nagaraju J (2008) Genetic evidence from Indian red jungle fowl corroborates multiple domestication of modern day

chicken. ***BMC Evolutionary Biology*** 8:174.

4. Mrinal N and Nagaraju J (2008) Intron loss is associated with gain of function in the evolution of the gloverin family of antibacterial genes in *Bombyx mori*. ***Journal of Biological Chemistry*** 283: 23376-23387.
5. Singh J and Nagaraju J (2008) In silico prediction and characterization of microRNAs from red flour beetle (*Tribolium castaneum*). ***Insect Molecular Biology*** 17: 427-436.
6. Johny S, Chakraborty S, Gadagkar R and Nagaraju J (2009) Polymorphic microsatellite loci for primitively eusocial wasp Ropalidia marginata. ***Molecular Ecology Resources*** 9: 2203-2205.
7. Arunkumar KP, Mita K and Nagaraju J (2009) Silkworm Z chromosome is enriched in testis-specific genes. ***Genetics*** (In Press).

Other Publications :

1. Nagaraju J (2008) Silks of India, Grace and Lustre. ***Biotech News*** 3: 4-7.
2. Nagaraju J (2008) Silkworm Breeding Multiplier Effect. ***Biotech News*** 3: 24-27.

Patents :

1. Nagaraju J. Novel FISSR-PCR primers and methods of identifying genotyping diverse genomes of plant and animal systems including rice varieties, a kti thereof. Indian Patent 219765, granted on 13 May, 2008.
2. Chandrashekharaiah, Rao CG, Sivaprasad V, Babu M R, Goel AK, Prasad J and Nagaraju J . Superior, Novel quality mulberry silkworm hybrids and a method thereof. Indian Patent 218895, granted on 16 April, 2008.

Laboratory of Genomics and Profiling Applications

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	Aravind Kumar	Project Assistant (until Oct, 2008)
	Anita Kumari	Project Assistant (until Oct, 2008)
Collaborators	SPR Prasad	CDFD
	Ch V Goud	CDFD

Objectives:

1. Development, standardization and validation of DNA markers for genetic fidelity testing of tissue culture raised plants and for phylogenetic studies
2. Referral Centre for detection of genetically modified foods employing DNA-based markers
3. Development of novel strategies/ methodologies for enrichment of human DNA from mixtures containing human and non-human DNAs for DNA profiling-based human identification
4. To study the human genetic diversity among various population groups of India

Project 1: Referral Centre for Genetic Fidelity Testing of Tissue Culture Raised Plants Employing DNA Markers.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

As a Referral Centre for the Genetic Fidelity Testing of Tissue Culture Raised Plants Employing DNA Markers, our laboratory's focus was to develop molecular markers which could be useful for true-to-type testing of important tissue culture raised crop plants like banana, black pepper, potato, sugarcane and vanilla. Previously, various tissue cultured samples of above crop plants were obtained from different authentic sources for the purpose of genetic fidelity testing, it was decided to isolate microsatellite or simple sequence repeat (SSR) markers, which are highly polymorphic, using a selective hybridization procedure, and to check their utility for the intended purpose. We had previously reported that few clones containing SSRs were obtained, for black pepper and potato by employing this method.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

(a) Inter-simple sequence repeats (ISSR) based assay for genetic fidelity testing:

While the isolation of SSRs from these crops are in progress, in the meantime, it was decided to check the suitability of ISSR-based assays for genetic fidelity testing of tissue culture raised plants based on some of the previous studies. ISSR markers, even though are dominant and anonymous markers, they are less demanding as compared to

SSRs, both technically as well as cost wise and therefore these could be preferred for large-scale screening of planting material.

Thirty ISSR primers derived from University of British Columbia (UBC) list were screened to select the most suitable primers which show good reproducibility and amplification. The selected primers were then used to screen for polymorphism between different varieties of each crop. The markers which show reproducibility and high polymorphism to distinguish different varieties are used for assessing the clonal fidelity of the micropropagated plants taken out at different passages of sub- culturing.

While using some of the UBC primers in ISSR assays, the number of amplified fragments ranged from 8-12, while in some they resulted in less than 4 bands. For ISSR based assays, it was decided to focus on the primers which yield an average of 8-12 bands per sample reproducibly. Based on this criterion, PCR conditions for about a dozen ISSR primers have been standardized for banana, black pepper, potato, sugarcane and vanilla crop plants. They are being checked for reproducibility and for polymorphism by comparing the banding pattern in different varieties of each species. Few of these primers were able to distinguish some but not all varieties. Further standardization and evaluation of results is under progress before using them for genetic fidelity testing.

(b) Isolation of microsatellite markers:

Microsatellites or SSRs are an extremely valuable tool for genome mapping, phylogenetic studies, marker-assisted breeding, etc. A method of microsatellite isolation by Glenn and Schable (*Methods in Enzymology*, 2005, 395: 202-222) was followed for microsatellite enrichment from potato, banana and sugarcane. Few clones for potato, sugarcane, banana, black pepper and vanilla were obtained from these enrichments, which contain SSRs along with flanking sequences. These SSR markers have to be tested and validated for polymorphism in different varieties of each species. Since it is desirable to isolate multiple SSR markers, which are polymorphic and informative, the above strategy would be adopted to isolate more number of microsatellites in these crop plants in future.

(c) Employing previously known banana SSR markers for genetic fidelity testing:

A total of forty-two SSR primers, which are reported in the literature as being polymorphic, are presently being tested for their utility in genetic fidelity testing. PCR conditions are being standardized and validated for these primer pairs. Once they are standardized, the informative SSRs would be tested in different varieties of tissue-culture raised banana plants for their utility in genetic fidelity testing. Some of these SSR markers have in our preliminary experiments have shown high degree of polymorphism in few of the varieties tested so far, and thus are very informative. Further standardization, validation, and testing in other varieties of banana are currently under progress.

Project 2: Referral Centre for Detection of Genetically Modified Foods Employing DNA-based markers.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

As a Referral Centre for detection of genetically modified (GM) foods employing DNA-based markers, our laboratory has standardized DNA based qualitative detection of transgenic elements in the numerous varieties of transgenic cotton (*Gossypium hirsutum*). These cotton varieties, which are either commercially released or under field trials in India, were obtained from different seed producing companies and comprised of four transgenic cotton events viz., MON 531 (*cry1Ac*), MON 15985 (*cry1Ac+cry2Ab*), Event 1 (*cry1Ac*) and GFM event (*cry1Ac+cry1Ab*). Multiplex PCR allows several target DNA sequences to be screened and detected in a single reaction and thus fewer reactions are needed to test a sample for presence of GMO-derived DNA. Various combinations of duplex and triplex PCRs were standardized for the detection of transgenic elements in these cotton varieties. Also, DNA extraction from various crop varieties like tomato, rice, papaya, corn, potato, brinjal and okra were standardized using different isolation protocols based on International Standards Organization (ISO) protocols for developing DNA based detection strategies for transgenic elements.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Event-specific detection of MON 531 and MON 15985 events in cotton were standardized using event-specific primers. Endogenous reference genes, being unique to a plant species, help to authenticate the crop variety being tested. These endogenous reference genes are also useful in the quantitative measurement of GMOs, as the amount of transgenic material present in a sample can be

directly correlated with the amount of endogenous reference gene in order to derive at a relative quantity of transgenic material present in the sample. For this purpose, PCR amplification of several endogenous reference genes belonging to various plant species was standardized. The following are the various crop varieties and their endogenous reference genes for which successful PCR assays were standardized: Cotton: *Sad 1* and *SAH 7* genes; Rice: Sucrose phosphate synthase (*sps)* gene and Root specific *gos 9* gene; Tomato: ascorbate peroxidase *apX* gene and Tomato anther specific *lat52* gene; Potato: Sucrose synthase *pss* gene and UDP-glucose pyrophosphorylase (*UGP*) gene; Papaya: *papain* gene; and Corn: High mobility group (*hmga*) gene.

In addition, we have also generated biological resources for quantitative detection of GM cotton using real-time PCR. We have developed indigenous reference plasmids containing the event-specific (junction) genomic regions of cotton GM events MON 531 and MON 15985. In addition, plasmids containing the endogenous reference genes were generated for the following crops: Cotton: *Sad 1* and *SAH 7* genes; Rice: *gos 9* gene; Tomato: ascorbate peroxidase *apX* gene; Potato: Sucrose synthase *pss* gene and UDP-glucose pyrophosphorylase (*UGP*) gene; and Papaya: *papain* gene. In future, we aim to develop tandem-marker plasmids containing specific target sequences for cotton and a species specific reference marker sequence in a 1:1 ratio. Once generated, tested and validated, these reference plasmids would be used as real-time PCR standards for quantification of transgenes.

Project 3: Development of novel strategies/ methodologies for enrichment of human DNA from mixtures containing human and non-human DNAs for DNA profiling-based human identification

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

The major reasons for contributing to failure of DNA profiling of skeletal exhibits for human identification purposes can be attributed to high degradation/ fragmentation and contamination of human DNA with non-human DNAs. To overcome these limitations in the forensic human DNA typing, we had reported in the previous year about the efforts to selectively enrich the human DNA from non-human DNA using human specific biotinylated oligonucleotide probes that are designed to the regions flanking the microsatellites in the human genome. In the second approach, we are trying to selectively enrich the human DNA from the mixture of microbial DNAs by employing antibodies directed against 5-methyl cytosine (5-mC), i.e., to specifically 'pull down' human DNA sequences containing 5-mC, followed by whole genome amplification (WGA) to amplify such DNA sequences. Subsequently, human short tandem repeat (STR)-containing fragments would be 'captured' and subjected to STR/mini-STR genotyping analysis to unambiguously identify the body parts/ skeletal remains of deceased persons.

Previously, we had shown, in pilot studies, that when four biotinylated STR oligonucleotides were used for 'pull-down', the precipitated DNA when subjected to STR-genotyping showed specific amplification of expected loci.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

To further improve on the biotinylated-oligonucleotide mediated pull-down of specific loci in human DNA, ten biotinylated oligonucleotides were designed in the genomic regions flanking ten different microsatellite regions in the human genome, with an aim to selectively enrich the target loci. Several combinations of different oligonucleotide probes were then tried to study the efficiency of enrichment of target regions using biotin-streptavidin affinity. This process involved hybridization of biotinylated oligonucleotides to the target regions in the human genome, followed by stringent washing to remove unbound human and non-human DNAs in order to enrich the target molecule of interest. The enriched fragments are then subjected to PCR amplification using AmpF/ STR® Identifiler® kit to identify the efficiency of enrichment. Initial attempts to selectively enrich human DNA was carried out on sonicated human DNA (~500 bp; to simulate the natural conditions), where the efficiency of enriching was tested for the ten different microsatellite loci using respective specific oligonucleotide probes. It was found that, all the ten targeted microsatellite loci were successfully enriched. When the same enrichment was performed with non-biotinylated oligonucleotides, it was observed that none of the ten microsatellite loci were enriched; showing clearly that the enrichment using biotinylated oligonucleotides is indeed specific. In future experiments, we aim to increase this biotinylated oligonucleotide mediated pull-down to all the 15 STR loci, in order to obtain a complete forensic profile in challenging skeletal samples.

In the second approach, to test whether the antibodies directed against 5-mC residue would enrich ('pull-down') human DNA sequences containing 5-mC, immunoprecipitation 'pull down' reactions were performed employing polyclonal and monoclonal antibodies directed against human 5-mC residue on sonicated human genomic DNA. The precipitated DNA when subjected to STR genotyping analysis showed amplification of only few of the 15 loci examined. Thus, to increase the efficiency of the STR analysis, in future, the above immunoprecipitated DNA would be subjected to WGA prior to STR genotyping to increase the starting template DNA.

Project 4: To study human genetic diversity in various populations groups in India

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

This is a new activity.

India represents a unique conglomeration of human genetic diversity. With a total population of ~1.2 billion, encompassing diverse genetic make-up, India is an ideal platform for performing population genetic studies. One focus of our laboratory is to perform genetic studies to understand the patterns of genetic variations in different population groups in India and use the knowledge gleaned from these studies to address questions pertaining to the occurrence, distribution, and functional effects of genetic variation/s within population groups in India; and also to address how such variation is correlated with human pre-history, evolution and migration, and demographic distribution/stratification. In the past year, our laboratory embarked on a study to understand the role of genetic variants / single nucleotide polymorphisms (SNPs) that causes skin pigmentation differences seen in different populations in India.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Skin pigmentation is a polygenic trait, and thus far, about thirty to forty genes have been identified to play a significant role in skin pigmentation in humans. Also, the recent advancements in human genomic research, especially, genome-wide association studies, and long-range haplotype structure analysis in different populations in the world have identified several genetic variants in the candidate skin pigmentation loci that play an important role in eliciting the skin pigmentation variation in human populations. Thus, our focus in this study is to validate the putative genetic variants that are associated with skin pigmentation variation in humans in Indian population – considering the observable skin pigmentation differences between South Indian and North Indian populations, as the people in the North tend to have a fairer skin tone than people in the South. Presently, we are in the process of standardizing two SNP genotyping methods, first, PIRA (Primer Introduced Restriction Analysis) PCR - a restriction fragment length polymorphism based approach, and the second is a single base extension based approach for genotyping nine variants in eight skin-pigmentation loci. Our preliminary analysis of few of these variants in South Indian and North Indian population has revealed interesting insights, and in the coming year, we aim to intensify this study by collecting large number of samples from South Indian and North Indian populations along with a precise measurement of skin- melanin index in order to correlate our genotypes to the actual phenotype to understand this complex phenotype in a better way.

Laboratory of Fungal Pathogenesis

Understanding the pathobiology of an opportunistic human pathogen, *Candida glabrata*

Principal Investigator	Rupinder Kaur	Staff Scientist
Ph D Students	Gaurav	JRF
	Maruti Nandan Rai	JRF
	Sapan Borah	JRF
Other members:	K G Rajaneesh	Project Assistant
	G Neelima	Project Assistant

Candida glabrata is an opportunistic human fungal pathogen that normally resides as a commensal in the mucosal tissues of healthy individuals but under conditions of immuno-compromise can cause both superficial mucosal and life threatening systemic infections. *C. glabrata* accounts for about 16-20% of total Candida blood stream infections worldwide and is the second or third most common cause of Candidiasis. In addition, *C. glabrata* infections are usually associated with a high mortality rate, probably in part, due to its innate resistance to the most widely used anti-fungal drug, fluconazole. Research in our laboratory is centered on a better understanding of *C. glabrata*'s interaction with host immune cells and its resistance towards anti-fungal drugs.

Project 1: Functional genomic analysis of *C. glabrata*-macrophage interaction

Objectives:

1. Analysis of intra-cellular behavior
2. Screening of *C. glabrata* mutant library for altered survival profiles
3. Identification and analysis of the genes required for survival *in-vitro*

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

An *in-vitro* system consisting of human monocytic cell line THP1 was established to study the molecular interactions of *C. glabrata* with macrophages which in conjunction with neutrophils constitute the first line of host defense against this pathogenic yeast. Infection studies of PMA (Phorbol-12 Myristate 13-acetate) differentiated THP1 cells with wild-type *(wt) C. glabrata* cells at a MOI (multiplicity of infection) of 10:1 revealed that instead of being killed by activated human monocytes (macrophages), *wt C. glabrata* cells undergo a moderate 5-7 fold replication over a period of 24 hours. However, a *C. glabrata* strain disrupted for putative cell surface associated aspartyl proteases was rapidly killed by macrophages displaying a viability of 1.34% after 24 hrs of co-incubation with macrophages.

Details of progress made in the current reporting year (April 1, 2008–March 31, 2009)

Since our objective is to screen a *C. glabrata* mutant library for altered survival profiles in macrophages *via* a modified version of signature tagged mutagenesis (STM) approach, we first standardized the conditions for such a screen using differentiated THP1 cells. The mutant library to be used for our modified version of STM is comprised of 18,432 mutants and was generated by homologous recombination of *in-vitro* generated Tn*7* insertions in *C. glabrata* genomic clones. 96 wild-type *C. glabrata* strains (each strain contains a unique sequence (about 60bp) flanked by common PCR priming sites integrated at the deleted *ura3* locus) were used to make this library. These mutants have been assembled in a total of 192 pools wherein each pool is comprised of 96 mutants. Each of these pools carries the same set of 96 tags but within a pool each mutant carries a different tag thereby allowing a parallel analysis of 96 mutants at once. For a pool of tagged mutants, the ratio of hybridization in the output and input pools reflects any shift in the representation of the corresponding mutant in the pool.

These 96 unique tag sequences have individually been cloned in pUC19 plasmids and plasmid DNA containing the tags was prepared from ninety six *E. coli* clones. DNA samples were quantitated, denatured, and spotted on Hybond nylon membranes (about 200ng per plasmid) using 96-well Dot Blot apparatus. The spotted DNA was cross linked by UV cross linker. To do quality control tests on these filters, membranes were either

hybridized with a mutant pool containing all 96 tags or with a pool containing only two tagged mutants (Tag12 and Tag96). As expected, the first set of hybridization yielded an almost uniform signal for all 96 tags while the second set of hybridization lighted up spots corresponding to Tag12 and Tag96 on the membrane thus excluding the possibility of any cross-hybridization amongst the tags.

After standardizing the membrane filter preparation and hybridization conditions, we started screening the mutant pools for altered survival profiles in THP1 cells. Briefly, *C. glabrata* mutant pools were grown in YPD at 30ºC for overnight and PBS washed overnight culture was used to inoculate input and output. For input, each mutant pool was grown for overnight in the YPD media at 37ºC so as to exclude temperature sensitive mutants from our screen. For output, each pool of 96 tagged mutants was used to infect differentiated THP1 cells ($1X10^6$) in 24 well plates at a MOI of 1:10. At 24 hrs post infection, intracellular yeast were recovered by lysing macrophages in water and the lysate was used to re-infect THP1 cells. A total of three rounds of macrophage infection were carried out so as to enrich for the desired mutants in the final population.

Cell pellets from both input and output (in duplicates) were collected, genomic DNA was prepared and the first round of PCR was carried out using a primer set that bind to the common region flanking each tag sequence. An aliquot of primary PCR was used as template for the second round of PCR to generate labeled probes with a PCR mixture containing P^{32} labeled á-dCTP. The labeled PCR product from both input and output was hybridized to the membrane filters blotted with 96 unique tag sequences (as described earlier). Ratio of output/input was quantitated by phosphorimager for each pool. The mutants with an output/input ratio of =3 and = 0.33 were selected as 'up' (increased survival) or 'down' (reduced survival) mutants respectively (Fig.1) We have screened 48 mutant pools (4800 mutants) in THP1 cells so far and interestingly, mutants carrying the same tag but representing different pools are coming through our screen multiple times displaying the same altered survival phenotype. Since during mutant library generation, a strain carrying a particular tag was transformed with the same mutagenized *C. glabrata* genomic fragment and multiple mutants (up to 96) carrying Tn*7* insertions in the corresponding genomic loci were selected, it's possible that these altered macrophage survival mutants have insertions in the same gene but at different positions. We are planning to test this by mutant rescue and sequencing analysis.

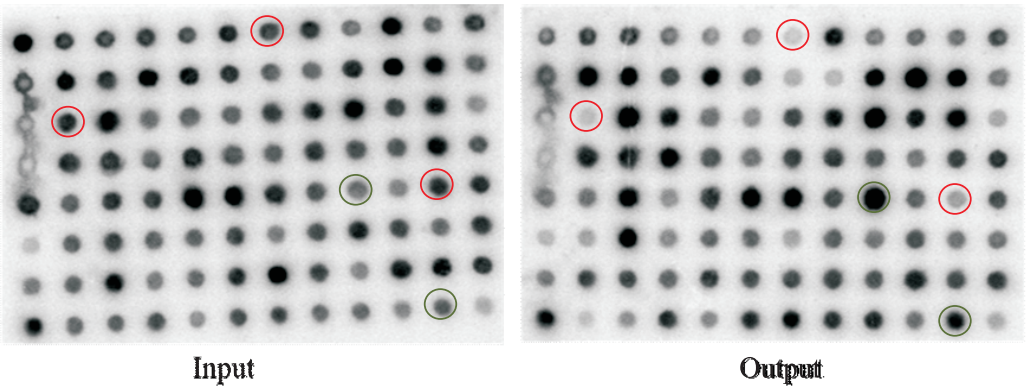

Figure1 : Filters hybridized with labeled PCR products amplified from mutant Pool 11 grown either in the media (input) or recovered after co-incubtion with THPI cells (Output). Up and Down mutants are encircled in green and red respectively.

Project 2: Innate resistance of *C. glabrata* to fluconazole

Objectives:

1. Identification and characterization of fluconazole sensitive mutants
2. Understanding the molecular basis of high innate resistance of *C. glabrata*

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

To better understand the inherent low fluconazole susceptibility of *C. glabrata*, fluconazole sensitive mutants were tested for their inability to survive fluconazole stress by CFU assay. One of such mutant, *flv1::Tn7* was found to rapidly lose viability upon fluconazole exposure and also showed sensitivity to other stresses such as high temperature (37ºC) and cell wall damaging agents (calcofluor white and caffeine). Since this mutant contained a Tn*7* insertion in the 3' region of *FLV1*, a complete knock-out was generated in the *wt* background by both one step and two-step gene replacement methodology using HYG cassette (*hph* gene (confers hygromycin resistance) flanked by 5' and 3' UTR of *FLV1*). The disruption of the gene was confirmed by PCR using appropriate primer sets. Surprisingly, the *flv1* deletion strains generated by both strategies showed resistance towards fluconazole and this resistance was not due to loss of mitochondrial function.

Details of progress made in the current reporting year (April 1, 2008–March 31, 2009)

The differential susceptibility of a *flv1::Tn7* insertional mutant and a *flv1* knock-out strain (*flv1?::hph*) towards fluconazole could be attributed to strain variations, disruption strategies, multiple integrations of the disruption cassette in the knock-out strains or presence of secondary site suppressor mutations. To address these possibilities, we have under taken following approaches.

1. Southern analysis to check for multiple integrations of the disruption cassettes in *flv1?::hph* strains
2. Creating *flv1* deletions in different strain backgrounds
3. Regeneration of the *flv1::Tn7* insertional mutant in the wild-type background
4. Complementation analysis of fluconazole sensitivity and fluconazole resistance phenotypes of *flv1* mutant strains by expressing *FLV1* gene from a plasmid

Firstly, to rule out the possibility of multiple integrations of the disruption cassette in the created *flv1* knock-out strains, that may have led to the disruption of another gene in addition to *FLV1* disruption, thus resulting in fluconazole resistance phenotype, we carried out Southern analysis on the genomic DNA of *wt* and *flv1?::hph* strains. Genomic DNA was digested with Sac*I*, blotted onto the membrane and hybridized with a probe specific either to *FLV1* or to *hph (*disruption cassette). As shown in figure 2, a band of 0.66kb corresponding to *FLV1* gene appeared only in the *wt* strain confirming the complete deletion of *FLV1* gene in all the knock-out strains. Hybridization with the *hph* cassette specific probe yielded a single band in all the *flv1* deletion strains (*flv1?::hph*) indicating that there are no multiple integrations of the *hph* cassette in any of the KO background. Similar results were obtained with Southern analysis of Hind*III* digested gDNA of various strains.

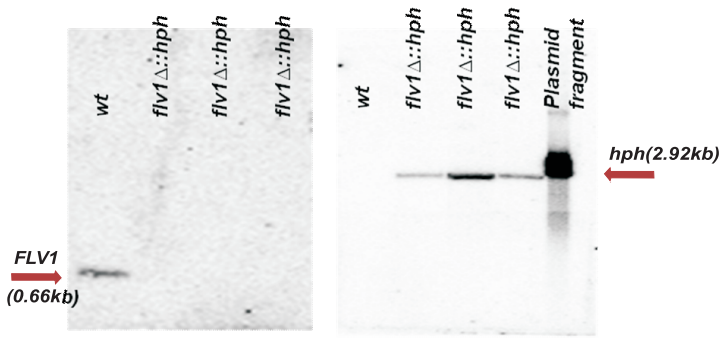

Figure 2. Southern blot analysis of SacI digested genomic DNA from wt and flv1 hphstrains demonstrating the disruption of FLV1 with hph gene

Next, to check if the difference in the fluconazole susceptibility of *flv1::Tn7* insertional mutant and *flv1?::hph* deletion strain is due to the difference in the parental background (Tn7 insertional mutant was originally made in URA$^-$ *wt* strain while the KO was constructed in URA$^+$ *wt* background), we disrupted *FLV1* gene in both URA$^-$ and URA$^+$ *wt* strain by one step methodology. A total of five URA$^-$ and three URA$^+$ *flv1?::hph* strains were selected and tested for their fluconazole susceptibility phenotype. Interestingly, all the strains showed a clear resistance towards fluconazole suggesting that the

fluconazole phenotype is independent of the parental strain background.

Thirdly, we decided to regenerate the original Tn*7* insertion in the wild-type background. For this, Tn*7*-*hph* cassette was recovered from *flv1::Tn7* strain by digesting the gDNA with Mfe*I* enzyme followed by self-ligation and transformation into *E.coli* BW24373 strain. The plasmids thus obtained were used to transform *wt C. glabrata* strain to uracil protrophy. Transformants were tested for Tn*7* insertion in the *FLV1* locus by PCR and sequencing analysis. As expected, the newly generated *flv1::Tn7* insertion mutants were found to be fluconazole sensitive.

Lastly, we amplified the full length *FLV1* gene from *wt* gDNA and cloned it under the PGK promoter. The complementation of fluconazole resistance phenotype of *flv1?::hph* strains by plasmids carrying *FLV1* ORF was tested by serial dilution spotting assay on plates. As shown in figure 3, *FLV1* expression restored the wild-type-like phenotype in the *flv1?::hph* strains.

Having established that both the fluconazole sensitivity of *flv1::Tn7* mutant and fluconazole resistance of *flv1?::hph* strain are the bona-fide phenotypes of *flv1* mutant strains, we are currently trying to understand the molecular basis of these phenotypes by conducting Northern analysis on genes that are known to be involved in fluconazole resistance and biochemical studies to check the functionality of Flv1p in *flv1::Tn7* mutant.

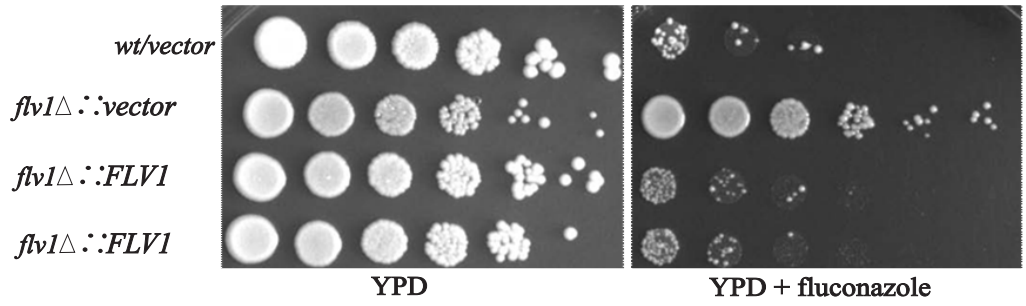

Figure 3. Growth profile of wt and flv hph on YPD and YPD containing 64mg/ ml fluconazole. Fluconazole resistance of flv1 hph strain is complement by plasmid-bome FLV1

Publications:

Kaur R (2009) Review of: *Lilavati's Daughters: The Women Scientists of India*, Edited by Rohini Godbole and Ram Ramaswamy, Indian Academy of Sciences, Bangalore, 2008, 369 pages. ***Current Science*** (In press).

Laboratory of Molecular Virology

Molecular Pathogenesis of lentiviruses

Principal Investigator	S Mahalingam	Staff Scientist
Ph D students	M K Subba Rao	SRF
	Gita Kumari	SRF

Objectives:

1. Understanding the mechanism of human immunodeficiency virus pathogenesis.
2. Functional analysis of putative Nucleolar GTPases.
3. Understanding the function of Ras effector proteins.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Recent reports suggest that Ras proteins are localized in different intracellular compartments in addition to their known association with the plasma membrane and the function of various tumor suppressor genes (TSGs) depends upon their sub-cellular localization. Proteins with molecular masses of less than 35-40 kDa can enter the nucleus by passive diffusion rather than by a signal-mediated process. To distinguish between these two possibilities, we evaluated the transport activities of Ras associated TSGs in the context of a chimeric protein designed to exceed the diffusion limit of the nuclear pore. Enhanced Green Fluorescent Protein (eGFP) fusion proteins of RASSF1A, RASSF1C, RASSF2, and RASSF5 were generated and expressed in Cos-7 cells to characterize their cellular localization. Results suggest that RASSF1A is associated with the microtubule assembly whereas RASSF1C, a variant of RASSF1, was localized in the nucleus as punctate pattern in addition to its microtubular association. RASSF2 was localized to the nucleus, and excluded from nucleolus. However, RASSF5 was localized both in the nucleus and nucleolus. In contrast, GFP was found to be distributed throughout the cell, consistent with its passive diffusion. We generated both N-terminal and C-terminal tagged fusion proteins with Flag or GFP and our results showed that the RASSF5 containing Flag or GFP at the N-terminus localized in the cytoplasm. In contrast, RASSF5 fusion protein containing tag (either GFP or Flag) at the C-terminus localized to the nucleus. Interestingly, we observed the cytoplasmic localization of RASSF5 in lung adenocarcinoma cell line, A549 consistent with the previous report by Moshnikova et al. Surprisingly, GFP-RASSF5 fusion protein translocated to the nucleus when A549 or Cos-7 were cells treated with well-known nuclear export inhibitor, leptomycin B (LMB) suggest that RASSF5 may be a nucleo-cytoplasmic shuttling protein. RASSF5 with C-terminal fusion of GFP was used for further analysis since this fusion protein localized to the nucleus and can be visualized directly in living cells without antibody staining. In conclusion, RASSF1C, RASSF2, and RASSF5 appear to possess specific signals and transported into nucleus by a signal-mediated process. Based on the basal distribution of GFP-fusion proteins, we used GFP as a living tracer to understand the mechanism(s) of RASSF1C, RASSF2, and RASSF5 nuclear transport.

Details of work done in the current reporting year (April 1, 2008 - March 31, 2009)

The following significant findings were made by the group in the current year.

HIV-1 Vpr: a closer look at the multifunctional protein from the structural perspective: The human immunodeficiency virus-1 (HIV-1) Vpr protein plays multiple roles in HIV-1 replication. In early infection, Vpr provides help in the nuclear localization of pre-integration complex. Subsequently, Vpr induces cell cycle arrest of infected cells at G2/M phase. Cell cycle arrest facilitates higher rate of viral gene transcription. Vpr is also capable of activating transcription of viral and heterologous genes. Vpr induces apoptosis in infected cells leading to loss of immune cells and onset of clinical AIDS. Interestingly, Vpr is also considered as a passenger protein in the virus particles as it is incorporated into the virus particles through interaction with Gag. The structure of full length Vpr has been resolved recently through NMR. In this review, we have analyzed the functions of Vpr using the available data from structural perspective. Packing of the three helices of Vpr around a core formed by hydrophobic side chains and integrity of helical domains are critical for Vpr functions. The distinct functions of Vpr have been attributed to

structural integrity of different domains. The unique distribution of acidic and basic residues in Vpr is an interesting feature. Two hydrophobic pockets on the structure of Vpr are proposed to be important targets for modulating Vpr functions. The inter-relationship between different functions of Vpr is discussed in the context of structure. . Based on bioinformatics analysis, we propose new targets for modulating Vpr functions, which need to be validated experimentally.

Evidence for Multiple Mechanisms Regulating Nucleolar Retention of Breast Cancer Autoantigen, NGP-1(GNL2):

NGP-1 is a putative GTPase, highly expressed in breast carcinoma and localized to the nucleolus. NGP-1 belongs to the MMR1-HSR1 family of large putative GTPases which are emerging as crucial coordinators of signal cascades orchestrating between nucleolus and nucleus and even between cytoplasm and nucleolus. As the members of this family share very closely related G domains with similar cellular localization, it is important to study whether these proteins are regulating same function(s) differentially or different functions in similar manner or involved in redundant cellular functions? To this end, we have characterized the pathways and the mechanism of NGP-1 nucleolar transport and whether the GTP binding play any role in its nucleolar retention. In the present study, we have demonstrated the presence of two functional Nucleolar Localization Signals (NoLS) signals within NGP-1 and both signals independently translocate NGP-1 as well as the heterologous proteins to the nucleolus. Site-directed mutagenesis analysis of both amino (N) and carboxyl (C) terminus NoLSs revealed that the presence of tandem repeats of positively charged amino acids contributing to the NoLS function. Interestingly, both signals are interacting with transport receptor, importin-b for efficient nucleolar retention of NGP-1. In addition, our data suggest that the nucleolar localization of NGP-1 is regulated by GTP gating mediated mechanism. Structural analysis and the alanines scanning mutagenesis of conserved residues within G-domains indicate that the G4 and G5 motifs are crucial for GTP binding and subsequent retention of NGP-1 into nucleolus. Upon actinomycin D treatment, NGP-1 showed a diffused distribution through out the nucleus suggesting that the on-going transcription play an important role in NGP-1 localization in the nucleolar compartment. Finally, our results indicate that the expression of NGP-1 was induced upon stimulation of primary human peripheral blood mononuclear cells (PBMC) with Concanavalin A. In addition, we have observed high level expression of NGP-1 in transformed cells suggesting that increased level of NGP-1 expression may induce cell proliferation. Together, our present data suggest that multiple mechanisms modulate nucleolar retention of NGP-1 to tightly regulate nucleolar function on cell growth and proliferation.

ERK-2 Mediated Phsophorylation Regulates Nucleo-Cytoplasmic Shuttling and Cell Growth Control of Ras Associated Tumor Suppressor Protein, RASSF2.

The Ras GTPase controls the normal cell growth through binding with an array of effector molecules, such as Raf and PI3-kinase in a GTP dependent manner. RASSF2, a member of the Ras association domain family, known to be involved in the suppression of cell growth and is frequently down regulated in various tumor tissues by promoter hypermethylation. In the present study, we demonstrate that RASSF2 shuttles between nucleus and cytoplasm by a signal mediated process and its export from the nucleus is sensitive to leptomycin B. Amino acid domain 240-260 in the carboxyl terminus of RASSF2 harbors a functional nuclear export signal which is necessary and sufficient for efficient export of RASSF2 from the nucleus. Substitution of conserved Ile254, Val257 and Leu259 within the minimal NES impaired RASSF2 export from the nucleus. In addition, wild type but not the nuclear export defective RASSF2 mutant interacts with export receptor, CRM-1 *in vivo* and exported to the cytoplasm. Surprisingly, we observed the nucleolar localization for the nuclear export defective mutant suggests the possibility that RASSF2 may localize in nucleolus, nucleus and cytoplasm and observed nuclear localization for the wild type protein may be due to the faster export kinetics from the nucleolus. Furthermore, our data suggests that inhibitors of MAPK/ERK-2 pathway block the phosphorylation and export of RASSF2 from nucleus clearly indicate that phosphorylation play a critical role in modulating the nucleo-cytoplasmic shuttling of RASSF2. RASSF2 induces the cell arrest at G1/S phase and apoptosis. Interestingly, nuclear export defective but not nuclear import defective mutant of RASSF2 maintain the wild type cell cycle arrest and apoptosis suggest that nuclear localization of RASSF2 is important for cell cycle regulation and apoptosis. Taken together, these data suggest that active transport between nucleus and cytoplasm

may constitute an important regulatory mechanism for RASSF2 function. Collectively, our data provided evidence for the first time that MAPK/ERK-2 mediated phosphorylation regulates RASSF2 nucleo-cytoplasmic shuttling and the nuclear localization is critical for regulating cell growth and apoptosis.

Nuclear localization of RASSF2 is required for its anti-proliferative activity: Nucleo-cytoplasmic transport has been described for several molecules that are involved in controlling cell growth and proliferation. Interestingly, many functions of tumor suppressor proteins such as p53, BRCA1, INI1 and ING1 are regulated through their localization within a specific sub-cellular compartment and the mislocalization resulted in alteration of these tumor suppressors function and leads to initiation and progression of cancer. In order to determine whether localization of RASSF2 in a specific cellular compartment is critical for its ability to regulate the cell cycle progression, we co-transfected wild type, nuclear import defective (RASSF2 ?NLS) and export defective (RASSF2 ?NES) mutants of RASSF2 with pBABE puro vector in Cos-7 cells. After three days of selection in puromycin (3µg/ml), cells were fixed, stained with propidium iodide and examined by Fluorescence Activated Cell Sorter (FACS) to analyze the cell cycle profile. Our results indicate that more number of cells accumulated in G1/S phase (2N amount of DNA) from wild type (57%) and nuclear export defective (RASSF2?NES) mutant (58%) transfected cells. However, nuclear import defective mutant of RASSF2 (RASSF2?NLS) failed to arrest the cells at G1/S as like the empty vector. These results clearly indicate that RASSF2 with wild type nuclear localization is critical for regulating cell cycle at G1/S phase.

We next investigated the RASSF2 induced apoptosis. To analyze the RASSF2 induced apoptosis, Cos-7 cells were transfected with wild type, nuclear import defective mutant, nuclear export defective mutant of RASSF2, and vector alone along with pBABE-puro plasmid. After three days selection with puromycin, cells were probed with FITC-conjucated annexin-V and propidium iodide. Annexin-V interacts with phosphatidylserine exposed in the plasma membrane of cells, which is considered an early apoptosis marker. We performed the double staining to distinguish viable cells from non-viable cells. Results in figure 6B indicate that significant number of cells transfected with wild type RASSF2 were annexin V-FITC positive and propidium iodide negative. However, we observed similar patterns of annexin V-FITC staining from the cells transfected with nuclear export defective mutant (RASSF2?NES) but not with nuclear import defective mutant (RASSF2?NLS) expressing cells. These data provided evidence that nuclear localization is critical for RASSF2 induced apoptosis and further suggest that the observed apoptosis may be a consequence of the cell cycle arrest induced by RASSF2.

Further evidence for RASSF2 induced apoptosis was obtained by caspase-3 activation and PARP cleavage assay. Caspase-3 is one of the major effector caspase in the apoptosis process and its cleavage is one of the classical apoptotic features. To determine whether RASSF2 induces caspase-3 activation, we assessed caspase-3 expression in cells transfected with nuclear import and export defective mutants of RASSF2 by Western blot analysis using caspase-3 specific antibodies. Our results indicate that a 17-kDa active fragment of caspase-3 appeared in the wild type and nuclear export defective mutant but not in nuclear import defective mutant of RASSF2 and GFP transfected cells. These data suggest that the observed apoptosis may be due to the induction of caspase-3 activation by RASSF2. Induction of apoptosis was further confirmed by PARP cleavage assay. PARP is an enzyme involved in DNA repair mechanism and is cleaved by caspase-3 from the native 115 kDa to 85-kDa protein. A significant increase in PARP cleavage was observed in nuclear export defective mutant similar to wild type RASSF2. However, no cleavage of 115 kDa PARP was observed in nuclear import defective mutant of RASSF2 and GFP transfected cells. Wild type, nuclear import and export defective mutants of RASSF2 and GFP were expressed efficiently and we used equal amount of cell lysates for determining the caspase-3 and PARP. These results clearly indicate that RASSF2 induces both early as well as late stages of apoptosis. Taken together, our results provided evidence that RASSF2 induces cell cycle arrest at G1/S and apoptosis in a nuclear localization dependent manner.

Laboratory of Immunology

Understanding and regulation of inflammatory and Tumorigenic responses

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	Sidharth Mahali	JRF
	S Adeel Husain Zaidi	JRF
Other Members	N Ravi Prakash	Project Assitant
	Damodar Edupalli	Project Assistant
	T Navneeta	Project Assistant
Collaborators	GT Ramesh	TSU, Texas, USA
	Chitta S Kumar	SK Univ., AP

Objectives

1. Designing and detection of novel pathway mediated by novel small molecules to induce anti-inflammatory and anti-tumorigenic responses.
2. Regulation of TNF receptors in different cell types.

Summary of work done until the beginning of this reporting year (April 1, 2007- March 31, 2008)

In the present study, we demonstrate the biological activity of esterified caffeic acid with methyl vanillate, methyl 4-[{(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl}oxy]-3-methoxybenzoate**,** also termed as caffeic acid methyl vanillate ester (CAMVE). Our study demonstrates that esterified caffeic acid with methyl vanillate, termed as caffeic acid methyl vanillate ester (CAMVE) down-regulates inflammatory responses through inhibition of NF-kB and dependent several genes expression, further suggesting its efficacy as a promising therapeutic agent (**J.Clin. Immunol., 2009, 29: 90-98**). CAMVE potentiated TNF-induced cell death. CAMVE inhibited cell proliferation by inhibiting G1 to S phase progression. It suppressed TNF-induced Bcl-2 expression and potentiated chemotherapeutic agents mediated cell death. CAMVE activated calcineurin and helped nuclear translocation of NF-AT that expressed FasL thereby induced cell death. Overall, our results indicate that the combinatorial synthesis of novel caffeic ester derivatives can be a good approach to bioactive chemotherapeutic development **(J. Cell. Physiol., 2009, 218: 653-662)**.

Details of progress in the current reporting year (April 1, 2008 - March 31, 2009)

(A) Decrease in RelA phosphorylation by inhibiting protein kinase A induces cell death in NF-kB-expressing and drug-resistant tumor cells

The RelA (p65) is a subunit of NF-kB and actively participates in inflammation and tumorigenesis. Hence, the regulation of p65 is an important strategy to regulate those responses. In this study we provide data that the dichlorophenyl derivative of 1,2,4-thiadiazolidine (known as P_3-25) induced cell death in NF-kB-expressing [U-937 (transfected with vector alone or *p65* constructs) and HuT-78 (constitutively expressed NF-kB)], but not in peripheral blood mononuclear cells (PBMC) isolated from fresh human blood (Fig.1A). P_3-25 potentiates different chemotherapeutic drugs-mediated cell death (Fig.1B) without increasing the levels of LDH in these treatments (data not shown). P_3-25 inhibited NF-kB DNA binding activity partially, but inhibited NF-kB-dependent genes expression completely. It inhibited phosphorylation of Rel A (p65) by inhibiting activity of protein kinase A (PKA), whereas partial inhibition of IKK activity was observed at 6 h of P_3-25 treatment (Fig.1C). The IC50 (50% inhibitory concentration) of P_3-25 was 10.5 nM for PKAa as detected by measuring the incorporated ^{32}P to kemptide by recombinant PKAa treated with P_3-25 (Fig.1D). The PKA inhibition was independent of adenylate cyclase activity or cAMP level. Our results suggest that P_3-25 inhibits PKA activity followed by decreased phosphorylation of p65 and transcriptional activity of NF-kB thereby decreasing antiapoptotic proteins resulting in induction of

apoptosis in NF-kB-expressing and Doxorubicin-resistant cells. The study might help to understand the mechanism of P_3-25-mediated apoptosis and to design it as new chemotherapeutic drug for tumor therapy.

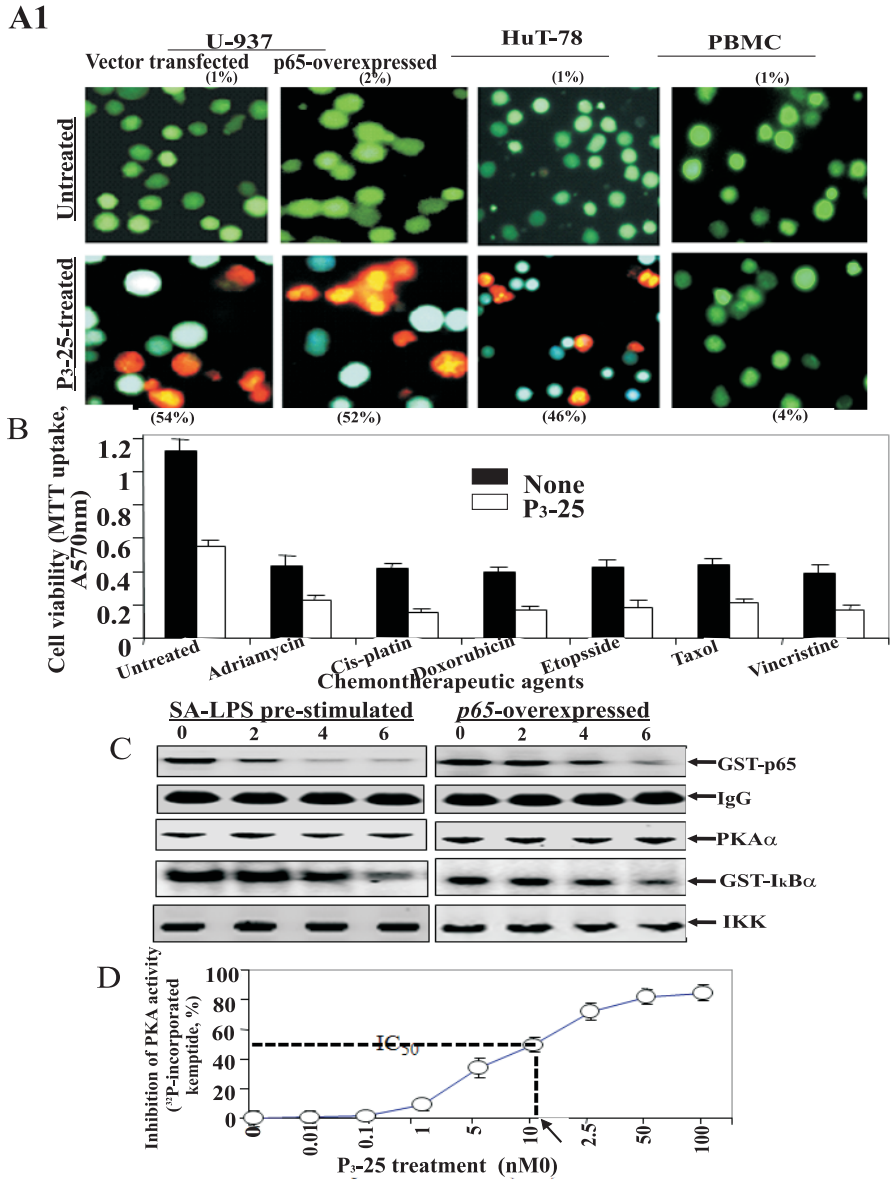

Figure-1: P_3-25 induces chemotherapeutic drug-iduced cell death and activates PKAa. P_3-25 induced cell death (red in color) as detected by 'Live & Dead assay' in NF-kB expressing cells (p65-transfected Jurkat and HuT-78) and PBMC (A). P_3-25 potentiated different chemotherapeutic-drugs mediated cell death as detected by cell viability assay (B). P_3-25 decreased PKAa, but not IKK activity in SA-LPS or p65-transfected cells as detected by the amounts of phosphorylation of substrate proteins (C). P_3-25 inhibited PKAa activity as shown by the amounts of ^{32}P-incorporated kemptide (D).

(B) Decrease in RelA phosphorylation by inhibiting protein kinase A induces cell death in NF-kB-expressing and drug-resistant tumor cells

Doxorubicin is one of the most effective agents used in the treatment of various tumors. Its use is restricted by the development of resistance to apoptosis, the mechanism of which is not fully understood. Nuclear transcription factor kappa B (NF-kB) has been shown both to block apoptosis and to promote cell proliferation, and hence has been considered as an important target for anticancer drug development. We found that in wild type and Dox-revertant MCF-7 cells, Doxorubicin induced NF-kB was transient and Dox-resistant cells showed high basal activity of NF-kB (Fig.2A)

and expression of genes dependent on it. Moreover, in resistant cells Doxorubicin was unable to induce apoptosis as detected by assays for reactive oxygen intermediates generation, lipid peroxidation, cytotoxicity, PARP degradation (Fig.2B) and Bcl-2 expression. High basal expressions of multi-drug resistant protein and transglutaminase were found in Dox-resistant cells and inhibition of NF-kB by multiple ways decreased those amounts (Fig.2C) and also sensitized these cells by Doxorubicin. The high basal expression of SOD1 was decreased in NF-kB-downregulated Dox-resistant cells (Fig.2D). The amount of Mdr decreased in Dox-resistant cells when NF-kB was downregulated (by incubating cells with BAY) (Fig.2E). The high basal expression of Mdr was decreased kinetically by BAY-treated Dox-resistant cells as shown by Western blot (Fig.2F). This study provides evidence in support of the concept that high levels of NF-kB confer resistance to Doxorubicin in breast tumor cell lines. Doxorubicin induced NF-kB transiently may in some way be responsible for some of the cells becoming resistance to cell death. Considering Doxorubicin's usefulness in treating different tumors alone or in combination generating resistance against it caused much distress to these patients. Understanding the mechanism of this resistance against Doxorubicin is of value.

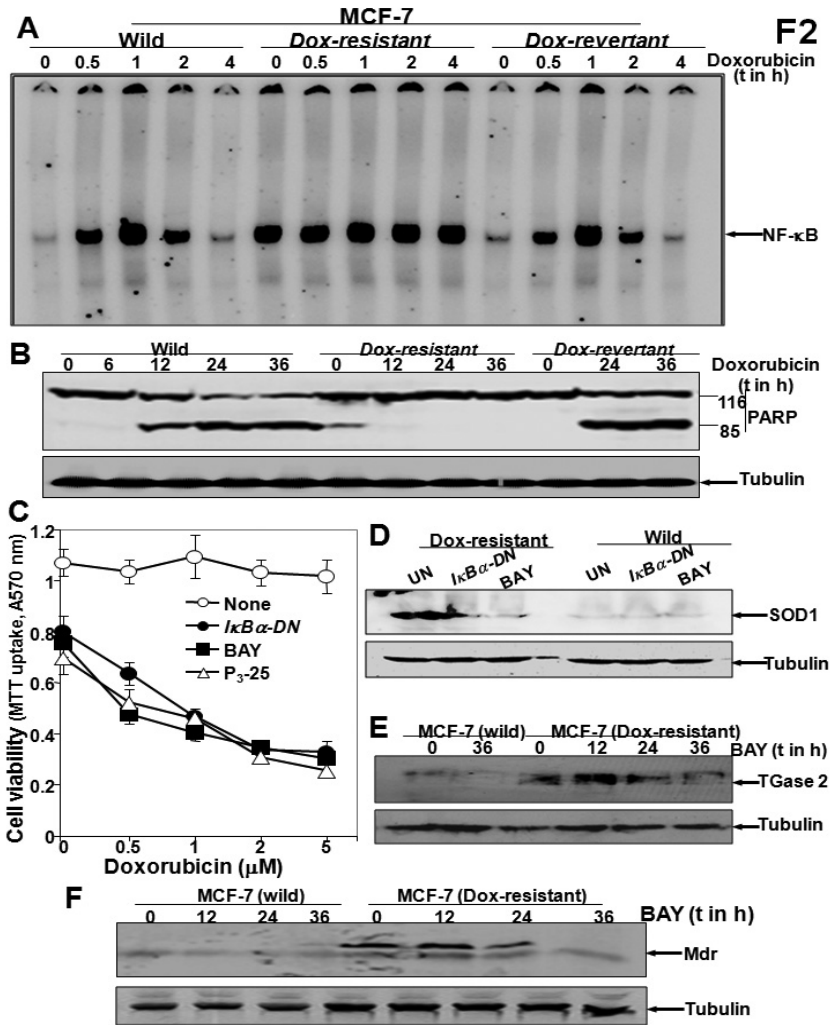

Figure-2: Decarese in NF-kB decreases SOD1, transglutaminase (TGTase) and Mdr and sensitizes Dox-resitant cells to apoptosis. Doxorubicin induced NF-kB in MCF-7 wild-type and Dox-revertant cells, but showed bigh basal activity in Dox-resistant cells as shown by gel shift assay (A). Doxorubicin treatment increased PARP cleavage in MCF-7 wild-type and Dox-revertant, but not in Dox-resistant cells (B). NF-kB downregulation (transfecting by IkBa-DN construct or treated with BAY or P_3-25) sensitized Dox-resitant cells to apoptosis as measured by cell viability (C). Dox-resistant cells showed high basal expression of SOD1, TGase 2, or Mdr and BAY treatment decreased the amounts of all these (D, E, & F).

(C). Interaction of dichlorophenyl form of thiadiazolidine with TRAF2 inhibits nuclear factor kappaB, but induces c-Jun N-terminal kinase-Activator protein-1 pathway

The compound 5-(4-methoxyarylimino)-2-N-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25) is known to possess anti-bacterial, anti-fungal, and anti-tubercular activities. In this report we provided evidences that P_3-25 inhibits nuclear transcription factor kappaB (NF-kB), which induces inflammatory and tumorigenic responses, but activates another transcription factor, activator protein (AP)-1. P_3-25 does not inhibit TNF receptor associated factor (TRAF) 6-mediated NF-kB DNA binding, but inhibits TRAF2-mediated NF-kB activation. P_3-25 interacts with TRAF2 and inhibits association of TANK (TRAF for NF-kB), but facilitates binding of mitogen activated protein kinase kinase kinase (MEKK)1 thereby activates c-Jun-terminal kinase (JNK) and AP-1. For the first time, we are providing evidences that suggest the interaction of P_3-25 with TRAF2 leads to inhibition of NF-kB and activation of AP-1 pathways. These results suggest novel approaches to design P_3-25 as an anti-cancer/-inflammatory drug for therapy through regulation of TRAF2 pathway.

P_3-25 inhibits TRAF2-, but not TRAF6-mediated DNA binding of NF-kB and activity of IKK. P_3-25 treatment showed decrease in NF-kB DNA binding in TRAF2-, but not in TRAF6-transfected or TRAF2 and TRAF6 co-transfected cells (Fig.3A). Transfection of *TRAF2* or *TRAF6* induced IKK activity. P_3-25 treatment of such cells decreased IKK activity indicated by decreased intensity of the labeled GST-IkBa substrate bands in a time-dependent manner (Fig.3C). The high IKK activity remained unaltered with increasing time of P_3-25 treatment in *TRAF6* transfected cells. These data suggest that P_3-25 interferes with TRAF2-mediated signaling at upstream of IKK level. These data suggest that P_3-25 might have other effect besides TRAF2 in inhibition of NF-kB.

P_3-25 induces TRAF2- or TRAF6-mediated activation of AP-1 and JNK. P_3-25 induced AP-1-DNA binding activity in vector-transfected cells. High basal AP-1-DNA binding remained unaltered by P_3-25 treatment in *TRAF2*, *TRAF6*, or *TRAF2* and *TRAF6* transfected cells (Fig.3B). Basal activity of JNK increased in TRAF2- or TRAF6-transfected cells. P_3-25 enhanced activation of JNK in a time-dependent manner in these cells (Fig.3D). These data further support the AP-1 activation upon P_3-25 treatment through JNK activation via TRAF2 and TRAF6 pathway.

P_3-25 interferes TRAF2-TANK, but not TRADD-TRAF2 or TRAF2-MEKK1 interaction. TRADD-TRAF2, TRAF2-TANK, and TRAF2-MEKK1 binding sites were identi?ed. The P_3-25 or 5-aryl TZD binding sites with TRADD-TRAF2, TRAF2-TANK, and TRAF2-MEKK1 complexes were predicted and shown by surface binding in Fig.3E. Auto Dock binding affinities of P_3-25 and 5-aryl TZD into TRADD-TRAF2, TRAF2-TANK, and TRAF2-MEKK1 were evaluated by the binding free energies (ÄGb, kcal/ mol), inhibition constants (Ki), hydrogen bonds, and RMSD values. On the basis of these values, we commented –

- interactions of TRADD-TRAF2 complex with P_3-25 or 5-aryl TZD are very weak.
- P_3-25 has more binding affinity than 5-aryl TZD TRAF2-TANK complex.
- the complex of TRAF2-MEKK1 with P_3-25 showed very less interactions in compare with TRAF2-TANK or TRADD-TRAF2 complex.
- synthetic P_3-25 molecule more potently inhibits the TRAF2-TANK complex than 5-aryl TZD

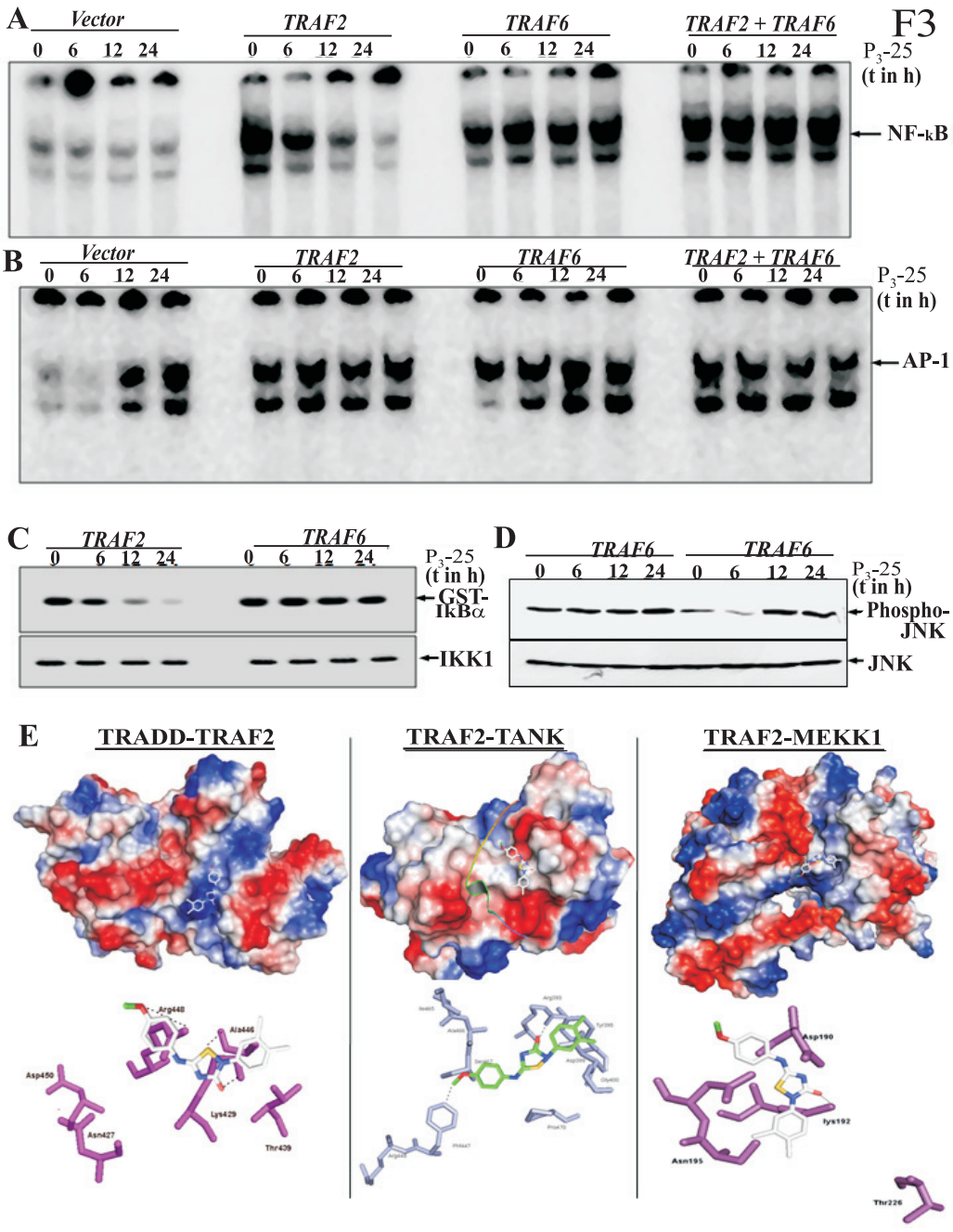

Figure-3: P_3-25 inhibits TRAF2-, but not TRAF6-mediated NF-kB and IKK activation; induces TRAF2- and TRAF6-mediated AP-1 and JNK activation; inhibits TRAF2-TANK, but not TRADD-TRAF2 or TRAF2-MEKK complex. P_3-25 inhibited NF-kB (A) and IKK activity (C) in TRAF2-, but not TRAF6-transfected U-937 cells. It was unable to inhibit AP-1 DNA binding (B) and JNK activation (D) mediated by TRAF2 or TRAF6. Docking study shows the inneraction of P_3-25 with TRADD-TRAF2, TRAF2-TANK, and TRAF2-MEKK1 complexes (E).

Publications :

1. Bose JS, Gangan V, Jain SK and Manna SK (2009) Downregulation of inflammatory responses by novel caffeine acid ester derivative by inhibiting NF-kappaB. ***Journal of Clinical Immunology*** 29: 90-98.
2. Bose JS, Gangan V, Jain SK and Manna SK (2009) Novel caffeic acid ester derivative induces apoptosis by expressing FasL and downregulating NF-kappaB: Potentiation of cell death mediated by chemotherapeutic agents. ***Journal of Cellular Physiology*** 218: 653-662.
3. Manna SK and Gangadharan C (2009) Decrease in RelA phosphorylation by inhibiting protein kinase A induces cell death in NF-kappaB-expressing and drug-resistant tumor cells. ***Molecular Immunology*** 46: 1340-1350.
4. Bose JS, Gangan V, Prakash R, Jain SK and Manna SK (2009) A dihydrobenzofuran lignan induces cell death by modulating mitochondrial pathway and G2/M cell cycle arrest. ***Journal of Medicinal Chemistry*** (In Press).
5. Gangadharan C, Thoh M and Manna SK (2009) Inhibition of constitutive activity of nuclear transcription factor kappaB sensitizes Doxorubicin-resistant cells to apoptosis. ***Journal of Cellular Biochemistry*** (In Press).

Laboratory of Bacterial Genetics

Studies on gene regulation, transcription termination, and amino acid and ion-transport in *Escherichia coli*

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	L Shanthy	JRF
	Amit Pathania	JRF
Other Members	V K Mishra	Staff Scientist
	K Anupama	Staff Scientist
	Abhijit A Sardesai	Staff Scientist
	R Harinarayanan	Staff Scientist (since Nov. 2008)
	J Krishna Leela	Technical Officer III
	T S Shaffiqu	Technical Assistant

Objectives

1. To study the ArgP regulon and the mechanism of ArgP-mediated transcrip-tional regulation of the arginine exporter ArgO
2. To test the model of and mechanisms mediating R-loop formation from nascent untranslated transcripts
3. To investigate an unusual phenomenon of K^+ toxicity in *hns trx* double mutant strains

Summary of work done until the beginning of this reporting year (April 1, 2007 – March 31, 2008)

1. A few years ago, this laboratory had identified in *E. coli* both a novel arginine exporter ArgO, as well as a regulator protein ArgP that was shown to be involved in the transcriptional regulation of *argO*. Mutations in *argP* also contributed to an osmosensitivity phenotype, which was inferred to be caused by a decrease in glutamate dehydrogenase activity in the mutant strains. We have since shown by in vitro experiments that ArgP binds the *argO* regulatory region to recruit RNA polymerase to the promoter in the presence of either arginine or lysine, but that whereas Arg-mediated recruitment leads to activation of *argO* transcription, Lys-mediated recruitment leads to trapping of RNA polymerase at the promoter and hence to *argO* transcrip-tional inactivation. We had therefore proposed that the *argO* case is the first known instance of an environmental signal regulating bacterial transcription at the final stage of promoter escape (ie., after open complex formation) by RNA polymerase.
2. Based on novel phenotypes identified by us earlier for *E. coli rho* and *nusG* mutants defective in factor-dependent transcription termination (the latter mutant had been isolated in this laboratory), we have proposed that nascent untranslated transcripts in bacteria are prone to generating toxic RNA-DNA hybrids (R-loops) in the DNA upstream of the moving RNA polymerase, and that indeed the purpose of bacterial transcription-translation coupling is to preclude the occurrence of such R-loops. We have been engaged in pursuing and testing the predictions of this model, and have obtained evidence that the essential endoribonuclease RNase E acts to promote R-loop formation in *rho* and *nusG* mutants while overexpression of enzymes that remove R-loops such as RNase H1, RecG or UvsW leads to suppression of several *rho*- and *nusG*-associated phenotypes.
3. We have identified a novel phenotype of potassium toxicity in *E. coli* strains doubly defective for the nucleoid protein H-NS and reduced cytoplasmic thioredoxin (through mutation in either the *trxA* or *trxB* genes encoding thioredoxin and thiore-doxin

reductase, respectively). These strains are unable to grow in minimal media containing 40 mM or more of K^+ (it may be noted that the routinely used minimal A medium has around 115 mM K^+), whereas they grow quite normally in media in which K^+ has been substituted with Na^+. We have been undertaking studies to understand the basis of this unusual phenotype.

Work undertaken in the current reporting year (April 1, 2008 - March 31, 2009)

1. *ArgP regulon and argO transcriptional regulation*

a. Experiments were undertaken to test the effect of ArgP on the transcriptional regulation of *gdhA*, encoding glutamate dehydrogenase which catalyses one of two pathways for glutamate synthesis in *E. coli* (the other being catalysed by *gltBD*-encoded glutamate synthase). In vivo transcription of *gdhA* was measured with the aid of *gdhA-lac* fusions, and the findings were as follows: (i) *gdhA* transcription is oppositely affected by growth in medium of elevated osmolarity (with 0.3 M NaCl), depending upon whether the strain is proficient or deficient for glutamate synthase – thus, *gdhA* expression at high osmolarity is reduced 3-fold and increased 4-fold, respectively, in *gltBD*$^+$ and *gltBD* mutant strains. (ii) In comparison with isogenic *argP*$^+$ strains, the *argP* mutants exhibited 3-fold decrease in *gdhA* expression irrespective of growth medium osmolarity and *gltBD* status, suggesting that ArgP is a transcriptional activator of *gdhA*. (iii) As mentioned above, the effects of ArgP and of culture osmolarity were independent and additive, such that *gdhA* expression was reduced 10-fold in the *argP gltBD* strain grown at high osmolarity in comparison to that in the *argP*$^+$ *gltBD* strain under the same conditions. This would perhaps explain why the former strain is osmosensitive, since glutamate accumulation through its increased synthesis is known to be an important feature in bacterial osmoregulation.

Further, in an attempt to map the functional promoter(s) of *gdhA*, experi-ments of primer-extension analysis on total cellular RNA were undertaken and two putative transcription-initiation sites were identified 63 bp and 165 bp, respectively, upstream of the start of the *gdhA* open-reading frame.

b. The ArgP protein has previously also been described as IciA, that is, inhibitor of chromosome replication initiation, and in this perspective it has been reported to bind to three 13-mer sequences in the *oriC* region as well as to control transcription of the *dnaA* and *nrdA* genes. One report has also mentioned that ArgP expression is autoregulated. We have performed electrophoretic mobility shift assay (EMSA) experiments with ArgP and the regulatory regions of the genes mentioned above, as well as with those of *argO* and *gdhA*, in both single gene- and competition-formats. Our results indicate that the rank order of affinity of ArgP binding is *argO* >> *gdhA* ~ *oriC* > *dnaA* ~ *nrdA* ~ *argP*, with the binding to *argO* being 10- to 100-fold stronger than to the other DNAs that were studied.

c. To delineate additional genes under ArgP control, we have performed microarray transcriptomics experiments with total RNA from *argP*$^+$ and *argP* mutant strains, and the interesting gene candidates so identified are being investigated further.

d. We have also initiated functional studies on the mechanism of ArgO-mediated export of arginine, by the approach of obtaining and characterizing *argO* mutants with altered transporter characteristics. ArgO derivatives with an haemagglutinin (HA) tag at either terminus have been constructed and shown to retain exporter function in complemen-tation assays. Mutagenesis studies on the HA-tagged ArgO proteins are in progress.

2. Studies on E. coli rho and nusG mutants and the R-loop model

a. As mentioned above, we had earlier found that non-lethal mutations leading to deletion of the C-terminal half of RNase E suppress R-loop related phenotypes of *rho* and *nusG* mutants. We had interpreted this observation to indicate that full-length RNase E, but not its truncated version, is able to cleave nascent untranslated transcripts and thus to promoter R-loop formation in the *rho* and *nusG* mutants.

RNase E preferentially cleaves 5¢-monophosphorylated mRNAs in what is referred to as the 5¢-end-dependent mechanism, and recently a novel enzyme RppH has been identified in *E. coli* that catalyses the removal of pyrophosphate from 5¢-triphosphorylated mRNA and so facilitates RNase E action on mRNAs in vivo. Null

mutants of *rppH* are neverthe-less viable.

b. We have now identified for the first time that whereas the single mutants carrying either the *rppH* mutation or the truncated version of RNase E are viable, the double mutant is inviable. Furthermore, the synthetic lethality observed in the double mutant is suppressed by *rho* or *nusG* mutations. Our data provide support for several hypotheses, namely, (i) RNase E has two mechanisms by which it cleaves mRNA, that include the 5¢-end-dependent mechanism which is dependent on RppH action, and an 'internal entry' mechanism on ribosome free RNA which is lost in the C-terminally truncated enzyme, and the presence of any one of these mechanisms is essential for viability; (ii) the *rho* and *nusG* mutations restore viability in the double mutant strain by providing an alternate route for mRNA degradation through R-loop formation; and (iii) therefore, one can infer that the reason why RNase E is essential in *E. coli* is because of its role in mRNA turnover. We are at present testing various additional predictions arising out of these models.

   We have also attempted to test the model for R-loop formation from nascent untranslated transcripts by a bisulphite reactivity assay. The rationale is that following RNA-DNA hybridization in the R-loop, the non-template DNA strand will be exposed as single-stranded DNA and hence be susceptible to bisulphite-mediated C-to-T conversions even without DNA denaturation.

   A nonsense mutation in *lacZ* and a frameshift mutation in *trpE* have been employed to generate locus-specific transcripts uncoupled from translation in the *rho* and *nusG* mutants. Following bisulphite treatment of genomic DNA + RNA and PCR of the *lacZ and trpE* loci, the techniques of denaturing gradient gel electrophoresis (DGGE) and sequencing of PCR clones have been used to determine the extent of C-to-T changes suffered in the different strains. Our results appear to indicate that the mutant *lacZ and trpE* loci from the *rho* and *nusG* strains are more susceptible to bisulphite action than those from the wild-type strain, and that this susceptibility is reduced upon overexpression of R-loop removing enzymes including RNase H1 and UvsW. Thus, these data offer support to our R-loop model.

c. We had earlier discovered that certain mutations in the nucleoid protein H-NS can reverse the transcription-termination defects in *rho* and *nusG* mutants, but the mechanism by which this is brought about is not clear. We have now sought to obtain and characterize suppressor mutations that nullify the effect of *hns* mutation in the *rho/nusG* strains; these suppressor mutants were selected on the basis of their predicted ability to restore relief of transcriptional polarity which is the hallmark of *rho* and *nusG* mutants and which is not manifest in the strains additionally carrying the *hns* mutation.

   Several suppressor mutants have been identified by this approach. Some of them are in *rho* itself and one is in *nusA* (encoding NusA, also involved in factor-dependent transcription and antitermina-tion); these mutations appear to be acting by exacerbating the transcription-termination defect to the extent that the *hns* mutation is not able to reverse the same. One more suppressor mutation has been mapped to the 19.9 min region of the *E. coli* chromosome and is being further characterized.

d. RNA decay proceeds by endonucleolytic cleavages followed by the action of several 3¢5¢exonucleases, but no 5¢-3¢ exonuclease has been identified in E. coli. One the other hand, a 5¢-3¢ exonuclease RNase J1 has recently been identified in Bacillus subtilis and shown to be essential for viability.

   We have initiated experiments for the regulated heterologous expression of RNase J1 in *E. coli*, on the rationale that the 5¢-3¢ exonuclease might act to provide an alternative to Rho and NusG functions in taking care of nascent untranslated transcripts. Our preliminary results indicate that RNase J1 expression is toxic in *E. coli* and that this toxicity is alleviated in strains deficient for RNase E. Further studies are in progress

3. Potassium toxicity in hns trx mutants

In earlier studies, we had identified that the potassium-toxicity phenotype in *hns trx* mutants can be alleviated by methionine supplementation or by mutations in *rpoS, ptsP, ahpC* or *metL* genes. Two additional suppressor loci were identified and characterized this year, namely, *spoT* involved in ppGpp metabolism and *acpP* involved in biosynthesis of fatty acids. The findings so far indicate a complex metabolic interplay that is responsible for the unusual potassium-toxicity phenotype whose unraveling would need additional genetic and biochemical studies.

Publications:

1. Akif M, Khare G, Tyagi AK, Mande SC and Sardesai AA (2008) Functional studies of multiple thioredoxins from *Mycobacter-ium tuberculosis*. ***Journal of Bac-teriology*** 190: 7087-7095.
2. Kumar S, Balakrishna K, Agarwal GS, Merwyn S, Rai GP, Batra HV, Sardesai AA and Gowrishankar J (2009) Th1-type immune response to infection by pYV-cured phoP-phoQ null mutant of Yersinia pseudotuber-culosis is defective in mouse model. ***Antonie Van Leeuwen-hoek*** 95: 91-100.
3. Dutta D, Bandyopadhyay K, Datta AB, Sardesai AA and Parrack P (2009) Properties of HflX, an enigmatic protein from Escherichia coli. ***Journal of Bacteriology*** (In Press).

Other Publications :

1. Gowrishankar J and Divakar P (2008) Scientometrics and modified h-indices. (correspondence) ***Current Science*** 95: 1656.
2. Sardesai AA and Gowrishankar J (2008) Joshua Lederberg – a remembrance. ***Journal of Genetics*** 87: 311-313.

Patents :

1 Gowrishankar J and Harinarayanan R. A method of altering levels of plasmids.

(i) Japanese Patent 4142649, granted on 20 Jun 2008.

(ii) German Patent DE 60224457 T2, granted on 5 Feb 2009.

(iii) European Patent 1551969 B1, granted on 2 Jan 2008.

2 Gowrishankar J and Nandineni M R. A microbial process for arginine production. Indian Patent 230540, granted on 27 Feb 2009.

Laboratory of Computational Biology

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Project 1: Knowledge based approach for protein fold-recognition

Objective: To develop a new fold-recognition tool with a sensitivity and specificity better than the existing tools

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

We developed a Support Vector Machine (SVM) based fold-recognition tool. For a given protein sequence the secondary structural and solvent accessibility state frequencies of amino acids and amino acid pairs are extracted and used them as the feature vectors for classification of the corresponding protein into one of the known protein folds. Training and testing using a bench mark set of protein folds showed that this tool performs better than any of the known fold-recognition tools in terms of prediction accuracy, sensitivity and specificity.

Detail of progress in the current reporting year (April 1, 2008 to March 31, 2009)

We continued our efforts to improve our fold-recognition tool in terms of fold-coverage. As mentioned in the last year's report our tool was trained and tested on a bench mark set comprising of 27 protein folds. In order to make it useful for fold-recognition applications the tool was trained and tested on different sizes of datasets. It was found that prediction accuracy decreases with the increase in the number of folds. This is expected because as the number of folds increases the number of pair-wise classes to be learned increases quadratically. To deal with this problem, we resorted to an hierarchical approach for protein fold recognition where we first predict the structural class (viz., a-class; b-class and a,b-class) of a given protein sequence and then predict the fold within that class. Here we had to train our tool separately for the three structural classes and thus reducing the complexity. Furthermore, we also included a number of so called 'Orphan folds' which increased the number of protein folds amenable for fold-prediction to 284. It may be worth noting that prediction of orphan folds is only possible by our method. Investigations revealed that the hierarchical approach gives rise to accuracies of 74%, 77% and 66% for a-class, b-class and a, b folds respectively. We are currently implementing a web-server version our tool.

Project 2: Studies on simple sequence repeats in prokaryotic genomes

Objective: Analysis of frequencies, abundance and polymorphism of simple sequence repeats (SSRs) in fully sequenced prokaryotic genomes

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

The whole genome sequences of five different strains of *Yersinia pestis* and its ancestor *Yersinia pertuberculosis* (YPTB) were surveyed for distribution and abundance of perfect polymorphic SSRs (PSSRs). We also studied the effects of the associated polymorphism events on the functions of the nearby genes.

Details of progress in the current reporting year (April 1, 2008 – March 31, 2009)

Since additional genome sequences of *Yersinia pestis* and of *Yersinia pseudotuberculosis* became available we performed our studies on all these

genomes. We found that distribution, abundance and enrichment of SSRs in these new genomes are very similar to the five genomes studied earlier. We further investigated relative preferences of protein coding regions to harbor SSRs by calculating their propensity values as follows.

$$PS = \frac{\frac{n}{N} * 100}{\frac{m}{M} * 100}$$

Where, PS = propensity of protein coding regions encoding a COG function; n = Number of nucleotides found in SSRs in ORFs assigned with a specific COG functional group, N = Number of nucleotides found in SSRs in all the ORFs with assigned COG functional group, m = Total number of nucleotides in ORFs assigned with a specific COG functional group, M = Total number of nucleotides in all the ORFs with assigned COG functional group. A propensity value greater than 1.0 indicates that the ORFs of a particular COG function have high preference to harbor SSRs whereas a value less than 1.0 indicates no such preference. Our study revealed that ORFs which encode proteins involved in defense, intracellular trafficking, secretion, and vesicular transport show high propensities for long SSRs (=6bp). It was also interesting to note that proteins involved in replication, recombination, repair, defense mechanism, cell wall/membrane/envelope biogenesis, cell motility, intracellular trafficking, secretion, vesicular transport and hypothetical proteins show high propensities (PS>1.0) for mono, di, tetra and penta SSRs which are capable of introducing frame-shift mutations.

We further made cross genome comparisons of equivalent SSRs and found a total of 739 SSRs showing length variation in various genomes. Of the PSSRs 636 were formed by mononucleotide tracts of which 61% were A|T tracts. Out of 739 PSSRs 425 PSSRs can be classified as those found in coding regions and of these 40% were mapping on to the N-terminal side of the translated protein product and about 30% were mapping on to the C-terminal side. The remaining were mapping on to the middle part of the translated protein products. As a consequence of polymorphism the coding regions have undergone changes such as length variation, premature termination and fission as depicted in Figure 1. These changes seem to underlie some of the observed phenotypic variations among the different strains of the pathogen in relation to adaptation and virulence.

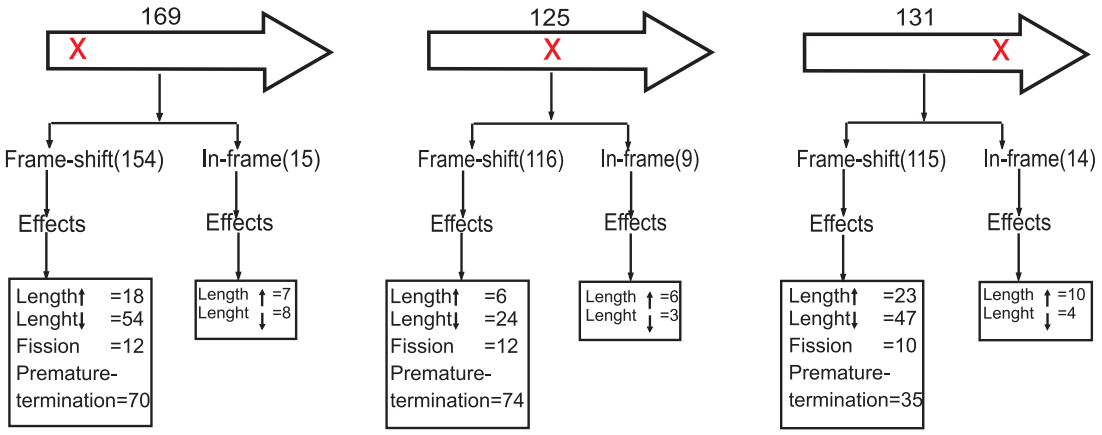

Figure 1: Schematic diagram illustrating the location (indicated by X) of PSSRs on ORFs (indicated by arrow) along with their effects to the corresponding translated products.

Project 3 : Analysis and modeling of luncharacterized mutations mapping on to the transmembrane regions of human membrane transport proteins

Objectives:

1. Analysis of known mutations in transmembrane helices of human membrane transporter superfamily to find membrane-specific sequence-structure features that can discriminate the disease associated mutations from the benign mutations
2. Development of a method to classify uncharacterized mutations into disease associated and benign mutations.
3. To estimate/model the effects of deleterious mutations at the protein structure and functional level.

Summary of work done until the beginning of this reporting year (April 1, 2007 – March 31, 2008)

SWISS-PROT database was surveyed for gathering known mutations both of disease associated as well as benign types mapping on to the transmembrane regions of the human membrane transport proteins. A total of 326 disease mutations and 333 benign mutations were identified. We analyzed solvent accessibilities and substitution scores from PHAT and GRANTHAM matrices for their discriminatory potential to classify disease and benign mutations.

Details of the Work during the current reporting year (April 1, 2008-March 31, 2009)

We investigated discriminatory potential of 19 diverse amino acid physicochemical properties as applicable to transmembrane regions (Table 1). These properties have been used in the prediction of transmembrane regions of membrane protein. It must be noted that deleterious or benign nature of a mutation depends on the nature of amino acid residue change as well as the position-specific preferences at the site of mutation. We therefore weighted the physicochemical property values of the wild-type and mutated amino acid residues by their estimated position-specific probabilities at the mutation sites. The distribution of weighted values of the various physicochemical properties of the disease associated and benign mutations were compared. It was found that, in general, all the properties give rise to good discrimination between disease and benign mutations. Of the various properties hydrophobicity related properties give rise to better discrimination between disease and benign mutations, in particular, the Engelman hydrophobobicity values give the best discrimination where 77% of disease-causing mutations are associated with scores <-0.5 and 71% of benign mutations have > 0. We are currently exploring the discriminatory potential of different combinations of physicochemical properties.

Table 1. The list of 19 membrane-specific physicochemical properties of amino acids

S.No.	Features
A)	**HYDROPHOBICITY SCALES**
1	Modified Kyte-Doolittle scale in an iterative procedure (MDK4). (Edelman, 1993).
2	Modified Kyte-Doolittle scale in an iterative procedure (MDK1). (Edelman, 1993).
3	Modified Kyte-Doolittle scale in an iterative procedure. Predicting membrane protein structure. (Juretic et al., 1993)
4	Modified Kyte-Doolittle scale in an iterative procedure (MDK0).(Edelman,1993).
5	Modified Kyte-Doolittle scale in an iterative procedure (MDK2).(Edelman,1993).
6	Membrane protein surrounding hydrophobicity scale. (Ponnuswamy and Gromiha, 1993)
7	Membrane protein surrounding hydrophobicity scale (combined membrane scale). (Ponnuswamy and Gromiha, 1993)
8	Weights from the Interfacial hydrophobicity scales (Jacobs and White, 1989)
9	Interfacial hydrophobicity scales (IFH (0.5)) (Jacobs and White, 1989)
10	Engelman hydrophobicity values of membrane proteins. (Engelman et al., 1986).
B)	**STRUCTURAL FEATURES**
11	Quadratic Minimization of predictors for protein secondary Structure. Application to Transmembrane Alpha helices. Edelman-25. (Edelman, 1993).
12	Quadratic Minimization of predictors for protein secondary Structure. Application to Transmembrane Alpha helices. Edelman-15. (Edelman, 1993).
13	Quadratic Minimization of predictors for protein secondary Structure. Application to Transmembrane Alpha helices. Edelman-31. (Edelman, 1993)
14	Quadratic Minimization of predictors for protein secondary Structure. Application to Transmembrane Alpha helices Edelman-21. (Edelman, 1993).
15	Linear optimization structure predictors applied to transmembrane proteins. (Edelman and White, 1989)
C)	**OTHER FEATURES**
16	Coil in water to helix in membrane values. (Heijne and Blomberg, 1979)
17	Membrane/Aqueous domains ratio of amino acid residues in membrane transport proteins. (Deber et al., 1986)

Publications:

1. Achary MS and Nagarajaram HA (2008) Comparative docking studies of CYP1b1 and its PCG associated mutant forms. ***Journal of Biosciences*** 33:699-713.
2. Achary MS and Nagarajaram HA (2009) Effects of Disease Causing Mutations on the Essential Motions in proteins. ***Journal of Biomolecular Structure and Dynamics*** 26:609-624.

Patents :

Shamim MTA and Nagarajaram HA. Fold-wise classification of proteins. USA Patent filed on 29 August, 2008.

Laboratory of Molecular Cell Biology

Molecular pathogenesis and functional genomics of *Mycobacterium tuberculosis*

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Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Objectives:

1. *In-vitro* Replication of *Mycobacterium tuberculosis* DNA
2. C-terminal domain of HU protein of *Mycobacterium tuberculosis* provides increased stability of binding and specificity in recognizing different structures of nucleic acid.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Many of the PE/PPE proteins are either surface localized or secreted outside and are thought to be a source of antigenic variation in the host. The exact role of these proteins are still elusive. We previously reported that the PPE41 protein induces high B cell response in TB patients. The PE/PPE genes are not randomly distributed in the genome but are organized as operons and the operon containing PE25 and PPE41 genes co-transcribe and their products interact with each other. We next described the antigenic properties of the PE25, PPE41 and PE25/PPE41 protein complex coded by a single operon. The PPE41 and PE25/PPE41 protein complex induces significant ($p<0.0001$) B cell response in sera derived from TB patients and in mouse model as compared to the PE25 protein. Further, mice immunized with the PE25/PPE41 complex and PPE41 proteins showed significant ($p<0.00001$) proliferation of splenocyte as compared to the mice immunized with the PE25 protein and saline. Flow cytometric analysis showed 15–22% enhancement of CD8+ and CD4+ T cell populations when immunized with the PPE41 or PE25/PPE41 complex as compared to a marginal increase (8–10%) in the mice immunized with the PE25 protein. The PPE41 and PE25/PPE41 complex can also induce higher levels of IFN-ã, TNF-á and IL-2 cytokines. While this study documents the differential immunological response to the complex of PE and PPE vis-à-vis the individual proteins, it also highlights their potential as a candidate vaccine against tuberculosis.

Mycobacterium tuberculosis (*M.tb*), the pathogen that causes tuberculosis, is capable of staying asymptomatically in a latent form, persisting for years in very low replicating state, before getting reactivated to cause active infection. It is therefore important to study *M.tb* chromosome replication, specifically its initiation and regulation. While the region between *dnaA* and *dnaN* gene is capable of autonomous replication, little is known about the interaction between DnaA initiator protein, *oriC* origin of replication sequences and their negative effectors of replication. By KMnO4 mapping assays the sequences involved in open complex formation within *oriC*, mediated by *M.tb* DnaA protein, were mapped to position -500 to -518 with respect to the *dnaN* gene. Contrary to *E. coli*, the *M.tb* DnaA in the presence of non-hydrolysable analogue of ATP (ATPãS) was unable to participate in helix opening thereby pointing to the importance of ATP hydrolysis. Interestingly, ATPase activity in the presence of supercoiled template was higher than that observed for DnaA box alone. *M.tb* rRv1985c, a homologue of *E.coli* IciA (Inhibitor of chromosomal initiation) protein, could inhibit DnaA-mediated *in-vitro* helix opening by specifically binding to A+T rich region of *oriC*, provided the open complex formation had not initiated. rIciA could also inhibit *in-vitro* replication of plasmid carrying the *M.tb* origin of replication. These results have a bearing on the

functional role of the important regulator of *M.tb* chromosomal replication belonging to the LysR family of bacterial regulatory proteins in the context of latency.

Details of progress made in the current reporting year (April 1, 2008 - March 31, 2009)

1. *In-vitro* Replication of *Mycobacterium tuberculosis* DNA

Ammonium sulphate (34%) fractionation of *M. bovis* BCG total cell extract yields maximal DNA replication: DNA replication was carried out with protein concentration varying from 60-200µg, at a temperature of 30 ^{0}C for 45 min. When DNA replication was carried out using 26% ammonium sulphate cutoff DNA replication could only be seen in the presence of higher concentration (150-200µg) of protein [Figure 1a]. Similarly when 28% cutoff was used DNA replication could be seen only at still higher concentration of protein with maximal DNA synthesis occurring in the presence of 150-200µg protein. Interestingly, replication using 28% ammonium sulphate cut off is significantly lower than seen when 26% cutoff was used. However, when 34% cut off of enzyme fraction II was used to carry out replication, more than 3-7 fold increase in DNA replication, as measured by nucleotide incorporation, could be seen [Figure 1a]. It could also be seen that maximal DNA synthesis occurs when 150 µg of the 34% cutoff fraction II is used. These results demonstrate that the 34% ammonium sulphate precipitation of the *M. bovis* BCG fraction II supports optimal DNA replication when *M.tb ori* sequence cloned in pUC vector is used as a template. Maximal DNA synthesis could be observed when replication was carried up to 90 min. [Figure 1b(B)] and maximal DNA synthesis occurs at 30 ^{0}C [Figure 1c] (B).

Replication once initiated at higher temperature continues even at low temperature. Results presented so far clearly show that 34% ammonium sulphate cut off fraction II of *M. bovis* BCG supports DNA replication, which is optimum at 30 ^{0}C and 90 min. It was also very clear that at 16 ^{0}C replication is very inefficient [Figure 1c(A) and 1c(B)]. We therefore determined whether the pre-priming complex formation alone requires higher temperature or the latter is required throught the replication cycle. Experiments were accordingly designed to ascertain whether replication once initiated at 30 ^{0}C can continue at lower temperature. For this, reaction was carried out using 100-200µg fraction II either at 30 ^{0}C for first 30 min and then continued at 16 ^{0}C for subsequent 60 min [Figure 1d(A), lanes 1-3] or at 30 ^{0}C for 90 min (lanes 4-6). The reaction products were fractionated by electrophoresis on a 5% PAGE and the appearance of the band was scored as a reflection of replication. The densiometric quantitation of bands [Figure 1d(B)] was carried out using Typhoon variable mode imager and image quant software. As can be seen [Figure 1d(B)] there is very less difference when reaction was first incubated at 30 ^{0}C for 30 min, followed by 16 ^{0}C for 60 min as compared to being carried out for 90 min at 30 ^{0}C. However, when the reaction was carried out solely at 16 ^{0}C, very less incorporation of á^{32}PdATP [Figure 1c(A) and (B)] could be observed. It therefore appears that initiation of DNA replication requires higher temperature (30 ^{0}C) and once the initiation of DNA replication has occurred the reaction can be continued even at low temperature.

Replication competent fraction II of *M. bovis* BCG prefers *M.tb oriC*. The template plasmid used in our DNA synthesis assays namely pUC_OriM.tb, contains two origin of replication sequences, pUC *oriC* and *M.tb oriC*. It was therefore considered necessary to determine which *oriC* is more efficiently recognized by the replication competent fraction II from *M. bovis* BCG. For this the replication was carried out in the presence of increasing protein concentration (60-200µg). After the usual phenol extraction the replication products were passed through Sephadex G_{50} spin columns which were pre-equilibrated with water. The elution products were restriction digested with *Hin*d III and *Bam*H1 (0.5 Unit/20µl) in a 20µl reaction mix. After heat inactivation of the restriction enzyme the digests were fractionated on 5% polyacrylamide gel (Figure 1e). As can be seen the intensity of the 827 bp band corresponding to *M.tb oriC* increases (lanes 2-6) with increasing protein concentration. In addition, a faint band could also be observed corresponding to the 2686bp pUC18 backbone which could be due to initiation from pUC18 *oriC*. It therefore appears that the replication competent *M. bovis* BCG fraction II preferentially recognizes the *M.tb oriC* during replication initiation.

Replication competent *M. bovis* BCG fraction II differentially recognizes other *ori* sequences. Having shown (Figure 1e) the preferred replication from *M.tb oriC* we next determined whether other *ori* sequences are differentially recognized by our *M. bovis* BCG replication competent fraction II. Accordingly *in-vitro* replication was carried out using different plasmids like pUC 18, pBSK II and pOK12

carrying pMB1, pBR322 and P15A *oriC* sequences and compared directly with *M.tb oriC*. DNA replication was carried out as usual using 150µg of fraction II, as it gave highest DNA synthesis. As can be seen (Figure 1f) all the above three plasmids were quantitatively far less efficient in terms of á^{32}PdATP incorporation in comparison to pUC_OriM.tb.

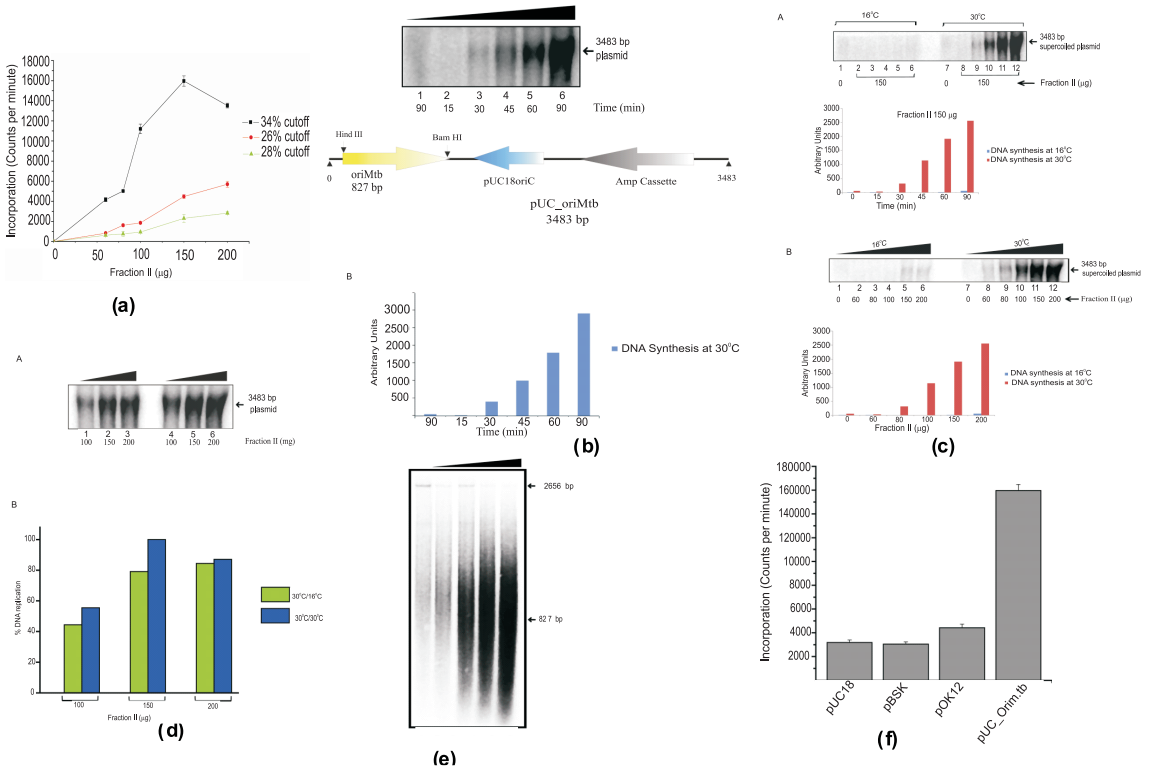

Figure 1 : in vitro DNA Replication. (a) DNA replication carried out with crude enzyme fraction made from M. bovis BCG using different ammonium sulphate cut off (Fraction II). (b) DNA replication on carried out with 34% cutoff with increasing time at 37 OC and (C) at two different temperatures. (d) DNA replication carried out first at 16/30 0C for 30 min and followed for another 60 min at 30 0C. (e) M bovis BCG replication competent fraction preferentially recognize M.tb oriC (f) DNA replication carried out on plasmids having different oriC sequences

2. C-terminal domain of HU protein of *Mycobacterium tuberculosis* provides increased stability of binding and specificity in recognizing different structures of nucleic acid.

Presence of a basic CTR in HupB paralogs from the mycobacterial clade. As noted previously a very visible feature of the polypeptide sequence of HupB paralogs belonging to the mycobacterial clade is the presence of a basic C terminal region (CTR). Member proteins of this clade are 214 amino acids long whereas *E. coli* HupA and HupB are 90 amino acids long. The N terminal region of HupB$_{Mtb}$ shows significant homology to enterobacterial nucleoid associated proteins such as HupA, HupB and IHF [Figure 2a(A)], whereas the CTR displays variation in amino acid composition (in contextual terms) even within the members of the mycobacterial clade. Amino acid sequence of the HupB$_{Mtb}$ CTR shows that it is rich in lysine and alanine. It has six PAKK and one KAAK repeats [Figure 2a(B)], repeats are marked by rectangular box) which are also present in histone H1, and are known to facilitate DNA binding. The CTR of HupB$_{Mtb}$ shows significant homology to sea urchin histone H1 [Figure 2a(B)]

primarily in terms of the presence of these tetrapeptide repeats. In order to determine the specific function related to this CTR, two *hupB*$_{Mtb}$ ORFs were generated by PCR; one bearing full length *hupB*$_{Mtb}$ DNA sequence (encoding HupB$_{Mtb}$) and another bearing DNA sequence of *hupB*$_{Mtb}$ (*hupB*$_{MtbN}$) that was capable of encoding an *hupB*$_{Mtb}$ variant bearing only the N-terminal 95 amino acids (HupB$_{MtbN}$). A hexa-histidine tag followed by a thrombin cleavage site was appended to the N-termini of both the polypeptides.

HupB$_{Mtb}$ and HupB$_{MtbN}$ exist in solution in a dimeric state. In order to assess the role of CTR (if any) in mediating oligomerization of HupB$_{Mtb}$ and to determine the oligomeric status of both HupB$_{Mtb}$ and HupB$_{MtbN}$, gel filtration was carried out. Gel filtration analyses showed that both proteins (HU-FL and HU-N) exist as dimers in solution [Figure 2b]. In *E. coli*, HU generally exits in solution as dimer and has four conserved structural regions; an á-helix 1, responsible for dimerization and the remaining three (â sheet 2, flexible arm 3 and á helix 4) constitute the DNA binding motif. The deletion of CTR of HupB$_{Mtb}$ did not impede HupB$_{MtbN}$ from existing in a dimeric state.These studies thus suggest that, the determinant(s) of dimerization in HupB$_{Mtb}$ lies within its N terminal and that the CTR has no apparent role in protein dimerization.

Interactions of HupB$_{Mtb}$ with DNA reveal that the CTR of HupB$_{Mtb}$ is required for recognizing unusual structures of nucleic acid. Under low salt conditions (10 mM NaCl or KCl) HupB$_{Mtb}$ bound to linear ds DNA (ds oligonucleotide A1) in a weakly cooperative manner with a Kd of 42 nM whereas even at 1200 nM HupB$_{MtbN}$ barely displayed 50% binding to A1. [Figure 2c(A)]. For HupB$_{Mtb}$ linear ds DNA interaction three retarded complexes [Figure 2c(A) shown by arrowheads] were apparent with complex 2 being the major species. Based upon the length of ds DNA used herein, that is 48 bp, and assuming that retardation is provoked by dimeric HupB$_{Mtb}$, one can roughly estimate that HupB$_{Mtb}$ binds to linear DNA with one dimer occupying DNA ranging from 16 to 24 bp. We studied HupB$_{Mtb}$ (at 50 nM) binding to A1 under increasing salt concentrations, and observed that HupB$_{Mtb}$ binding was greatly impaired at salt concentrations above 150 mM [NaCl or KCl; Figure 2c(C)]. *E. coli* HU is known to bind with duplex DNA containing a nick or a gap of one or two nucleotides with high affinity. A specific DNA protein complex (Kd 65 nM), under high salt conditions was detected when the interaction of HupB$_{Mtb}$ with linear DNA bearing a nick (ds oligonucleotide A2), was studied [Figure 2c(D)]. One other hand binding of HupB$_{MtbN}$ to A2 was highly impaired under high salt conditions [Figure 2c(E)]. In low salt HupB$_{MtbN}$ bound to nicked DNA with a Kd of 1000 nM. HU is known to bind specifically DNA containing either nick or a gap or DNA junction (cruciform DNA) without sequence preference. These structures are associated with the DNA damage and repair.

We studied the interaction of HupB$_{Mtb}$ and HupB$_{MtbN}$ with linear ds DNA bearing a one, two nucleotide gap and cruciform DNA, specified by oligonucleotides A3, A4 and A5 respectively, in the presence of low salt (10 mM KCl), to find that HupB$_{Mtb}$ bound with higher affinity to the said structures than HupB$_{MtbN}$ [Figure 2d]. From the above mentioned studies it emerges that the CTR of HupB$_{Mtb}$ is required for specific recognition of a duplex DNA containing a nick or a gap of one or two nucleotides and cruciform bearing DNA, with high affinity.

Interaction of HupB$_{Mtb}$ with supercoiled plasmid DNA. HU is a nucleoid associated protein and binds to single stranded and double stranded DNA and stabilizes DNA against thermal denaturation. HU along with IHF, H-NS and SMC proteins facilitate the compaction of chromosomes. When HU binds to DNA, several dimers of HU also bind to the same DNA sequentially in a non-cooperative or weakly cooperative fashion with one dimer per ~ 9bp irrespective of the length of DNA. The interaction of HupBMtb and HupBMtbN with supercoiled pBluescriptSK plasmid DNA was studied in buffer conditions employed in figure 9 wherein the molar ratio of protein to DNA ranged from 15:1 to 180:1. Viewed at a purely qualitative level HupBMtbN displayed a markedly reduced interaction with supercoiled plasmid DNA [Figure 2e(A), lanes 1-7]. Even at the highest protein to DNA molar ratio sufficient amount of unbound pBluscriptSK plasmid was visible [Figure 2e(A), lane 7]. HupB$_{Mtb}$ on the other hand bound very proficiently with supercoiled DNA, in the sense that at intermediate protein to DNA molar ratio the DNA protein complex was large and the DNA remained lodged in the wells [Figure 2e(A), lane 11]. Furthermore, the interaction of HupB$_{Mtb}$ with supercoiled DNA was more avid than with linear DNA of the same size [Figure 2e(B), lanes 8-14].

HupB$_{Mtb}$ displays greater affinity towards AT rich DNA. Since the average GC content of the *Mtb* strain H37Rv genome works out to around 65%, we wondered whether the CTR of HupB$_{Mtb}$ endows

it with the ability to interact more proficiently with GC rather than AT rich DNA. To assess if $HupB_{Mtb}$ bore this attribute, interaction of the said protein was studied with ds DNA oligonucleotides A13, whose GC content was 84% and A14 whose AT content was 64%. Surprisingly, $HupB_{Mtb}$ interacted proficiently with AT rich DNA forming two gel retarded complexes (marked by arrowheads) whereas its interaction with GC rich DNA was negligible [Figure 2f(B)]. On the other hand $HupB_{MtbN}$ [Figure 2f(A)] while retaining its ability to interact more proficiently with AT rich DNA, forming a single gel retarded complex, displayed at a lower level an ability to interact with GC rich DNA. We determined by circular dichroism studies the secondary structure parameters of the two proteins which showed that the two proteins exhibited some differences in the content of alpha helix and beta sheet structures (figure not shown). Perhaps these differences may account for the ability of $HupB_{MtbN}$ to display binding to GC rich DNA.

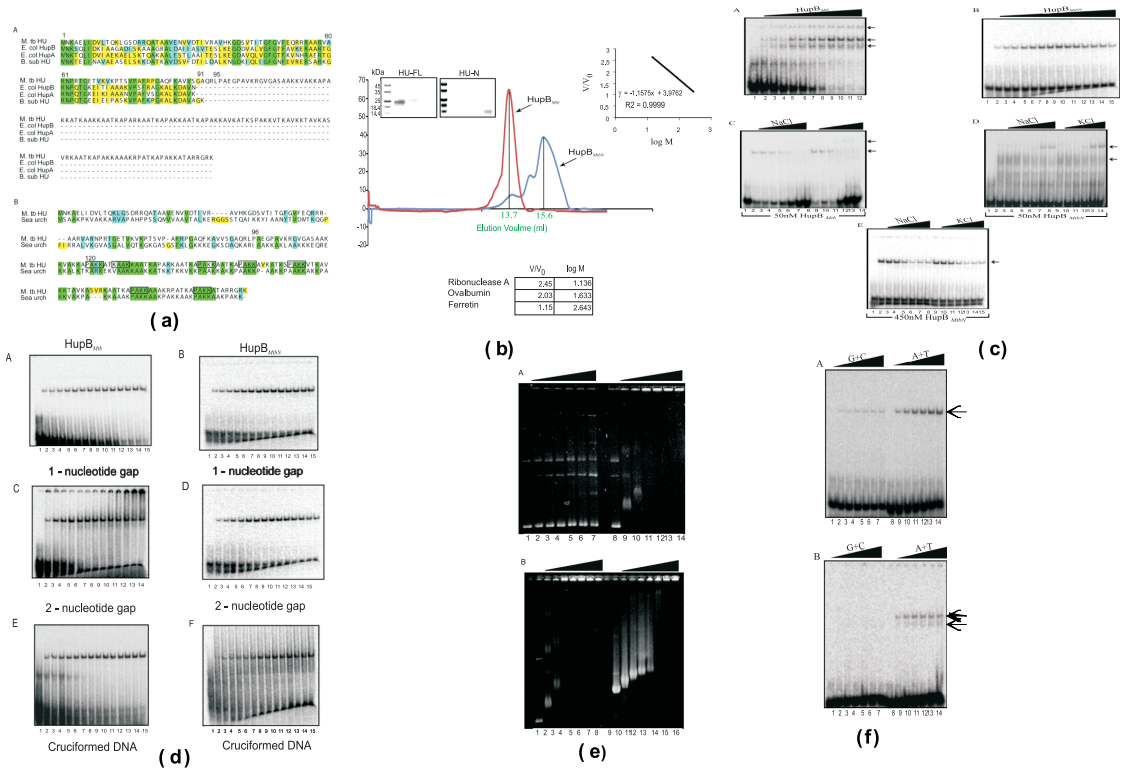

Figure 2 : Importance of C terminal of HupB. (a) Sequence alignment of HupB Mtb with different other bacterial species. (b) Size exclusion chromatography of HupB (c) Binding of HupB Mtb and HupBMtbN to ds and nick DNA under low and high salt condition. (d) Binding of HupBMtb and HupBMtbN to different structure of DNA. (e) Binding of HupBMtb and HupBMtbN to supercoiled and linear DNA (f) Binding of HupBMtb and HupB MtbN to GC and AT rich Oligos.

Publications :

1. Ahmed N, Dobrindt U, Hacker J and Hasnain SE (2008) Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. ***Nature Reviews Microbiology*** 6:387-94.

2. Farhana A, Kumar S, Rathore SS, Ghosh PC, Ehtesham NZ, Tyagi AK and Hasnain SE (2008) Mechanistic insights into a novel exporter-importer system of Mycobacterium tuberculosis unravel its role in trafficking of iron. ***PLoS ONE*** 7;3 (5):e2087.

3. Khan N, Alam K Mande SC, Valluri VL, Hasnain SE and Mukhopadhyay S (2008) Mycobacterium tuberculosis heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. ***Cellular Microbiology*** 10:1711-1722.

4. Smanla T, Niteen P, Ramanadham M, Sangita M, Murthy KJR, Nasreen EZ and Hasnain SE (2008) The Co-Operonic PE25/PPE41 protein complex of Mycobacterium tuberculosis elicits increased humoral and cell mediated immune response. ***PLoS ONE*** 3(10):e3586.

5. Ahmed N, Ehtesham NZ and Hasnain SE (2009) Ancestral Mycobacterium tuberculosis genotypes in India: implications for TB control programmes. ***Infection, Genetics and Evolution*** 9:142-146.

6. Kumar S, Farhana A and Hasnain SE (2009) In-vitro helix opening of M. tuberculosis oriC by DnaA occurs at precise location and is inhibited by IciA like protein. ***PLoS ONE*** 4(1): e4139.

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Patents :

1. Hasnain SE, Banerjee S, Ashok N, Raviprasad V, Katoch VM and Murthy KJR. A method of diagnosing tuberculosis. Singapore Patent 129179, gramted on 29 Feb, 2008

2. Hasnain SE and Prachee C. Peptide antigens which elicit high humoral Immune response and T-cell response. Indian Patent 227179, granted on 5 Jan, 2009.

3. Hasnain SE, Prachee C, Nagalakshmi Y, Aruna B, Murthy KJR and Katoch VM. Antigenic Peptides. Singapore Patent 129060, granted on 31 Jan, 2008.

Laboratory of Molecular and Cellular Biology

Macrophage biology and host-pathogen interaction in tuberculosis

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Objectives:

We aim to study the signaling pathways in macrophages regulating its innate-effector functions and how various candidate proteins of *Mycobacterium tuberculosis* interfere with macrophage-signaling cascades and suppress protective functions of host

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

1. There are indications that T-helper 1 (Th1) immune response is downregulated in patients with active tuberculosis (TB) infection. We found earlier that some mycobacterial proteins (found to be overexpressed in macrophages during infection) are involved in downregulating anti-PPD Th1 immune responses. Although, a role of IL-12 was indicated, it is not clear the mechanisms how various mycobacterial proteins modulate macrophage signaling to affect IL-12 and other innate-cytokine inductions and the Th environment. Interestingly, studies reveal that TB patients have altered glutathione balance indicating the possibility that mycobacterial proteins may alter the macrophage-glutathione redox balance which could be involved in altered cytokine profiles. Therefore, in this study direct effect of the glutathione redox in macrophage-innate cytokine balance was investigated.

2. We have shown earlier that the PE/PPE family proteins with SH3 binding domain can play important roles in modulation of immune responses. While comparing differential gene expression between *M. tuberculosis* and *M. bovis*, a recent study indicates one of the PPE proteins (Rv1196) to be highly expressed in *M. tuberculosis* as compared to *M. bovis*. These observations prompted us to speculate Rv1196 could play important role in *M. tuberculosis* pathogenesis. Emerging evidence suggests that one of the major pathogenic potentials of *M. tuberculosis* lies in its capability to deliberately evoke a T helper (Th) 2, mainly by upregulating IL-10 cytokine which in turn downregulates the protective Th1 response. Because *M. tuberculosis* infects and resides inside the macrophages, it is likely that the bacteria modulate macrophage innate-cytokine induction. Therefore, modulations in the innate-cytokines released by the

macrophages in response to Rv1196 were checked.

Details of progress made in the current reporting year (April1, 2008 - March31, 2009)

Project 1: Regulation of macrophage innate-effector function by glutathione redox: Implication in anti-tuberculosis therapy

N-acetyl-cysteine is known to increase the reduced gluthathione level and changes the reduced (GSH) and oxidized (GSSG) ratio in cells. Therefore, we next treated macrophages with increasing concentrations of NAC to alter the intracellular glutathione redox balance and induction of innate cytokines like IL-12, IL-10 and TNF-alpha was measured by EIA. It was observed that treatment of NAC resulted in increased intracellular GSH in a dose-dependent manner upto a concentration of 3 mM, but the intracellular GSSG levels were not significantly affected. However, higher concentrations of NAC (10 or 20 mM) increased GSSG levels by about 3 or 4 fold when compared with the medium-treated control macrophages. The intracellular glutathione-redox balance indicated as GSH/GSSG showed predominantly a reducing state when macrophages were treated with NAC upto 3 mM due to increased levels of intracellular GSH. However, the redox balance shifts towards more oxidizing state at higher concentrations of NAC (10 and 20 mM) due to increase in intracellular GSSG levels.

Interestingly it was observed that NAC activated induction of IL-12 p40 and IL-12 p70 in dose-dependent manners. Maximum induction of IL-12 p40/p70 was observed at 3 mM NAC. However, these levels were decreased with subsequent higher concentrations of NAC, i.e., 10 and 20 mM. The decrease was not due to cell cytotoxicity by higher concentrations of NAC used, since cell viability remained unchanged as revealed by MTT assay. Exogenous treatment of RAW 264.7 macrophages with glutathione reduced ethyl ester (GSH-OEt), a permeable form of glutathione alters the intracellular GSH and GSSG levels and similarly influences IL-12 p40. Pre-treatment of RAW 264.7 macrophages with BSO, a pharmacological inhibitor of GSH synthesis inhibited IL-12 p40 induction confirming that GSH has a positive effect on IL-12 p40 induction. A dose-dependent effect of NAC on IL-12 p40 induction was also observed in peritoneal macrophages from BALB/c mice and in monocyte-derived macrophages. No effect of GSH/GSSG was noticed in the induction of IL-10 and TNF-á.

IL-12 p40 is mainly regulated by c-rel transcriptoon factor. It was found that nuclear c-rel level was markedly increased in the cells treated with 3 mM NAC and gradually decreased with higher NAC concentrations (10 and 20 mM NAC). The total c-rel level was not affected by the changes in the GSH/GSSG balance. These data indicate that intracellular glutathione balance probably affects nuclear translocation of the c-rel transcription factor. The upstream pathways involved in c-rel signaling revealed that IêBá phosphorylation was increased by both 1 and 3 mM NAC.

It is interesting to note that although nuclear c-rel and IL-12 p40 were upregulated in macrophage rich in reduced glutathione (treated with 3 mM NAC), their levels were decreased in macrophage treated with 10 and 20 mM NAC. Therefore, we speculated that the resultant decrease in the level of IL-12 p40 was due to inhibition of IêBá phosphorylation by NAC at higher concentrations (treated with 10 and 20 mM NAC). However, levels of IêBá phosphorylation were actually increased in these cells. These results hint that the oxidized state of glutathione probably regulates nuclear c-rel levels by targeting different signaling cascades in macrophages. It is known that calmodulin protein (a highly conserved, ubiquitously expressed, intracellular sensor for calcium) interacts with IêBá-released c-rel and inhibit its nuclear transport. Therefore, we speculated that macrophages rich in GSSG may also have higher levels of CaM. When CaM levels were measured by EIA as well as by immunoblotting, it was found that NAC at 10 and 20 mM but not at 3 mM increased CaM expression. Co-immunoprecipitation as well as inhibition assay using trifluoperazine (TFP, a known pharmacological inhibitor of CaM activity) confirmed that oxidized glutathione targeted the CaM signaling to inhibit c-rel translocation to nucleus, and consequently IL-12 p40 gene transcription.

This study hints at the possibilities how macrophage activation status is regulated differently by the GSH/GSSG redox balance. A reducing glutathione environment can increase IêBá phosphorylation and degradation without much affecting CaM expression. This net effect positively

regulates the c-rel level important for switching on the transcription of the IL-12 p40 gene. However, on the other hand, when GSSG concentration is higher resulting in a shift in the GSH/GSSG ratio towards a more oxidized state, the CaM expression is increased, reversing the net effect on the levels of c-rel and thereby downregulates IL-12 p40 induction due to the sequestering effect of CaM on the IêBá-released c-rel. This can interestingly constitute a point of manipulation by the pathogens. Towards this context, our results revealing that NAC at 3 mM concentration (that increases intracellular GSH level) can improve anti-BCG Th1 response of PBMC from TB patients (in vitro recall assay) is interesting to devise strategies to improve the anti-BCG Th1 response in TB patients.

Project 2. Understanding roles of the PE/PPE family of proteins to modulate host protective responses

We overexpressed the *M. tuberculosis* Rv1196 protein with an N-terminal 6X histidine tag in *Escherechia coli* and purified the soluble protein to homogeneity of greater than 95%. The preparation had a very low endotoxin content (< 0.05 U/ml as determined by Limulus amebocyte lysate assay and was used for all subsequent experiments. The rRv1196 protein was found to specifically increase IL-10 production in a dose-dependent manner without eliciting detectable levels of other cytokines like TNF-alpha (TNF-á), IL-8 and IL-12 in phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophages. The effect was specific to Rv1196 as proteinase K digestion or heat denaturation of the protein abrogated IL-10 production. Further, preincubation of rRv1196 protein (3 ìg/ml) with F(ab)2 fragments of the anti-Rv1196 antibody (Ab) but not of the normal mouse serum (NMS) abrogated its ability to induce IL-10. Similar results were obtained when human monocyte-derived macrophages were stimulated with Rv1196. The Rv1196 protein stimulated THP-1 macrophages to express IL-10 mRNA transcripts.

Antibody-mediated blocking experiments indicated that Rv1196 interacted with toll-like receptor (TLR) 2 but not with TLR4, TLR6 and CD14. To substantiate that Rv1196 interacts with TLR2, we transfected HEK293 cells that naturally lack TLRs to express specific TLRs. The cells transfected with TLR2 and TLR4 constructs showed expression of these proteins (Fig. 1 A, i and ii). As expected, Rv1196 binding could be detected in HEK293 cells overexpressing TLR2 (Fig. 1 A, iii), but not in HEK293 cells overexpressing TLR4 (Fig. 1 A, iv). Again pull-down assay confirmed that Rv1196 interacted with TLR2

An insight into the way TLR2 and Rv1196 interact with each other was obtained by performing computational docking studies. We used 3D-dock, a molecular docking program that uses shape complementarity to assess the interaction of protein molecules, to predict possible sites for interaction between TLR2 and Rv1196. Interestingly, the top ranking docking scores for Rv1196 was found to be located at the TLR2 convex region that stretches the border of the central and C-terminal domain overlapping with the leucine rich repeat (LRR) 11~15 of TLR2 (Fig. 1 B). To further confirm that Rv1196 binds to the TLR2 LRR domain 11~15, we next transfected HEK293 cells with TLR2 LRR/LRR like motif deletion constructs and assessed for Rv1196 binding. It was found that a deletion of LRR 10~15 named as Mut3, abolished the binding of Rv1196 while other deletion mutants like Mut1, Mut2 and Mut4 encompassing LRR 1~5, LRR 5~10 and LRR 15~20 respectively showed similar binding as that of wild-type TLR2 construct (Fig. 1 C).

Further studies indicated that the Rv1196 specifically interacted with the TLR2 and led to an early and sustained activation of p38 MAPK, which was critical for IL-10 induction. In contrast Pam_3CSK_4 mainly activates ERK ½ and pro-inflammatory responses. This information indicates a novel signaling involving the TLR2-LRR domain in triggering IL-10 and involvement of PPE protein to activate a Th2-type signaling by specific recognition of the LRR 11~17 domain of TLR2 which may be useful in understanding the host-bacilli interaction and immune evasion strategy in tuberculosis.

Future plans and direction

1. Glutathione redox regulation and macrophage signaling in tuberculosis.
2. Detailed molecular signaling downstream of TLR2-LRR domain involved in the activation of IL-10 by Rv1196. Also, it will be studied whether Rv1196 play any physiological role in the pathogenesis of tuberculosis.
3. Role of PE/PPE family proteins in modulation of macrophage innate-effector functions

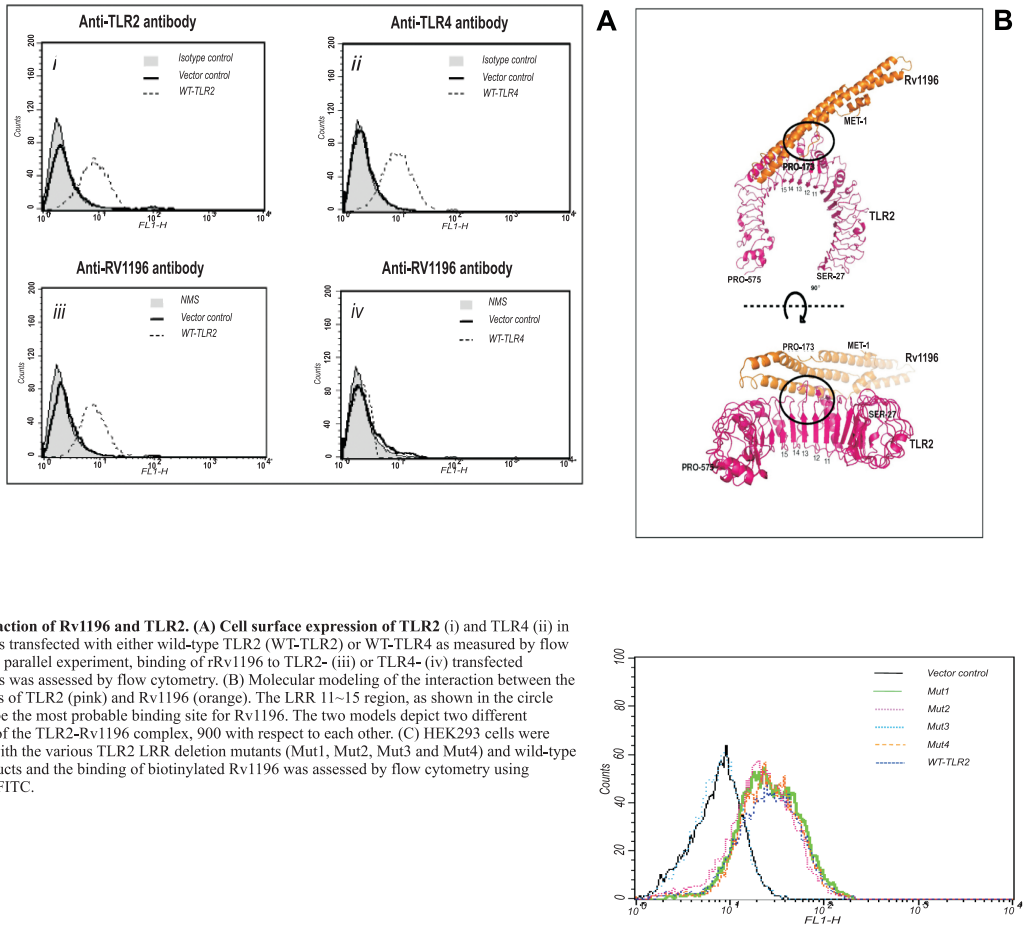

Fig. 1. Interaction of Rv1196 and TLR2. (A) Cell surface expression of TLR2 (i) and TLR4 (ii) in HEK293 cells transfected with either wild-type TLR2 (WT-TLR2) or WT-TLR4 as measured by flow cytometry. In parallel experiment, binding of rRv1196 to TLR2- (iii) or TLR4- (iv) transfected HEK293 cells was assessed by flow cytometry. (B) Molecular modeling of the interaction between the LRR domains of TLR2 (pink) and Rv1196 (orange). The LRR 11~15 region, as shown in the circle appeared to be the most probable binding site for Rv1196. The two models depict two different orientations of the TLR2-Rv1196 complex, 900 with respect to each other. (C) HEK293 cells were transfected with the various TLR2 LRR deletion mutants (Mut1, Mut2, Mut3 and Mut4) and wild-type TLR2 constructs and the binding of biotinylated Rv1196 was assessed by flow cytometry using streptavidin-FITC.

Publications:

1. Hussain MA, Naveed SA, Sechi LA, Ranjan S, Alvi A, Ahmed I, Ranjan A, Mukhopadhyay S, and Ahmed N (2008) Isocitrate dehydrogenase of Helicobacter pylori potentially induces humoral immune response in subjects with peptic ulcer disease and gastritis. ***PLoS ONE*** 3:e1481.
2. Khan N, Alam K Mande SC, Valluri VL, Hasnain SE and Mukhopadhyay S (2008) Mycobacterium tuberculosis heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. ***Cellular Microbiology*** 10:1711-1722.
3. Khan N, Alam K, Nair S, Valluri VL, Murthy KJR and Mukhopadhyay S (2008) Strong immune responses to the PPE protein Rv1168c are associated with active tuberculosis disease. ***Clinical and Vaccine Immunology*** 15:974-980.
4. Smanla T, Niteen P, Ramanadham M, Sangita M, Murthy KJR, Nasreen EZ and Hasnain SE (2008) The Co-Operonic PE25/PPE41 protein complex of Mycobacterium tuberculosis elicits increased humoral and cell mediated immune response. ***PLoS ONE*** 3(10):e3586.

Patents :

1. Mukhopadhyay S, Bhat KH and Khan N. A novel protein as potential candidate for development of anti-tuberculosis therapeutics. Indian Patent filed on 24 November, 2008.
2. Mukhopadhyay S and Khan N. A novel candidate protein to diagnose patients with active tuberculosis. USA Patent filed on 19 December, 2008.

Laboratory of Molecular and Cellular Biology

Genomics and biology of important bacterial pathogens

Principal Investigator	Niyaz Ahmed A S	Staff Scientist and Head *(Until December, 2008)*
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	Md Rizwan	PDF
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	Manjula Sritharan (University of Hyderabad)	
	P Vijayachari (RMRL, Port-Blair)	

Objectives

On a broader canvas, our group is interested in addressing fundamentally important questions in pathogen biology such as - how virulence evolves as a function of genome optimization under different compulsions offered by a colonized niche; how microbes regulate their genomic streamlining; what environmental stimuli are responsible for the diversification and stratification of microbial lineages; what is the functional significance of prokaryotic genomic diversity especially in the context of host and tissue tropism and towards understanding parasitism versus commensalism; and how can microbial genome data and the observed diversity gauged though community genotyping be experimentally harnessed for the generation and selection of optimally adapted microorganisms? We use a host of genomic and cellular microbiology experiments to try and address these issues. Important baseline studies from our group addressing these issues include analyses of diversity, evolution and functional biology of pathogenic Mycobacteria, *Helicobacter pylori* and *Leptospira* in the context of evolution of survival mechanisms, acquisition and optimization of virulence, and their impact on dissemination dynamics, invasion, persistence, signaling events, molecular pathogenesis, strain evolution and prevention measures. Most of these studies are collaborative meaning that we strive to involve strongly networked and community level interactions to address our envisaged research goals – such community approaches also involve public engagement through e-science, semantic web, and web 2.0 based tools. Therefore, most of our findings and observations are published through journals of mass appeal which harness functionality of these new generation applications *via* Open Access and community enabled platforms.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Last year we discussed leads obtained on putative roles of two of the novel *H. pylori* proteins that might be involved as apart of survival mechanism at the base of chronic gastritis – a precancerous sequel of *H. pylori* infection (Rizwan *et al*., 2008). More specifically the proinflammatory and proapoptotic roles of the plasticity region encoded proteins were discussed in relation to their being part of putative survival mechanisms that the organism harnesses to form a persistent niche. In addition we discussed possibility of mycobacterial antigens being environmental triggers of the type-1 diabetes mellitus.

Details of progress made in the current reporting year (April 1, 2008- December 10, 2008)

We continued efforts to understand various underlying pathways pertaining to proinflammatory and apoptotic signaling of the HP986 protein and further characterized its interaction with TNFR1 and Fas. To rule out signaling through TLR2 and TLR4, specific antibodies directed against TLRs were used to block any possible interaction of HP986 with the TLRs. We found no contribution of TLR induced signaling to our observed pro-inflammatory and pro-apoptotic effects which were indeed triggered through TNFR1 mediated pathway. These observations are being verified by siRNA mediated silencing of various competing receptors. Computational modeling and docking simulation studies were carried out to verify molecular interactions related to the interaction of HP986 with TNFR1. Our laboratory moved and completely relocated at the University of Hyderabad in December 2008 and was since rechristened as Pathogen Biology Laboratory at the School of Life Sciences (http://www.pathogen-evolution.org).

Publications

1. Ahmed N, Dobrindt U, Hacker J and Hasnain SE (2008) Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. ***Nature Reviews Microbiology*** 6:387-94.
2. Hussain MA, Naveed SA, Sechi LA, Ranjan S, Alvi A, Ahmed I, Ranjan A, Mukhopadhyay S, and Ahmed N (2008) Isocitrate dehydrogenase of Helicobacter pylori potentially induces humoral immune response in subjects with peptic ulcer disease and gastritis. ***PLoS ONE*** 3:e1481.
3. Rosu V, Ahmed N, Paccagnini D, Pacifico A, Zanetti S and Sechi LA (2008) Mycobacterium avium subspecies paratuberculosis is not associated with type-2 diabetes mellitus. ***Annals of Clinical Microbiology and Antimicrobials*** 7:9.
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5. Victoria B, Ahmed A, Zuerner R, Ahmed N, Bulach D, Quinteiro J and Hartskeerl R (2008) S10-spc-alpha is a conserved locus within highly plastic Leptospira genomes. ***PLoS ONE*** 3(7):e2752.
6. Ahmed N, Ehtesham NZ and Hasnain SE (2009) Ancestral Mycobacterium tuberculosis genotypes in India: implications for TB control programmes. ***Infection, Genetics and Evolution*** 9:142-146.
7. **Rosu V, Ahmed N, Paccagnini D, Gerlach G, Fadda G, Hasnain SE, Zanetti S and Sechi LA (2009) Specific immunoassays confirm association of *Mycobacterium avium* Subsp. paratuberculosis with type-1 but not type-2 diabetes mellitus***. PLoS ONE*** 4:e4386.
8. **Stavrum R, Myneedu VP, Arora VK, Ahmed N and Grewal HM (2009) In-depth molecular characterization of *Mycobacterium tuberculosis* from New Delhi - predominance of drug resistant isolates of the 'modern' (TbD1) type. ***PLoS ONE*** 4:e4540.

**from work performed in CDFD, but institutional affiliation of CDFD not listed.

Laboratory of Structural Biology

Structural and Biochemical Characterization of some *M. tuberculosis* Proteins

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	Eleanor Dobson, University of York, UK	

Objectives

1. Identification of important proteins of *Mycobacterium tuberculosis* for crystallographic and biochemical analysis
2. Expression and biochemical characterization of the chosen proteins. X-ray structural analysis of the chosen proteins
3. Develop applications of graph theory to understand genome-wide protein: protein interactions

Summary of work done until the beginning of this reporting year (April 1, 2007 – March 31, 2008)

Three broad categories of proteins from *Mycobacterium tuberculosis* were chosen for biochemical and structural work. They are:

Redox proteins:	●	Thioredoxins (A, B and C)
	●	Thioredoxin reductase
Heat Shock Proteins:	●	Chaperonin-60 family (Cpn60.1, Cpn60.2 and Cpn10)
	●	Heat shock protein 70 family (Hsp70, Hsp40)
Other Protein including Proteins involved in transcription processes:	●	HrcA and HspR: transcriptional repressors of the Hsp60 and Hsp70 operons respectively
	●	YefM:YoeB toxin-antitoxin complex

Thioredoxins and Thioredoxin reductase:

The three thioredoxins (Trx's A, B and C) encoded by the Rv1470, Rv1471, and Rv3914 were cloned and expressed in *E. coli*. All the three proteins were purified to homogeneity. We observed that TrxB and TrxC are able to reduce model substrates in vitro, such as insulin, via the electron transfer partner, TrxR, however, TrxA does not bear the same capability. Measured redox potentials of TrxB and TrxC showed

that these are -262±2 mV and 269±2 mV respectively, making them slightly more oxidizing than the *E. coli* thioredoxins. The redox potential of TrxA is -248±3 mV. These results suggest that *trxA* might be a pseudogene, while TrxB and TrxC might be the canonical thioredoxins.

Chaperonins:

We had demonstrated earlier using heterologous complementation and biochemical studies that the loss of chaperoning ability of the mycobacterial GroEL-1 is indeed due to its inability to form canonical tetradecamers. A chimeric *groEL-1* ORF bearing the DNA sequence corresponding to the equatorial domain of *E. coli groEL,* unlike the unmodified *groEL-1* ORF is able to complement the loss of cell viability phenotype conferred by a conditional allele of *groEL*, in *E. coli*. The corresponding polypeptide, *in vitro* is capable of existing, principally in a tetradecameric state, a canonical feature of *E. coli* GroEL. Complementary studies show that an *E. coli* GroEL variant, displaying properties similar to that of *Mtb* GroEL-1 can be obtained provided it bears the amino acid sequence corresponding to *Mtb* GroEL-1 equatorial domain. These experiments provided direct evidence for the importance of oligomerization in biologically relevant GroEL function.

Interestingly we observed that GroEL-1 is capable of binding to DNA without any sequence specificity. The affinity of DNA recognition by GroEL-1 is sufficiently high in the range of 100-200 nM suggesting that the protein has naturally evolved to bind DNA. Testing a range of oligonucleotide sequences of varying lengths revealed that GroEL-1 binds DNA in a sequence-non-specific manner. The *groEL-1* gene of *M. tuberculosis* was suggested to have acquired a nucleoid association function through loss of oligomerization, by utilizing its fundamental property as a non-specific substrate binding protein.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Project 1: Molecular characterization of Chaperonins

We had earlier reported a detailed study to address why the *M. tuberculosis* chaperonins are unable to complement the loss of GroEL in *E. coli*. Through these studies, we had concluded that the recombinant *M. tuberculosis* chaperonins do not form canonical tetradecamers, and that by facilitating their tetradecamer formation, these attain an identical function as that of *E. coli* GroEL. Surprisingly, sequence analysis of the available GroEL sequences reported by us earlier had indicated that the *M. tuberculosis* groEL genes have not turned into pseudo genes, despite one of them exhibiting accelerated evolution. During the period under review, we carried out experiments to test oligomeric properties of *M. tuberculosis* GroELs in their native condition. For this, the whole cell lysates of *M. tuberculosis* were fractionated by gel filtration chromatography, and various fractions were tested for the presence of GroELs by Western blotting. Interestingly, it was observed that GroEL1 exists as a monomer, dimer, heptamer and tetradecamer. Further by probing with anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine antibodies, it was revealed that only the tetradecameric fraction is phosphorylated on a Ser-residue. We thus believe that oligomerization of GroEL1 under native condition is mediated by specific phosphorylation on Ser-residues.

In the previous report, we had also shown that *M. tuberculosis* GroEL-1 possesses an unusual property as a DNA-binding protein. We had hypothesized that this property might be due participation of GroEL-1 in nucleoid formation in *M. tuberculosis*. In the period under review, we purified the nucleoids of *M. tuberculosis* and probed them with anti-GroEL1 antibodies. Presence of GroEL1 in the purified nucleoids was thus confirmed. Imaging by fluorescence microscopy further confirmed that GroEL1 of *M. tuberculosis* colocalises with its nucleoids (Fig. 1).

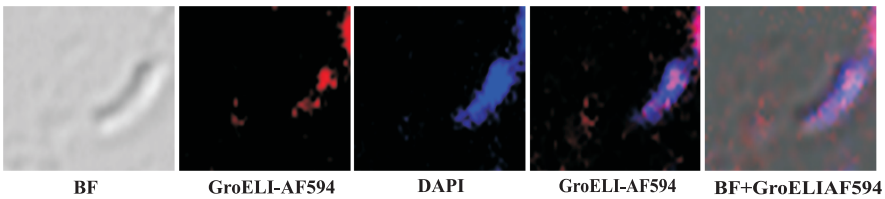

Figure 1: Mtb GroEL1 is associated with the nucleoid of Mtb: Brightfield image of *M. tuberculosis* H37Rv is shown. Cells were stained with DAPI to visualize the nucleoid (blue) and Alexa fluor 594 conjugated secondary antibody against *M. tuberculosis* GroEL1 monoclonal antibody (red) to visualize *M. tuberculosis* GroEL1. The superimposition of DAPI stained nucleoid and Alexa fluor 594 stained *M.tuberculosis* GroEL is also shown. The pink colour clearly indicates the colocalization of GroEL1 with *M. tuberculosis* nucleoids.

Project 2: Structural studies on toxin-antitoxins

The *yefM:yoeB* toxin-antitoxin gene was PCR amplified during the period under review. Crystal structure of the YefM antitoxin was determined. In order to demonstrate if the *M. tuberculosis* YefM antitoxin is able to neutralize YoeB toxin of *E. coli,* complementation study was carried out. The results of this study clearly demonstrate that the YefM of *M. tuberculosis* possesses the ability to neutralize YoeB of *E. coli*, and vice-versa (Fig. 2).

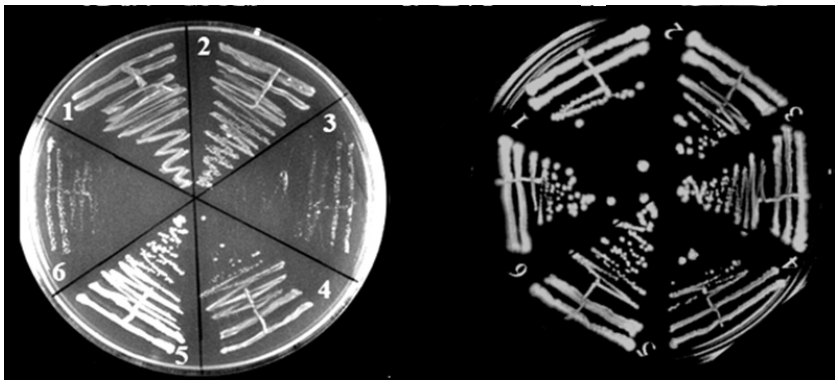

Figure 2. Complementation of *M. tuberculosis yefM* with *E. coli yoeB* : The *E. coli* strain SC36 (MG1655?*yefM-yoeB*)[8] harbouring the plasmids as described below were streaked on LB (ampicillin + chloramphenicol). The results of these are shown as (a) in presence of 0.1% arabinose and 2mM IPTG and (b) 0.2% glucose. It is clear that *E. coli yoeB*, when expressed in the presence of arabinose, is toxic for growth (sectors 3 and 6 in panel a). Its toxicity is effectively neutralized by the *yefM* genes of *E. coli* (sector 1 in panel a) as well as that of *M. tuberculosis* (sector 2 in panel a). Sectors represent: (1) *E. coli yefM* + *E. coli yoeB* (2) *M. tuberculosis yefM* + *E. coli yoeB* (3) pNDM220 vector + *E. coli yoeB* (4) *E. coli yefM* + pBAD33 vector (5) *M. tuberculosis yefM* + pBAD33 vector and (6) pNDM220 vector + *E. coli yoeB*

Project 3: Application of graph theory to genome-wide protein:protein interactions

We had earlier proposed a set of genome-wide functional linkage in *E. coli*. Using this data, during the period under review, we have proposed a method, where the gene expression data can be understood in the context of genome-wide functional linkages. Application of this method has been demonstrated for microarray data on UV exposed *E. coli*, and the corresponding microarray data on the *E. coli lexA* mutant.

Future plans and direction

We plan to undertake analysis of the heat shock repressor proteins of *M. tuberculosis*, as well as study properties of *M. tuberculosis* GroELs. These will also be undertaken for crystallographic analysis.

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Laboratory of Mammalian Genetics

Epigenetic mechanisms underlying developmental pathways

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Project 1: *DNMT3L*: epigenetic correlation with cancer

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Previously, we had reported loss of DNA methylation at the promoter of the DNA methyltransferase, *DNMT3L*, in cervical cancer samples. \We had observed loss of DNA methylation at the promoter of *DNMT3L* gene in more than 90% of the cancer cervical samples in comparison to normal samples which showed promoter hypermethylation. This loss of *DNMT3L* promoter DNA methylation correlated with its expression. Furthermore, we had found that *DNMT3L* overexpression stimulated cellular proliferation in HeLa and SiHa cells.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

DNMT3L and Nuclear Reprogramming

The correlation of *DNMT3L* promoter hypomethylation and its increased expression suggested that *DNMT3L* could be overexpressed in cervical cancer. To test if *DNMT3L* overexpression leads to neoplastic changes, it was decided to check whether *DNMT3L* overexpression could cause epigenetic reprogramming, which is an important correlate of most cancers. Our results showed that *DNMT3L* overexpression can induce nuclear reprogramming. Importantly, we found that the nuclear reprogramming by DNMT3L was a gradual process. This was evident not only at the molecular level where genome-wide gene expression level changes were observed upon overexpression of *DNMT3L* in HeLa cells but was also apparent from the morphological changes observed in *DNMT3L*-overexpressing HeLa cells (figure 1a). Morphological changes were apparent only around p20. Genome-wide changes in gene expression were clearly visible concomitant with morphology changes (Figure 1b). This gradual nature of reprogramming suggested a cascading effect of *DNMT3L* overexpression on the transcriptional machinery of the cell. As a regulator of *de novo* DNA methyltransferases and not as an actual DNA methyltransferase itself, it is possible that a change in the expression levels of *DNMT3L* is only able to alter DNA methylation and expression of a small subset of genes. But the change in expression level of these genes could trigger downstream events, eventually leading to genome-wide changes. This gradual amplification of the initial alteration caused by *DNMT3L* overexpression is very similar to what is known of events during carcinogenesis.

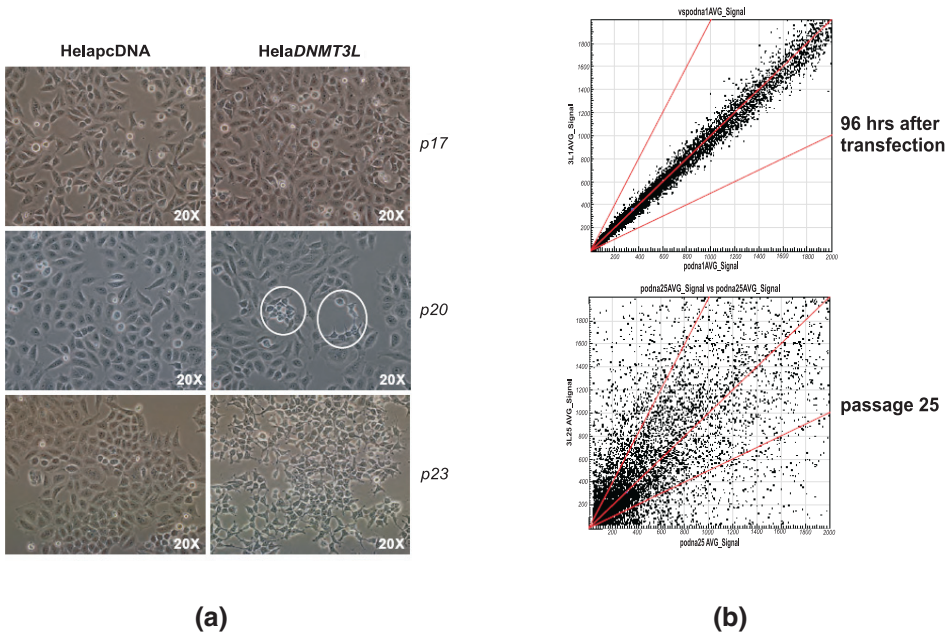

Figure 1: *DNMT3L and Nuclear Reprogramming.* (a) Altered cell morphology in *DNMT3L*-overexpressing HeLa cells. *DNMT3L*-overexpressing HeLa cells showed a gradual change in morphology with increasing passage (left panel). p17, p20 and p23: passage numbers. Circled regions in p20 show cells with the altered morphology. (b) Scatter plots comparing gene expression profiles between control vector and *DNMT3L* transfected HeLa cells with after 96 hours of transfection and 25th passage upon selection.

Project 2: Role of *Dnmt2* in mammalian cells

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Though it has all the domains specific for methyl transferases, *Dnmt2* has failed to show significant DNA methylation *in vitro* and *in vivo* conditions. Recently, *Dnmt2* was shown to methylate tRNA in mice but its role in mammals remains a mystery as *Dnmt2* Knock-out mice are viable and do not show any phenotypic abnormalities. As reported in the last report we had identified several proteins involved in RNA processing to be the interacting partners of DNmt2 by Yeast-two hybrid and Immuno-precipitation experiments.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Dnmt2 is part of RNA processing during cellular stress

Overexpression of *GFP-Dnmt2* in our study and sub-cellular localization studies for Dnmt2 by others previously had shown it to be predominantly localized in the cytoplasm. However, examination of the endogenous localization of Dnmt2 and its interacting partners revealed that Dnmt2 localises to cytoplasm only during cellular stress and under normal conditions it is localized in the nucleus (figure 2). Interestingly, for a DNA methyltransferase, mice knockout of which showed no phenotypic defects, our results show that *Dnmt2*-overexpression causes cell lethality. *Dnmt2* overexpression also leads to increase in the number of cells that were multinucleated. Microarray analysis showed that Dnmt2 overexpression deregulates several gene families. Most conspicuous genes to be deregulated were those involved in RNA processing, cellular stress, apoptosis and host-response to viral infection.

Since *Dnmt2* is the only known DNA methyltransferase to be conserved across various species, it is possible that methylation function of *Dnmt2* is part of a primitive mechanism by which a cell deals with environmental challenges especially viral infections. This possibility is based on the observations that viral infections can induce stress granule formation and our own microarray data which showed change in expression of several genes, involved in host response to viral infection, upon *Dnmt2* overexpression. It is possible that pathways and mechanisms involving Dnmt2 were used to deal with environmental cues causing cellular stress (especially in the context of viral infections) in organisms like Drosophila lacking the other DNA methyltransferases. But with the

evolution of other DNA methyltransferases, which have the capability to silence foreign DNA introduced into the cells during viral infections, the role of *Dnmt2* has probably become redundant. Examination of this hypothesis would be useful in understanding the functional capabilities of *Dnmt2*. Moreover, investigating whether localization of Dnmt2 to stress granules is due to its DNA and tRNA methylation function or because of a novel property would be important to unravel the role of Dnmt2 during cellular stress.

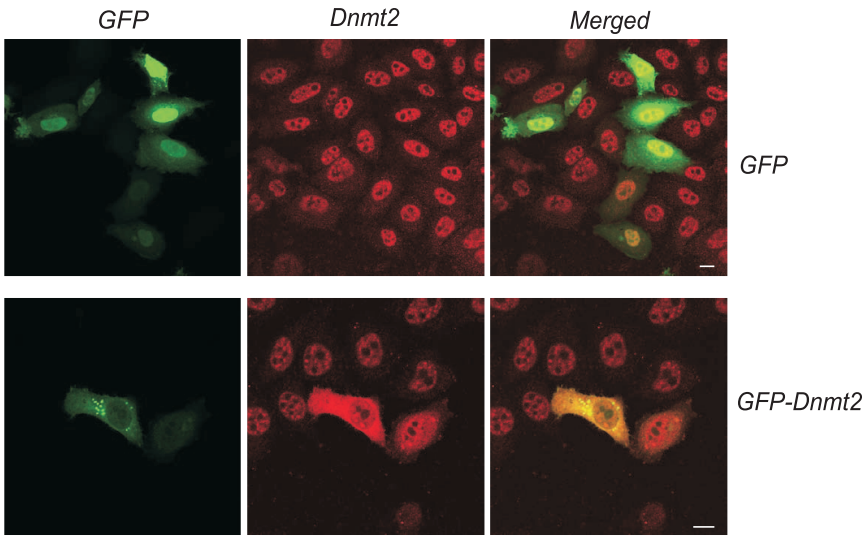

Figure 2: Relocalization of endogenous Dnmt2 to cytoplasm. HeLa cells transfected with *GFP-Dnmt2* or *GFP* alone were immunostained with Dnmt2 antibody. Secondary antibody conjugated to Alexafluor594 was used. Observe the difference in localization of endogenous Dnmt2 (red) in GFP-Dnmt2 positive and untransfected cells.

Project 3: Host epigenetic response to infection (New)

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Infection by *M. tuberculosis* is analogous to an environmental cue to which the host cell(s) respond by various mechanisms. Presence or absence of certain gene products in the host may be required, as these might enhance the process of infection. This would mean transcriptional activation or repression at the genetic level. The role of epigenetic events in the regulation of gene expression at the level of DNA methylation, histone modifications etc. are well known. In order to respond to the challenge by an environmental cue (in this case infection by *M. tuberculosis*), the cells would have to reprogram the epigenetic markings at several loci in the genome so that the affected gene may be appropriately modulated. One of the aims of our study is to examine these epigenetic changes and identify the genetic loci where these changes are brought about. This, we believe, will provide us important evidence on the genes in the host that might be participating in a response to mycobacterial infection.

The closely related species of *M. tuberculosis*, which are likely to exert similar effects on host gene expression, are *M. smegmatis* and *M. bovis BCG*. *M. smegmatis* are the fast growing relatives of *M. tuberculosis*, and are cleared rapidly from the host upon infection. These can thus be considered incompetent in modulating the host immune response. *M. bovis BCG* are evolutionarily closely related to *M. tuberculosis*, and are the derivatives of the *M. tuberculosis* complex. The crucial difference between *M. bovis BCG* and *M. tuberculosis* relates to the ability of *M. bovis BCG* in reprogramming the host immune response, such that subsequent infection by *M. tuberculosis* is not sustained. Moreover, *M. bovis BCG* are incapacitated in establishing an infection. Thus, even though their might be similarities in the host response to the three species, viz. *M. tuberculosis*, *M. smegmatis* and *M. bovis BCG*, possibility that subtle difference in which they evoke a (epi)genetic reprogramming within the host exists. Understanding of these subtle differences in the reprogramming event might lead to the understanding the components of host and *M. tuberculosis* interactions that are required to

establish dormant infection. We would therefore like to explore the differential effects of the three species on reprogramming the host (epi)genetic response.

As a part of this project, we decided to explore if any DNA methylation changes occur in macrophage cell lines upon infection with *M.bovis*. As shown in figure 3, difference were found between infected and uninfected cells using AIMS (Amplification of InterMethylated Sites) technique which can identify DNA methylation differences on a genome-wide basis. We are now in the process of confirming these findings using DNA methylation ChIP-on-Chip technology.

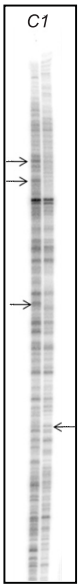

Figure 3: Representative gel showing DNA methylation difference between uninfected (C) and infected (I) Thp1 cells using AIMS. Arrows indicate differentially methylated DNA fragments.

Publications:

Sowpati DT, Thiagarajan D, Sharma S, Sultana H, John R, Surani A, Mishra RK and Khosla S (2008) An intronic DNA sequence within the mouse Neuronatin gene exhibits biochemical characteristics of an ICR and acts as a transcriptional activator in Drosophila. ***Mechanisms of Development*** 125:963-973.

Laboratory of Molecular Oncology

Genomics and molecular genetics of cancer and human genetic disorders

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Objectives

1. Identification of important deregulated genes/ pathways in cancers prevalent in India
2. Identificatoin of disease causing mutations and their characterization in genetic disorders

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

(a) Colorectal cancer : Work on the identification and characterization of major deregulated pathway(s) in young colorectal cancer patients was initiated. The strategy involved identification of status of Wnt signaling pathway (through beta catenin-IHC and APC mutation screening) and identification of status of microsatellite instability (MSI). Analysis of 103 samples revealed Wnt deregulation in 75% of elder patients but only in 40% of young patients, a statistically significant difference. In addition, several novel APC mutations were identified from Wnt+ tumors. Validation of HEEBO arrays from Stanford University was undertaken for carrying out comparative genomic hybridization as well as for transcript profiling. In addition, 6 CRC samples were analyzed by aCGH and a novel amplification located at 17p11.2 was identified.

(b) Esophageal cancer : Beta Catenin IHC based analysis of Wnt signaling was carried out on 54 esophageal cancer samples. Five out of fourteen adenocarcinonma samples exhibited deregulated Wnt activation where as in squamous cell carcinoma samples, Wnt activation was seen only in three out of fourty samples. Screening for microsatellite instability was also initiated but only 5 samples were analyzed. P53 inactivation was shown

to be a common event based on IHC results; nuclear stabilization of the protein was detected in twenty out of fourty three samples. P53 mutaton analysis was also initiated; out of 8 samples screened, mutation was detected in six samples out of which three harbored novel mutations. Array based CGH revealed two recurrent amplicons, one located at 11q13 that harbored the Cyclin D1 gene and the other, a novel amplification located at 10q21.

(c) **Hypohidrotic/anhidrotic ectodermal dysplasia (HED) :** HED is a rare genetic disorder that affects tissues of ectodermal origin including hair, teeth/ sweat glands, etc. Analysis of eight patients revealed four novel mutations while the other four harbored known mutations. Two novel mutations in the EDA gene were characterized by sequence and structural analysis and the results revealed that the mutations either destabilized the functional EDA trimer or perturbed the electrostatic surface charges.

(d) **Familial Hypertrophic Cardiomyopathy :** We detected mutations in the MyBPC3 gene in six families; p.C1124X in one sample; p.G758D in two samples, IVS28+1 (G to A) in one sample and a novel mutation IVS19+7 (G to A) in two samples.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Project1: Molecular genetic analyses of sporadic colorectal cancer occurring in the young

During the period under review, further analysis of CRC samples has revealed a statistically significant difference in the proportion of Wnt positive tumors between young (<50 yrs) and older (>60 yrs) patients. In order to understand the biology of tumors in young patients that do not exhibit deregulated Wnt signaling, we have carried out analysis of copy number alterations using array based comparative genomic hybridization. Six samples were analyzed in the previous reporting year and a further nine samples have been analyzed in the current year. Overall, genome-wide copy number analysis has been performed for seven Wnt negative tumors, three moderately Wnt positive tumors and four highly Wnt positive tumors. Differential copy number alterations included gain of Wnt5B and Cyclin D2 in Wnt positive tumors and the co-amplificatioon of ErBB2 and Grb7 in Wnt negative tumors. Wnt5b has been shown to be previously upregulated in several other cancers including Leiomyomas and esophagous and pancreatic cancer. Grb7 and ErBB2 are co-amplified in several cancers; antibody based therapy targeted against ErBB2, is routinely used for treatment of a subset of breast cancer patients that harbor amplification of this important oncogene. It would be important to analyze a larger cohort of CRC samples to detect whether these changes are recurrent and to identify other relevant deregulated CRC genes. Analysis of Wnt positive tumors has yielded APC mutations in several samples; novel mutations detected in the reporting year include p.L1511P, p.R1378K and the p.L1342X mutations.

Project 2: Analysis of DNA copy number alterations in esophageal cancer

We had earlier commenced molecular analysis of differences between esophageal adenocarcinoma and squamous cell carcinoma. We have now extended the analysis to a larger cohort of samples. P53 immunohistochemistry revealed nuclear staining in a significant proportion of adenocarcinoma (50%; 14/28) and squamous cell carcinoma samples (64%; 48/75), confirming earlier observations that p53 inactivation is a common event in both subtypes of esophageal cancer. We detected a significant difference (p=0.0001; fisher's exact test) in the status of Wnt signaling between the two subtypes of esophageal cancer; ESCC (6.3%; 5/79), Ea (50%; 15/30). In order to identify other significant differences between the two subtypes of cancer, we have extended analysis of copy number alterations (CNA) to a total of 14 samples (three Ea and eleven ESCC samples). Several interesting amplicons were identified; importantly, CNA profiles differed between the two subtypes. These results support our hypothesis that the biology of squamous cell carcinoma, more common in India, may differ significantly from adenocarcinoma, more common in western countries. Therefore, application of therapeutic strategies based on studies carried out in the West on adenocarcinoma may not be applicable to squamous cell carcinoma, thereby necessitating designinig of novel strategies to combat this major cancer in our country. Genome-wide transcript profiling was carried out on a total of ten samples including the two samples that harbored a novel amplicon at 10q21, reported last year. Interestingly, probes located within the amplification also exhibited significant increase in transcript levels (Figure 1). The average expression level for all the probes located within the amplicon was 24-fold

higher than the average expression level for all probes specific for other regions. In comparison, the difference was only 1.5-fold for another sample which did not harbor the amplicon, a statistically significant difference. Therefore, copy number gain is leading to a corresponding increase in expression of genes located within the amplified region; and one or more of the genes could be important for maintanence of the tumor phenotype.

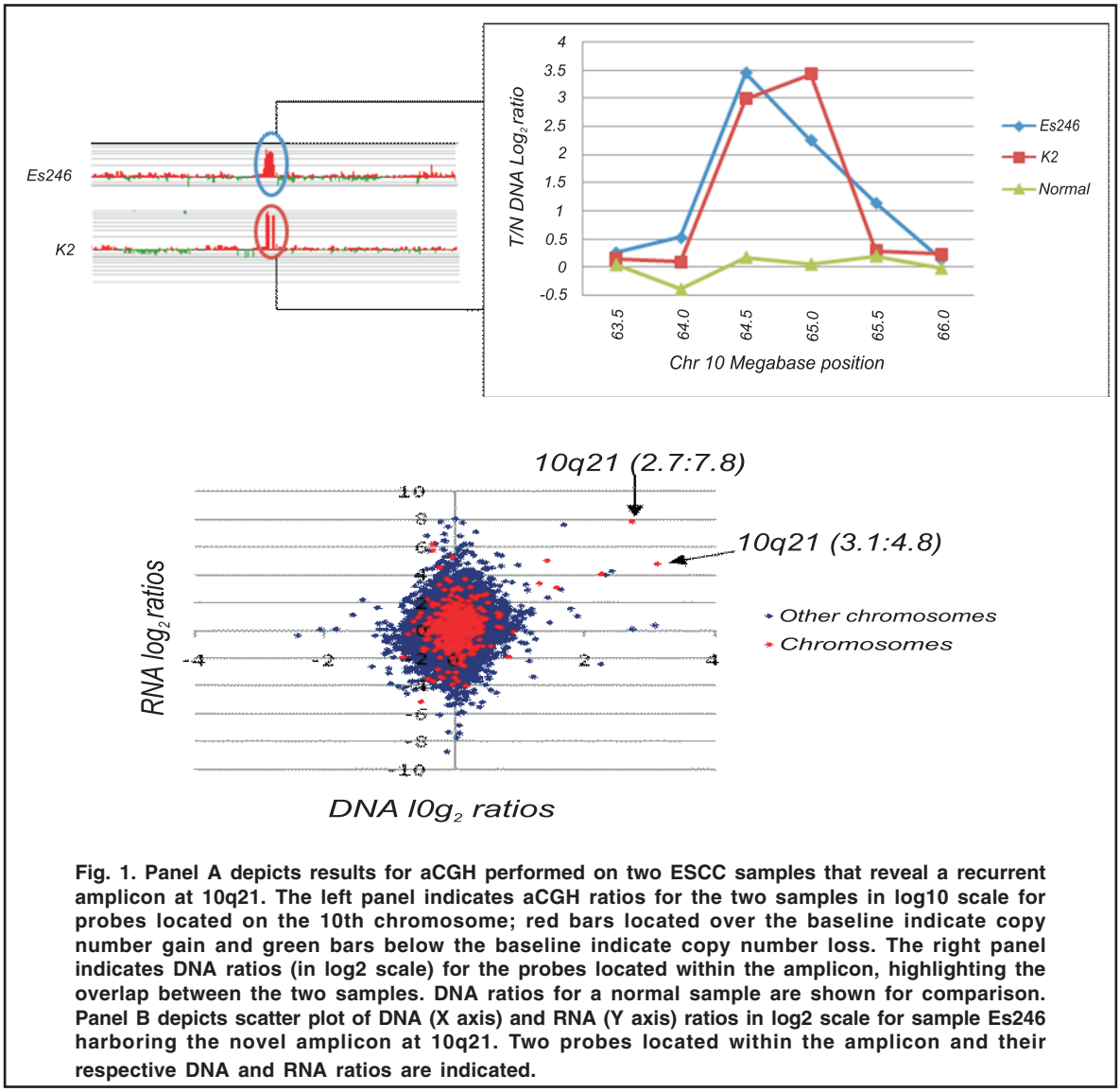

Fig. 1. Panel A depicts results for aCGH performed on two ESCC samples that reveal a recurrent amplicon at 10q21. The left panel indicates aCGH ratios for the two samples in log10 scale for probes located on the 10th chromosome; red bars located over the baseline indicate copy number gain and green bars below the baseline indicate copy number loss. The right panel indicates DNA ratios (in log2 scale) for the probes located within the amplicon, highlighting the overlap between the two samples. DNA ratios for a normal sample are shown for comparison. Panel B depicts scatter plot of DNA (X axis) and RNA (Y axis) ratios in log2 scale for sample Es246 harboring the novel amplicon at 10q21. Two probes located within the amplicon and their respective DNA and RNA ratios are indicated.

Project 3: Molecular genetic analyses of hypohidrotic or anhidrotic ectodermal dysplasia (HED)

Last year we reported identification of mutations in eight HED patients. During the current reporting period, we have screened an additional five patients and identified the p.G291W and p.R244X mutations in the EDA gene and the p.V340L (novel), P.C71Y (novel) and the p.G382S mutations in the EDAR genes, respectively. Interestingly, we have detected the EDAR p.G382S mutation in four independent families, out of the total eleven families that we have screened so far. In adition, the same mutation has also been reported from Pakistan. It is therefore possible that this could be a founder mutation in the Asian population. We have initiated haplotype analysis to confirm the same.

Project 4: Molecular genetic analyses of Familial Hypertrophic Cardiomyopathy (FHC)

We had earlier reported identification of the MYH7 p.R787H mutation in four independent families. In two of the four families, no additional member

harbored the MYH7 p.R787H mutation. Interestingly however, the probands in these two families exhibited compound heterozygosity; one harbored a novel MYBPC3 p.S1212X mutation (family 3) and the other harbored the MYBPC3 p.G758D mutation (family 4), in addition to the MYH7 p.R787H mutation. None of the other members tested harbored either of the two mutations in family 3. Five members in addition to the proband harbored the MYBPC3 p.G758D mutation in family 4. Our work has highlighted the importance of screening all important genes in FHC patients. Last year we reported the identification of a novel MyBPC3 IVS19+7 (G to A) mutation in two samples. A careful survey of the literature however revealed that mutations that affected the 5' splice site were restricted to the first 5 bases of the intron; in fact there was no mutation reported for any genetic disorder that affected the 7th base of the intron. Is it then possible that this could be a novel SNP in the India population? To answer this question, we have screened one hundred and fourty normal samples (two hundred and eighty 'normal' chromosomes) in addition to eighty patient samples (one hundred sixty patient chromosomes). Interestingly, the mutation was detected in a heterozygous condition in three normal and two patient samples, providing an overall allele frequency of 1.1%, indicating thereby that this could be a novel SNP in the Indian population. We also identified the MyBPC3 25 bp deletion located in the 34th intron that has been shown to occur with a frequency ranging from 2-8% in the Indian population. The deletion was present in a heterozygous condition in four out of eighty patient samples and in five out of 100 normal samples; an allele frequency of 2.5%. Further screening of patient samples identified the MyBPC3 IVS8+1 (G to A) mutation (two samples) and a novel MYH7 p.S322F mutation (one sample).

Future plans and direction

1. We plan to screen more CRC samples to determine status of Wnt signaling and microsatellite instaibility in young and older patients. Microarray based CGH and expression profiling will be carried out to deterimine deregulated pathwasy in Wnt negative CRC samples.
2. We plan to screen more esophageal cancner samples to determine status of Wnt/MSI/EGFR/p53 pathways. In addition, microarray based CGH and expression profiling will be undertaken to determine important deregulated pathways in squamous cell carcinoma of the esophagous and to classify tumors based on EGFR/p53/Wnt/MSI status.
3. Haplotype analysis will be carried out to derermine whether the p.G382S mutation, that cuases hypohidrotic ectodermal dysplasia, could be a founder mutation in India.
4. Fifty FHC patient samples will be screened for mutations in sarcomere genes.

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1. Kwei KA, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi Y, Chatterjee S, Karikari CA, Salari K, WangP, Hernandez-Boussard T, Swarnalata G, Rijn MV, Maitra A and Pollack JR (2008) Genomic profiling identifies GATA6 as a candidate oncogene amplified in pancreato-biliary cancer. ***PLOS Genetics*** 4:e100081.
2. Bashyam MD (2009) Studies on nonsense mediated decay reveal novel therapeutic options for genetic diseases. ***Recent patents on DNA & Gene Sequences*** 3:7-15.
3. Kumar S, Ramachandran A, Hasnain SE and Bashyam MD (2009) Octamer and heat shock elements regulate transcription from the AcMNPV polyhedrin gene promoter. ***Archives of Virology*** 154:445-456.
4. Angalena R, Chaudhary A, Bashyam MD and Dalal A (2009) Hemoglobin D (Iran) masquerading as Hemoglobin E: An interesting case report. ***Newsletter of Genetics Chapter of Indian Academy of Pediatrics*** (In Press).
5. Bashyam MD (2009) Nonse-mediated decay: linking a basic cellular process to human disease. ***Expert Review of Molecular Diagnostics*** (In Press).

Laboratory of Cancer Biology

Principle Investigator	Gayatri Ramakrishna	Staff Scientist
Ph D Students	Arvind Singh	SRF
	Shashi Kiran Sinha	JRF
	Babul Moni Ram	JRF
Others	Jayashree Dolpady	PDF
Collaborators	Pavani Sowjanya, Mediciti group, Medchal	

Project 1: Understanding the biology of Ras-mediated signaling events

Objective:

The major focus is to (a) delineate the signaling pathways between the various point mutants of Ras, (b) understand if there are any cell-type specific differences between fibroblasts and epithelial cells using both an *in vitro* and in *vivo* approach.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

1. An unusual downregulation of Ras-p21 in hepatocellular carcinoma (HCC) and this correlated well with upregulation of the iNOS pathway in the same tumors.
2. We created stable Tet-inducible K-*ras* mouse lung epithelial cells with various point mutants of K-*ras* and compared the growth profiles which showed only subtle changes.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Using the various conditionally expressing mutant form of K-*ras* viz., codon 12 mutant (12V), codon 13 mutant (13V) and codon 61 mutant (Glu 61) we evaluated in detail the various (a) growth parameters and (b) downstream signaling events. Comparative growth profile assays showed increased proliferative efficiency of 13V mutant (Fig. 1a). It has been reported that mutation in K-*ras* leads to transformation of epithelial and fibroblast cells. When we carried out the soft agar assay in early passage cells none of the K-ras mutant conferred anchorage independent growth advantage to the lung epithelial cells, however the late passage cells of 61R mutant of K-*ras* showed colonies in soft agar (Fig.1b). We next analysed the activation of two classical Ras-signalling cascades in the K-*ras* mutant Tet-inducible cell lines. While in all three stable cell lines we found an activation of the MAPK and AKT no appreciable differences were seen amongst the three mutants. However we made an interesting observation that following serum stimulation ERK1 activation is transient in nature in all the three mutants, however the ERK2 pathway remains *ras* actively sustained. This is indicative of differential role for the two kinases in cell signaling and further alterations of the ratio of ERK1/ERK2 within the cell could act as a sensor in affect the threshold properties of the Ras-signaling event controlling differential cellular responses. However our attempt to prove this using the ERK2 knockout based approach using the adenoviral expression system was not very successful and work is in progress to use an alternative RNAi strategy to test the above hypothesis.

Conclusions:

1. Amongst the three different K-ras mutants only the codon 61R Mutant Tet-inducible cell lines showed anchorage independence at higher passage.
2. Irrespective of point mutation in K-ras all the three mutants of K-ras showed altered ratio of ERK1/ERK2 following serum stimulation.

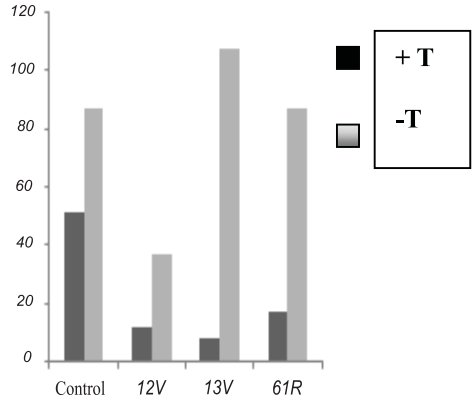

Fig.1 Colony formation assay in presence and absence (-T) of Tetracycline (+T) in control and different point mutants of ras (12Vm 13V, 61R) (The number of colonies are more when the gene is expressed in absence of tetracycline).

Project 2: Screening of Human papilloma virus (HPV) for cervical cancer screening programme and understanding the role of host biomarkers in cancer cervix progression.

Objective

In an ongoing collaborative project with a rural hospital based in outskirts of Hyderabad an attempt is being made to (a) understand the prevalence and genotype distribution of high risk HPV types in and (b) identify new biomarkers which can be used as an adjunct to HPV testing.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

From the start of the project in 2004 till date we have screened a total of 2374 women attending the rural cancer detection and HPV screening clinic of mediciti hospital at Medchal Mandal. Of these a total of 232 women were positive for HPV indicating HPV prevalence close to 11%. Majority of the women showed a clearance on follow up. Also we had reported that it is feasible for the women to self-collect vaginal samples for screening of HPV. Besides HPV testing we also found that detection of free circulating hTERT and hTR correlate with the cervical cancer disease burden.

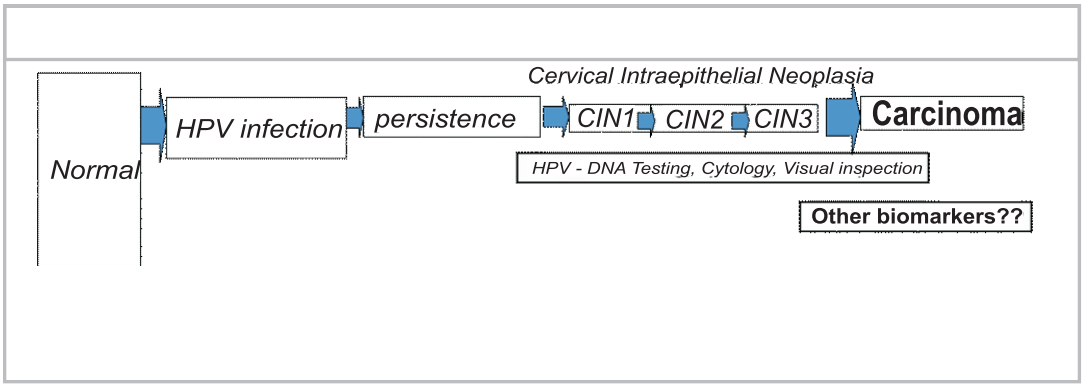

Fig. 2 Various stages in the cancer cervix progression following infection with HPV

Summary of work done until the beginning of this reporting year (April 1, 2008 - March 31, 2009)

1. Feasibility of home-based cervical cancer screening:

Objective: Our aim was to determine if (1) Hybrid Capture 2 and a PCR-based method were comparable for detection of high-risk HPVs, (2) clinician-collected and self-collected samples were equally efficient to detect HPV and cervical cancer precursor lesions and (3) if participation rates improved with home-based vs. clinic-based self collection.

Methods: Samples were selected from women participating in a cervical cancer screening study for both human papillomavirus (HPV) and Pap-test screening. Besides the clinician also performed visual inspection with acetic acid (VIA) for detection of any lesion. For 432 of 892 selected women, split sample aliquots were tested for HPV DNA using both the Hybrid Capture 2 assay (HC2) and the Roche prototype line blot assay. Women from a subset of villages were recruited at two separate time points for clinic-based self-collection and home-based self-collection, and participation rates were compared.

Results: Pairwise agreement between self- and clinician-collected samples was high by both HC2 (90.8% agreement, kappa=0.7) and PCR (92.6% agreement, kappa=0.8), with significantly increased high-risk HPV detection in clinician-collected specimens (McNemar's $p<0.01$). Ability to detect precursor lesions was highest by PCR testing of clinician-collected samples and lowest by Hybrid Capture 2 testing of self-collected samples (11/11 and 9/11 cases of cervical intraepithelial neoplasia grade 2/3 and cancer detected, respectively). Participation in home-based screening was significantly higher than clinic-based screening (71.5% and 53.8%, respectively; $p<0.001$) among women 30-45 years old.

Conclusion: The combination of improved screening coverage and a high single test sensitivity afforded by HPV- DNA testing of home-based self-collected

swabs may have a greater programmatic impact on cervical cancer mortality reduction compared to programs requiring a pelvic exam.

2. Activation of NFATc1 in cancer cervix:

Infection with high risk HPV-types is a major etiological risk factor for cervical neoplasia. Majority of the women infected with HPV show effective clearance of the virus, however in a subset of women with persistent infection the high risk HPV types lead to neoplastic changes of cervical epithelium. HPV alone is insufficient for causation of cervical disease and other molecular events are essential for progression towards invasive cancer.

Objectives: It has been noted that the HPV infection leads to a strong inflammatory response in the cervix. We therefore wanted to check if proteins belonging to , the nuclear factor of activated T cell (NFAT) family which play a central roles in inflammation and T lymphocyte activation are activated in the cervical cancer infected with Human papilloma virus. The NFAT family has five major members viz, NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5. We checked for the expression and activation of the three major members using *in vitro* model systems and also in the cervical neoplasia.

Results: NFAT2 expression was checked in four different cell line viz. normal immortalized keratinocytes (HaCaT) and cervical cancer cell lines which are infected with HPV (Hela and Siha) and uninfected with HPV (C33A). Of the three isoforms of NFAT, we found a significant upregulation in expression and activation of NFAT2 in the HPV infected cell lines (Hela and Siha) in comparison to the HPV negative cell lines (HaCaT and C33A) (Fig. 3). We found a similar increase in both expression and activation of NFAT2 in biopsy samples obtained from squamous cell carcinoma. Interestingly when we knocked down NFAT2 by RNAi approach the growth of Siha cells were retarded. This was also evident by the FACS anlaysis where the subG0 population goes up following knockdown of NFAT2. Besides the involvement of NFAT2 our results are also indicative of an involvement of both calcineurin and RCAN1 (negative regulator of calcineurin) in cervical cancer.

Conclusion: Our results demonstrate activation of NFAT2 and involvement of HPV E6/E7 proteins in activation of the Ca^{2+}/calcineurin signaling pathway in cervical cancer.

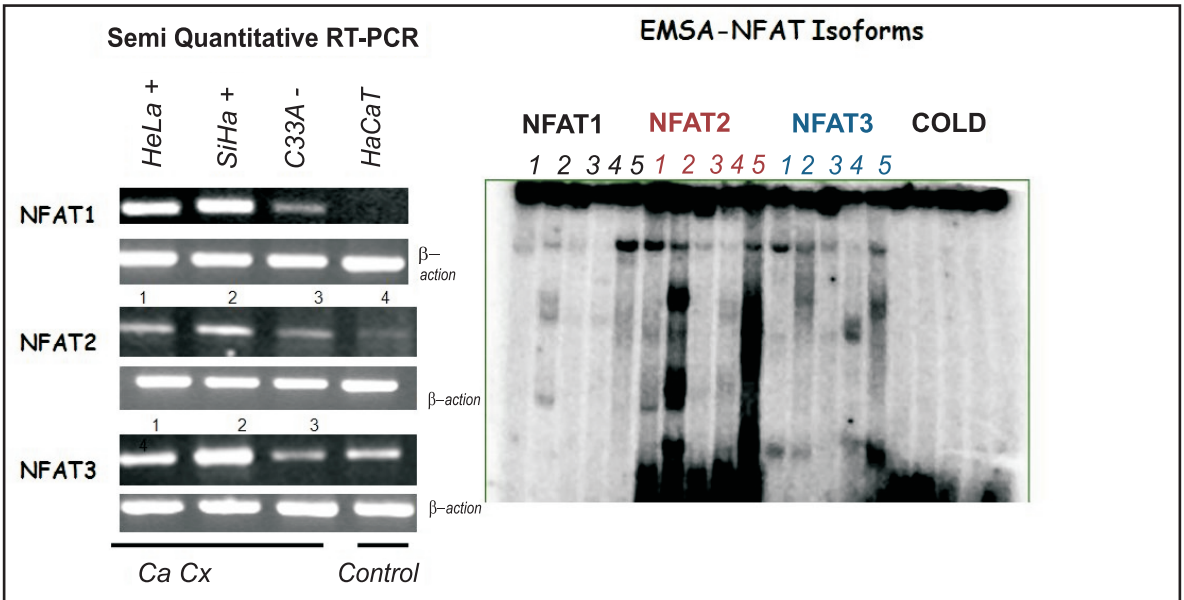

Fig. 3: Expression and activation of NFAT isoforms in normal (HaCaT) and cervical cancer cell lines (Hela, Siha and C33A). 1: HaCaT, 2: C33A, 3: Siha ,4: Hela and 5: Thp1

Project 3: Understanding the biology of Sirtuins, Sirt7, in context of cellular proliferation and senescence *(New project started)*

Objective:

Besides the genetic make-up of the cell, the epigenome plays a crucial role in gene regulation. The Epigenome in turn is maintained by acetylation and methylation of the chromatin and its associated protein. Chromatin modification are brought about by a DNA methyl transferases (DNMTS) and Histone deacetylases (HDACS). Amongst the various HDACS known , members of the silent information regulator 2 (Sir2) family are conserved from yeast to humans and regulate lifespan in various

organisms. Some of the recent reports point to role of Sirtuins as critical regulators at the crossroads between cancer and aging. However the exact functions of the various isoforms in context of cell proliferation and ageing is still unclear in higher organisms. In this context we intend to take a detailed study on the role of Sirtuins and in particular Sirt7 in relation to cell growth and senescence.

Details of progress made in the current reporting year (April 1, 2008- March 31, 2009)

Rationale of the study

The rationale for selecting SirT7 is it's (a) nucleolar localization and (b) role in ribosome synthesis and the main objectives of the study is to investigate the role of SirT7 in cell cycle progression and cellular proliferation and its role in context of cellular senescence and cancer.

Results:

To check if any mutations can be detected in SirT7 in cancerous cells we designed two different approaches : (a) we sequenced SirT7 cDNA (from 400bp to 1200bp) from various cancer cell lines of different origin viz., lung carcinoma (A549) , colon cancer HCT116; cervical cancer (Hela, SiHa, C33A) ; Lymphoid (U87, U373), immortalized keratinocyte (HaCaT); mammary (MCF-7) and hepatocellular carcinoma (HepG2 and Hep3B). Further sequencing of the region from 1- 400 is being carried out now. We didn't find any mutation in region 400bp to 1200bp in any of cell lines except A549 which showed a mutation E256K (G to A transition) in exon 7 in lung carcinoma cell line A549 (Fig. 4). To check if this mutation causes any change in deacetylation we plan to the clone this mutant form of SirT7 and transfect in mammalian cells and check for the growth properties. (b) we also sequenced exon 5, 6, 7 from DNA of primary human colon cancer, as these exons encompass the active site of the enzyme responsible for deacetylation. We did not find mutation in any of these exons atleast in the colon cancer that we analyzed. Future studies are underway to understand the importance of the above non-synonymous mutation in SirT7 in relation to cancer and in context of both cellular proliferation and senescence.

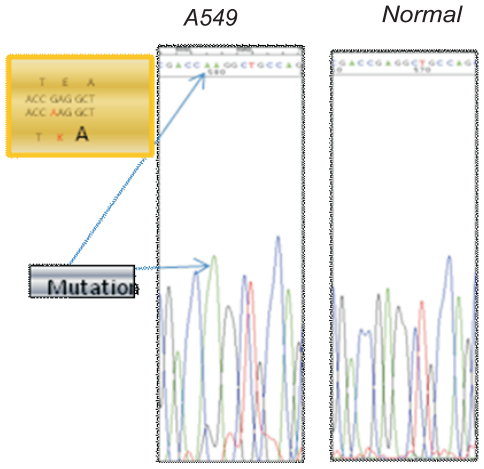

Fig.4 Mutation at site amino acid position 256 (Glutamic acid to Lysine; GAG-AAG transition in A549 cell line

Computational and Functional Genomics of Microbial Pathogens

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Project I: Genome analysis and functional characterization of *Plasmodium falciparum*

1. Amino Acid Substitutions in *Plasmodium falciparum*

The extreme compositional bias (AT bias) of genomes like *P. falciparum* has considerably complicated the sequencing and annotation efforts.

Objectives

- To use the improved substitution matrices for assigning functions to the hypothetical proteins of this parasite.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

We examined the amino acid substitution in *P. falciparum* and found significant differences compared to other genomes. Three different series of matrices; symmetric, non-symmetric and a specialized non-symmetric matrix were generated at different clustering percentages of the protein blocks.

Details of progress in the current reporting year (April 1, 2008 – 31, March 2009)

Using the new improved matrices we are attempting the annotation of *P. falciparum* proteins and an effort is being made to assign functions to the hypothetical proteins of this parasite. The functional pathways have been mapped for some of the hypothetical proteins that failed to give potential orthologs with the standard matrices. These proteins fall under important pathways like cell cycle, DNA repair,

pathogenesis and cell death (Figure 1). A further effort in this regard is being made to see if these proteins are gap fillers of metabolic holes in *P. falciparum,* and hence their role in Gene Ontology pathways is being analyzed.

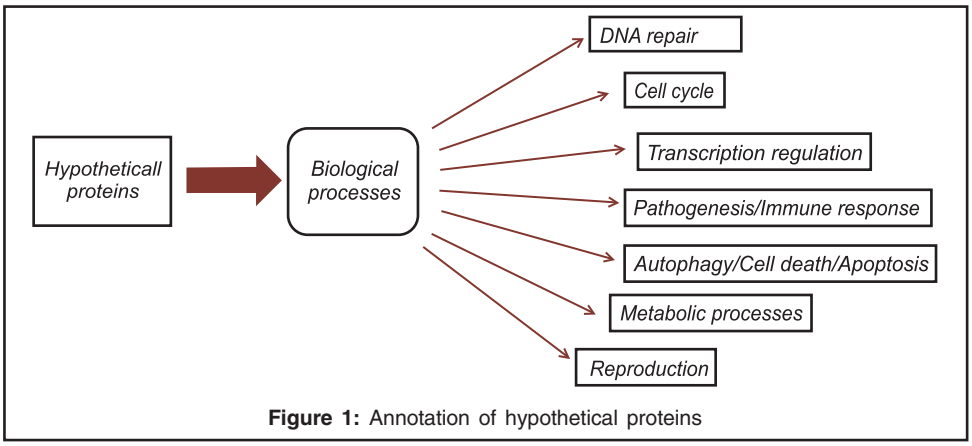

Figure 1: Annotation of hypothetical proteins

2. Nucleotide correlations between exon junctions of an AT biased genome: *Plasmodium falciparum* as a case study

Objectives

The nucleotides at the end of 5' exons and the beginning of 3' exons might contain some correlations in them that are required for the two exons to be joined.

- To study the nucleotide correlations between the exon junctions of an AT biased genome taking *Plasmodium falciparum* as a model organism

Details of progress in the current reporting year (April 1, 2008 – 31, March 2009)

We have downloaded the gene sequences of *P. falciparum*, from PlasmoDB (http://PlasmoDB.org) and have generated datasets of base size 20, (Figure. 2). The relative abundance ® of each of the dinucleotides is calculated by following the method of Karlin Burge (1995) and have observed the correlations (R>1.2) in the dinucleotides of the three datasets for phase 0, 1 and 2.

Our results shows that there is an increase in the correlations from phase 0 to 2, which suggests that phase 2 shows maximum number of correlations between the nucleotides of the exons. We are further exploring if this can be used to effectively identify splice sites in AT biased genome.

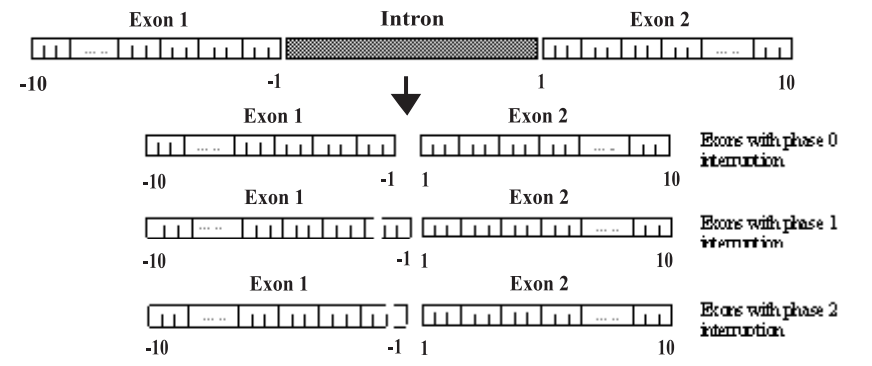

Figure.2. The construction of datasets generated from the exon sequences obtained from PlasmoDB. Each dataset (size 20) was generated by considering ten bases at the end of exon1 and the same number of bases at the beginning of exon2. The three datasets were generated depending upon the interruption of their codons (phase 0, 1 and 2).

3. Role of multiple Acyl CoA binding protein paralogues in *Plasmodium falciparum*

Objectives

- To study the binding preferences of all *Pf* ACBPs and to study stage specific expression of different Pf ACBP genes.
- To study the cellular localization of these ACBPs in *P. falciparum* infected human erythrocytes

Details of progress in the current reporting year (April 1, 2008 – 31, March 2009)

Acyl-coenzyme A binding proteins (ACBPs) are a family of 86 to 103 residues (~10 kD) proteins with conserved amino acid sequences. There are experimental evidences from diverse sources suggesting their role in modulation of fatty acid biosynthesis, regulation of the intracellular acyl-CoA pool size and many more. At present there are four small size ACBPs (10 kD) in *P. falciparum*. Do all Pf ACBPs have similar binding preferences for shorter chain acyl CoAs or some of them prefer longer acyl CoAs like bovine ACBP? To address this question we have cloned and expressed all the four ACBPs as histidine-tagged recombinant proteins and have purified then by Ni-NTA affinity chromatography. The ACBPs were further purified by gel filtration on Superdex-75 column (Figure 3 and 4).

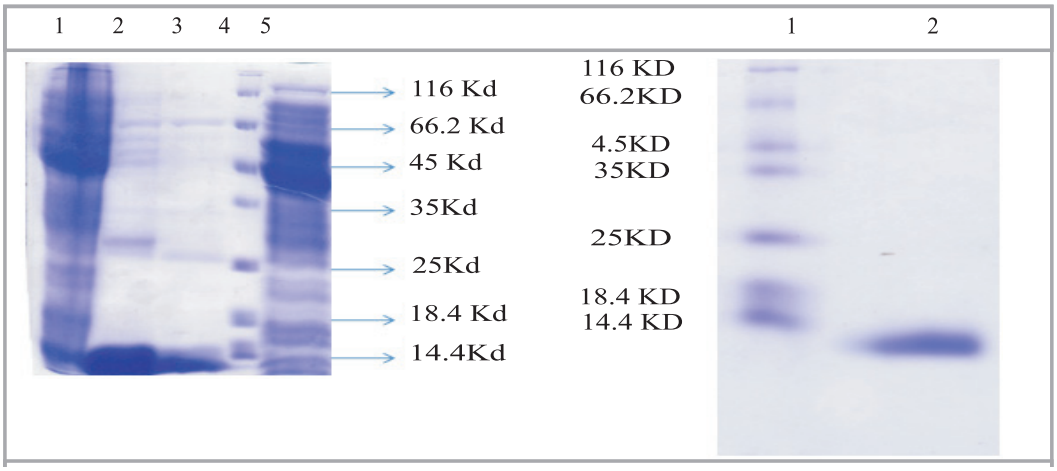

Figure 3: SDS-PAGE of Pf ACBP (PF14_0749) prior to superdex-75. Lane 1- induced culture, lane 2 and 3- purified protein, lane 4- protein markers, lane 5- uninduced culture Figure 4: SDS-PAGE of Pf ACBP (PF14_0749) after gel filtration. Lane 1- protein markers, lane 2- protein purified with Superdex-75

At present we are standardizing the protocols to remove the ligand (acyl CoAs) from ACBPs by reverse-phase HPLC on C18 column followed by visualization of liganded and unliganded ACBPs on isoelectric focusing gels and determination of dissociation coefficient (K_d) of these ACBPs for different acyl-CoAs by Biacore.

Project II: Genome analysis and functional characterization of the genomes of microbial organisms

1. Characterization of metabolite driven transcriptional regulators from *Mycobacterium tuberculosis*.

Objectives

Previous *in silco* analysis revealed that five genes encode FadR-like transcriptional regulators. The objective of this work is:

- To identify operator sites for GntR like transcriptional regulator from *M. tuberculosis* and their experimental validation.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

We have characterized one of the seven GntR-like transcriptional regulators, Rv0586 (mce2R) that was classified as FadR-like transcriptional regulator. We identified putative orthologs of Rv0586 in the genome of other sequenced mycobacteria and closely related non-mycobacterial species like *Nocardia farciana*. All upstream *mce2R* regions were aligned to find conservation in their operator sites in case they are recognized by a similar DNA binding domain. Amongst all the identified operator sites DNA motifs for Rv0586 and Mb0601; Mkms_2771 and Mjls_2751 were found to have

identical sequence. This protein was subjected to electrophoretic mobility shift assays (EMSA), which suggested that Rv0586 binds specifically to its upstream region to a conserved motif across the related species.

Details of progress in the current reporting year (April 1, 2008 – 31, March 2009)

Next we have characterized the HutC like regulator Rv0792. We have identified an upstream sequence that show HutC like binding pattern Figure 5A. and Figure 5B. Our preliminary experimental studies show that Rv0792 show electrophoretic mobility shift with motif in EMSA.

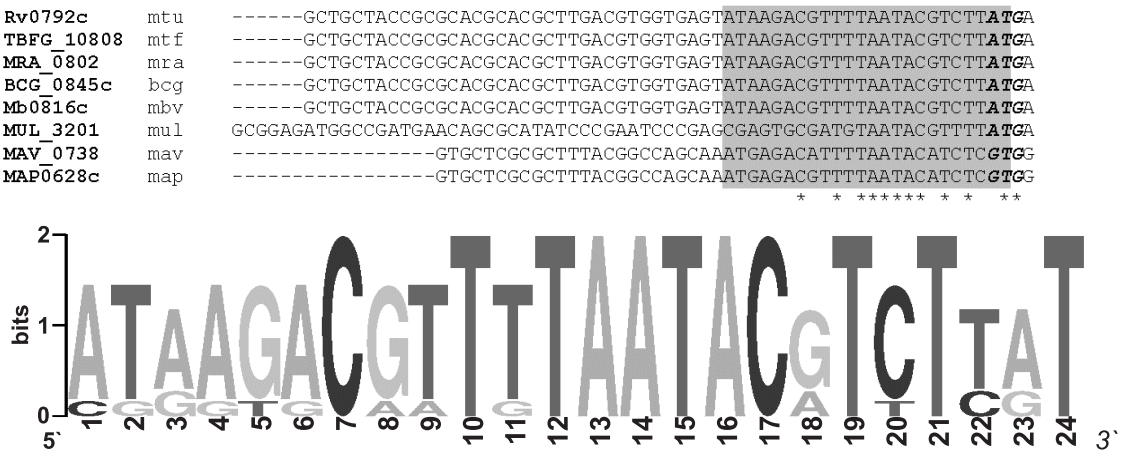

Figure 5. A Sequence alignment of upstream sequence. * marks the conserved position. B Seq Logo representation of this site.

2. Cyclic-Di-GMP signaling: A secondary metabolite driven signaling network in bacteria

The bis-3'-5'-cyclic dimeric guanosine monophosphate (c-di-GMP) is identified as a universal secondary messenger in bacteria and plays important role in cellular signaling and its regulation. The intracellular c-di-GMP concentration depends on the activity of GGDEF and EAL or HD-GYP domain containing proteins.

Objectives

- To understand how the various sensor domains combine with the GGDEF and the EAL domain and are integrated into complex regulatory and signaling networks in bacteria.

Details of progress in the current reporting year (April 1, 2008 – 31, March 2009)

In this study, we first systematically identified GGDEF, EAL, HD-GYP and PilZ domain containing proteins and other additional domains co-occuring with them on the same proteins in 779 bacterial species. This results in the identification of total 124 domains in 16179 bacterial proteins. We determine the sequential order of these 124 domains along a 16179 proteins often termed as domain architecture or organization. Topological properties show that the network is scale-free. The association of few domains at C-terminal with the functionally diverse adjacent domains can propagate a wide variety of downstream signals using few domains at the same time which gives robustness to the system. For example, GGDEF, GerE is a regulator and HisKA is involved in signal transduction mechanism (Figure 6a,b). The addition of domain order to construct linkage network has allowed us to establish novel insights in c-di-GMP signaling. SpoIIE, sporulation related domain has a fair number of linkages and suggests the possible link between sporulation and c-di-GMP signaling (Figure 6a). On the other way, interaction with MCP signal indirectly connect it with Ammonium_transp and hence nitrogen metabolism (Figure 6b).

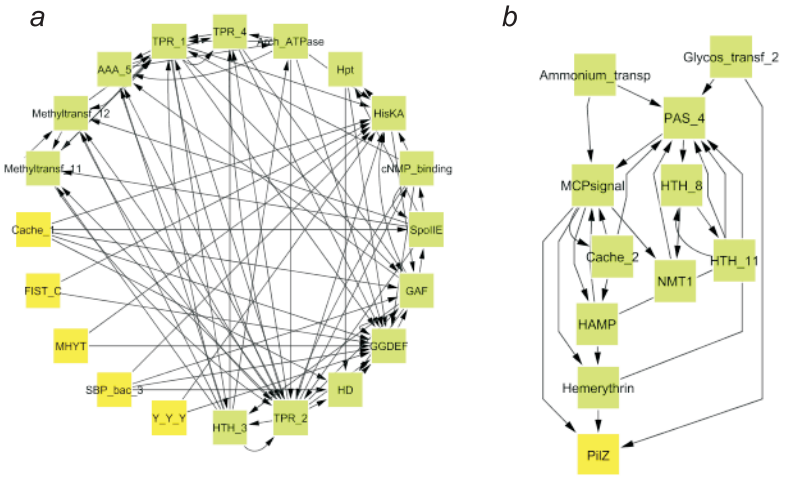

Figure 6. A directed domain architecture graph of C-di-GMP signalling associated domains. a) C-di-GMP synthesis module b) C-di-GMP mediated signalling module. Box represents the domain (nodes in graph) and edges (ponting arrow) indicates N-C terminal arrangement of domains within proteins.

Publications :

1. Paila U, Kondam R and Ranjan A (2008) Genome bias influences amino acid choices: analysis of amino acid substitution and re-compilation of substitution matrices exclusive to an AT-biased genome. ***Nucleic Acids Research*** 36: 6664 - 6675.
2. Rajesh V, Singamsetti VK, Vidya S, Gowrishankar M, Elamaran M, Tripathi J, Radhika NB, Kochar D, Ranjan A, Roy SK, and Das A (2008) *Plasmodium falciparum*: genetic polymorphism in apical membrane antigen-1 gene from Indian isolates. ***Experimental Parasitology*** 119:144-151.
3. Vindal V, Ashwantha Kumar E, and Ranjan A (2008) Identification of operator sites within the upstream region of the putative mce2R gene from mycobacteria. ***FEBS Letters*** 582:1117-1122.

Laboratory of Transcription

Mechanism of transcription termination and antitermination in *Escherichia coli.*

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	Sourav Mishra	JRF
Other Members	C Nisha	PDF
	Kalyani	Senior project assistant
	V Jisha	Project Assistant
	Nanci R. Kolli	Technical Assistant
	Gowresh	Lab Attendant.

Objectives

Transcription must terminate at the end of each operon. In E.coli, end of 50% of the operons consist of intrinsic termination signal that codes for a hairpin followed by a U-rich stretch in mRNA. Rest of the operons does not have any signature sequence and it is possible that termination of these operons depend on a factor called Rho. On the other hand, these termination signals can be overcome in response to certain type of modifications in the elongation complex and process is termed as antitermination. Mechanism of these termination and antitermination processes is still not very clear and offers an exciting subject for study. In my laboratory, studies in the following area are in progress.

1) Mechanism of action of transcription termination factor, Rho.
2) Molecular basis of Rho-NusG interaction.
3) Mechanism of transcription antitermination by N protein at Rho-dependent terminators.
4) Mechanism of action of transcription antititermination of Rho-dependent termination by an anti-rho factor Psu.
5) Physiological significance of Rho dependent termination.

Summary of the work done until the beginning of this reporting year (April 1, 2007- March 31, 2008)

1) By measuring the rate of RNA release by Rho from different stalled elongation complexes we have proposed that Rho exerts a brute force to dislodge the elongation complex (JBC, 2008).
2) We have described the isolation of and characterization different Psu (an inhibitor of Rho) mutants both *in vivo and in vitro*. These mutants together with cross-linking data will be useful to understand the interacting surface of Psu required in complex formation with Rho.

Details of the progress in the current reporting year (April 1, 2007 - March 31, 2008)

(a) Interaction surface of bacteriophage P4 protein Psu required for the complex formation with the transcription terminator Rho. Rho-dependent transcription termination is an essential function in prokaryotes and the transcription terminator Rho is highly conserved among different species. Bacteriophage P4 capsid protein, Psu, specifically interacts with and inhibits the function of E.coli Rho. Interaction surface of Psu involved in interacting with Rho is not known, knowledge of which is not only important to understand the mechanism of its action but also will be useful to design peptide inhibitor(s) for Rho. We have isolated and characterized seven Psu mutants defective in interacting with Rho and in exerting anti-Rho activity. Conformational probing of Psu revealed that the N-terminal region of the protein folds over onto its central part forming a globular domain leaving a solvent exposed “tail” in the C-terminus. The mutations are located in both of these two domains. N-terminal mutants are instrumental in disrupting the N- to C-terminal “cross talk” in Psu which is required for its structural integrity and its function. Site-specific cross-linking experiments

showed that the C-terminal "tail" preferentially cross-links to Rho and also this region of Psu was protected from limited proteolysis when bound to Rho. Therefore, the mutations in this region may have affected the direct interaction of Psu with Rho. We propose that the globular N-terminal domain of Psu confers structural integrity to the functionally important C-terminal "tail" which directly interacts with the hexameric Rho (Figure 1).

(b) Interaction surface required for the complex formation between a transcription terminator and an antiterminator.

The transcription terminator Rho interacts with the transcription elongation factor (also an antiterminator) NusG to bring about the termination process efficiently. Interaction surface involved in the Rho-NusG complex is not known, knowledge of which is essential for understanding the mechanism of this process. Using genetics and biochemical techniques here we demonstrate the following. 1) The C-terminal domain (domain II, 117-181) of NusG can form stable and specific complex with Rho in isolation, whereas the N-terminal domain (domain I, 1-122) does not have this property. However, domain II in isolation cannot functionally compete out the WT NusG and domain I which interacts with RNA polymerase, can dislodge WT NusG from the elongation complex. 2) Micro deletions in the four b-sheets of domain II not only affected the interaction with Rho but also affected the viability of the cells. 3) Out of twelve point mutations in the well-conserved amino acid positions located in these b-sheets of domain II, five of them (G146D, V148N, L158Q, V160N and I164A) were found to be specifically defective in binding to Rho both *in vivo* and *in vitro*. These point mutations also caused cold-sensitive phenotype to the cells. 4) Site-specific cross-linking experiments revealed that these specific regions of domain II directly interacts close to the dimeric interface of Rho in the region between amino acids 202-230, which is incidentally part of P-loop and located close to the ATP and bicyclomycin domains (Figure 2).

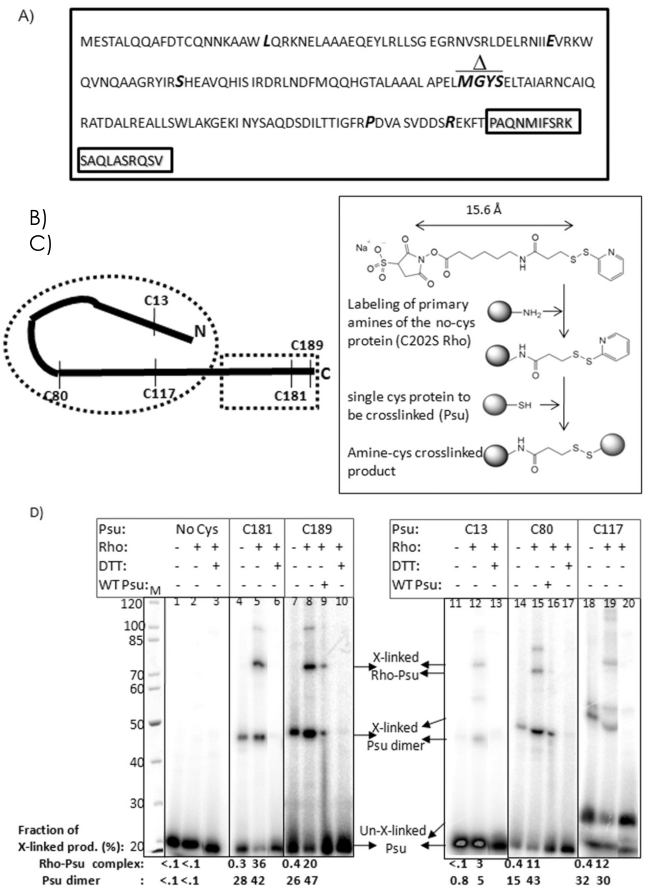

Figure 1. A) Location of the Psu mutants in the primary sequence. B) Folded structure of Psu dimeras proposed from the intra molecular cross linking and protease digestion exps Singel derivatives are also indicated. C) The cross linker SPDP used to cross link primary amines and residues. D) Preferential cross linking of C terminal tail of Psu to Rho.

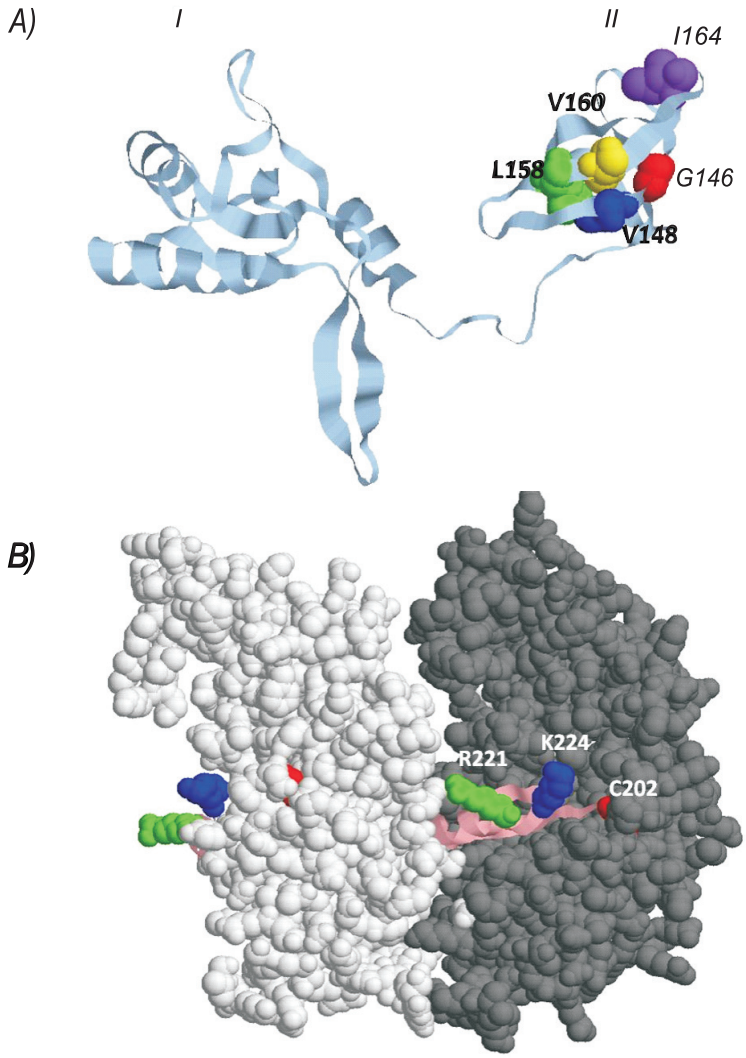

Figure 2.: a) Amino acids in the domain II of NusGerponsible for interaction with Rho. b) The dimeric interface region (190-130) of Rhois the likely interacting surface for NusG.

Publications

1. *Komissarova N, Velikodvorskaya T, Sen R, King R A, Banik-Maiti S and Weisberg R A (2008) Inhibition of a Transcriptional Pause by RNA Anchoring to RNA polymerase. ***Molecular Cell*** 31: 683-694.

2. Dutta D, Chalissery J and Sen R (2008) Transcription termination factor Rho prefers catalytically active elongation complex for releasing RNA. ***Journal of Biological Chemistry*** 283(29): 20243-20251.

3. Sen R, Chalissery J and Muteeb G (2008) Chapter 4.5.3.1, Nus Factors of Escherichia coli. In: Böck A, Curtiss III R, Kaper JB, Karp PD, Neidhardt FC, Nyström T, Slauch JM, and Squires CL (ed.), EcoSal—Escherichia coli and Salmonella: cellular and molecular biology. http://www.ecosal.org. ***ASM Press, Washington, D.C.***

4. Pani B, Ranjan A and Sen R (2009) Interaction surface of bacteriophage P4 protein Psu required for the complex formation with the transcription terminator Rho. ***Journal of Molecular Biology*** (In Press).

5. Sen R and Muteeb G (2009) Random mutagenesis using mutator strain. ***Methods in Molecular Biology, Humana Press*** (In Press).

* Work done elsewhere

Laboratory of Cell Signalling

Investigating the role of inositol pyrophosphates in eukaryotic cell physiology

Principal Investigator	Rashna Bhandari	Staff Scientist
Ph D Students	Swarna Gowri Thota	JRF
	Jadav Rathan Singh	JRF
Other Members	P Srilakshmi	Research Associate
Collaborators	Umesh Varshney, IISc, Bangalore	
	Satish Kumar, CCMB, Hyderabad	
	Keykavous Parang, University of Rhode Island, USA	

Objectives

1. Examine the role of inositol pyrophosphates in eukaryotic cell growth, proliferation and ribosome biogenesis.
2. Understand the cellular functions of mammalian inositol hexakisphosphate kinase 1 (IP6K1).
3. Generation of tools to detect inositol pyrophosphate mediated protein pyrophosphorylation.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Inositol pyrophosphates, exemplified by diphosphoinositol pentakisphosphate (PP-IP_5, or IP_7) and bis-diphosphoinositol tetrakisphosphate ($[PP]_2$-IP_4 or IP_8), are a class of inositol phosphates that contain pyrophosphate or diphosphate moieties. They are implicated in diverse biological functions, including growth, vesicular trafficking, apoptosis, telomere length maintenance and insulin secretion. We have earlier demonstrated that the beta phosphate group of inositol pyrophosphates can be transferred to pre-phosphorylated serine residues on proteins to form pyrophosphoserine (Science, 2004, 306: 2101-5; Proc. Natl. Acad. Sci., 2007, 104: 15305-10). This novel modification, *pyrophosphorylation*, occurs on several proteins within the cell, including proteins involved in ribosome biogenesis and vesicular trafficking.

5PP-IP_5 is synthesised from inositol hexakisphosphate (IP_6) and ATP by IP_6 kinases, three isoforms of which are present in mammals (IP6K1, IP6K2 and IP6K3). We have recently characterised IP6K1 knockout mice, and shown that they have low body weight compared with wild type mice, display low insulin levels and defective spermatogenesis (Proc. Natl. Acad. Sci. USA, 2008, 105: 2349-53).

Our aim is to understand the biochemical link between protein pyrophosphorylation and cellular phenomena regulated by inositol pyrophosphates. We have begun by utilising *S. cerevisiae* and mammalian cell lines as model systems to investigate the signalling pathways that are altered when inositol pyrophosphate levels are perturbed.

Details of progress made in the current reporting year (April 1, 2008 - March 31, 2009)

Project 1. Regulation of cell proliferation and ribosome biogenesis by IP_7

At the organism level, knockout experiments reveal that inositol pyrophosphates regulate cell proliferation in yeast and body size in mammals. At the molecular level, inositol pyrophosphates pyrophosphorylate proteins involved in ribosome biogenesis. Ribosome biogenesis regulates cell growth/cell size/organism size, and cell proliferation/cell cycle progression. Therefore, there is likely to be a biochemical link between protein pyrophosphorylation, the control of ribosome biogenesis, and the regulation of cell growth and proliferation by inositol pyrophosphates.

Two model systems are being used in our laboratory to study the role of inositol pyrophosphates in regulating cell growth and ribosome biogenesis. These are (a) *S. cerevisiae* (strains BY4741 and DDY1810): wild type vs *kcs1Ä* that displays substantially reduced levels of inositol pyrophosphates, and (b) Mouse embryonic fibroblast (MEF) cell lines derived from wild type and IP6K1 knockout mouse embryos, immortalised using SV40 Large T antigen. IP6K1 knockout MEFs display 70% lower levels of IP_7 compared with wild type MEFs.

To examine whether *S. cerevisiae* strains with low levels of inositol pyrophosphates display any defects in ribosome synthesis, WT and *kcs1Ä* yeast were

tested for their sensitivity towards antibiotics that inhibit ribosome function. Yeast lacking IP_7 displayed growth sensitivity to low doses of hygromycin, G418 and paramomycin, whereas WT yeast were insensitive (Fig. 1). Polysome analysis using sucrose density gradient centrifugation was carried out in collaboration with Dr. Umesh Varshney, IISc. When compared with wild type yeast, the *kcs1Ä* strain displays low levels of 40S and 60S ribosome subunits, as well as 80S monosomes and polysomes. Cell proliferation and cell cycle were monitored in WT *vs* IP6K1 knockout MEFs. Preliminary data suggest that IP6K1 knockout MEFs display abnormalities in cell cycle progression.

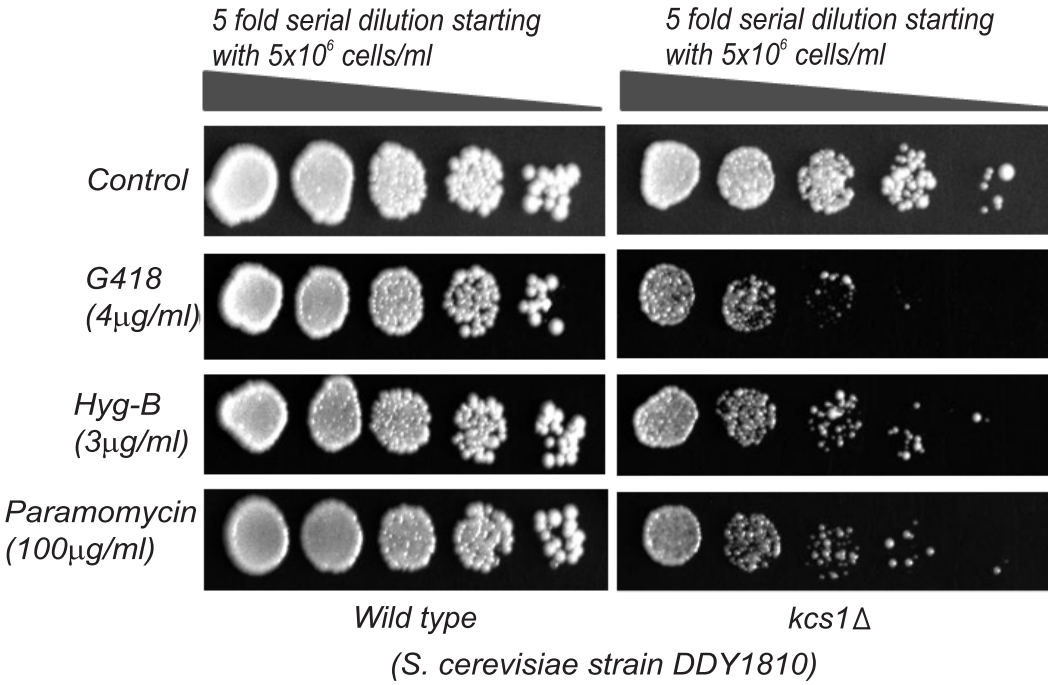

Figure 1. Yeast lacking IP_7 are sensitive to inhibitors of ribosome function.

Future direction: The exact stages of ribosome synthesis that are regulated by IP_7 will be investigated. Pre-rRNA transcript levels and rRNA processing will be monitored in WT and *kcs1Ä* yeast. Further characterisation of cell cycle defects will be performed in IP6K1 knockout MEFs.

2. Cellular functions of mammalian IP6K1

Our goal is to utilise IP6K1 knockout mice, as well as MEFs derived from these mice to understand the physiological role of inositol pyrophosphates in mammals. As the first step towards this, we plan to raise a polyclonal antibody that will specifically recognise IP6K1, but will not cross-react with the other isoforms, IP6K2 and IP6K3. Towards this end, we identified a 70 amino acid protein fragment in mouse IP6K1 that is most divergent in sequence from the other two IP_6 kinase isoforms. We have cloned and over-expressed this protein fragment as a fusion to GST, purified the fusion protein, and will use this as an antigen to raise polyclonal antibodies in rabbits.

Future direction : IP6K1 specific antibodies obtained from immunised rabbits will be used to examine the tissue distribution of IP6K1 in mice and its subcellular localisation in MEFs, using tissues from IP6K1 knockout mice as a negative control. Tissues expressing IP6K1 at high levels will be used for gene expression profiling experiments to compare the levels of different transcripts in wild type and IP6K1 knockout mice.

Project 3. Generation of tools to detect protein pyrophosphorylation

Serine pyrophosphorylation is an enzyme-independent reaction, requiring only the inositol pyrophosphate donor, the pre-phosphorylated protein acceptor, and divalent cations such as Mg^{2+}. The acceptor serine residues are pre-phosphorylated by a protein kinase, usually CK1 or CK2, and occur in acidic serine sequences, *i.e.* a stretch of two or more serine residues interspersed with Glu and/or Asp residues. Such sequences occur commonly throughout the proteome of all eukaryotic organisms.

To search for potential substrates that may be pyrophosphorylated by IP_7, we designed the consensus sequence $[E/D]_{=1}[S]_{=1}[E/D]_{=20}[S]_{=1}[E/D]_{=1}$, and used it to search the *Saccharomyces* Genome Database, which lists 5884 annotated ORFs. We obtained 204 total hits in 162 unique sequence entries. These proteins were classified according to their Gene Ontology terms based on their cellular function and subcellular localisation. A large number of potentially pyrophosphorylated yeast proteins are nucleolar in localisation and involved in ribosome synthesis.

Future direction : To examine the phenomenon of protein pyrophosphorylation it is now imperative to obtain reagents that detect pyrophosphoserine residues in cellular proteins. We will use pyrophosphoserine synthesised by our collaborator, Dr. Keykavous Parang, University of Rhode Island, USA, as the starting material to obtain specific antibodies or aptamers that recognise pyrophosphoserine, but not phosphoserine. With these tools we will map the 'pyrophosphoproteome' in *S. cerevisiae*, using our list of potential pyrophosphorylation substrate proteins as the starting point.

Publications

1. *Bhandari R, Juluri KR, Resnick AC and Snyder SH (2008) Gene deletion of inositol hexakisphosphate kinase 1 reveals inositol pyrophosphate regulation of insulin secretion, growth, and spermiogenesis. ***Proceedings of the National Academy of Sciences of the USA*** 105: 2349-2353.

1. *Draskovic P, Saiardi A, Bhandari R, Burton A, Ilc G, Kovacevic M, Snyder SH and Podobnik M (2008) Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. ***Chemistry and Biology*** 15: 274-286.

2. *Ponnusamy S, Alderson NL, Hama H, Bielawski J, Jiang JC, Bhandari R, Snyder SH, Jazwinski SM and Ogretmen B (2008) Regulation of telomere length by fatty acid elongase 3 in yeast: Involvement of inositol phosphate metabolism and ku70/80 function. ***Journal of Biological Chemistry*** 283: 27514-27524.

*Work done elsewhere

Laboratory of Plant Microbe Interaction

Understanding virulence mechanisms of *Xanthomonas* plant pathogens and interaction with host plants

Principal Investigator Subhadeep Chatterjee Staff Scientist

Objectives

1. Identification and characterization of virulence factors of *Xanthomonas*
2. Role of cell-cell communication in *Xanthomonas* colonization and virulence
3. Function of protein secretion system in *Xanthomonas* and role in virulence
4. Role of PAMP in pathogen recognition and plant defense response

Background : *Xanthomonas* group of plant pathogens causes more than 350 different plant diseases. Several of the members infect commercially important plants like-Rice, cabbage, citrus and tomato. We are trying to understand the virulence mechanisms of important *Xanthomonas* pathogens like, *Xanthomonas campestris* pv. *campestris* (Xcc; a pathogen of crucifers), *Xanthomonas oryzae* pv. oryzae & *Xanthomonas oryzae* pv. *oryzaecola* (Xoo, Xola; pathogens of rice) and *Xanthomonas axonopodis* pv. *citri* (Xac; pathogen of citrus).

Project 1 : Extracellular cell-cell communication system in plant pathogenic bacteria

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

New activity started last year

Work done in the current reporting year (April 1, 2008 - March 31, 2009)

Xanthomonas makes an extracellular fatty acid like signaling molecule called as **Diffusible Signaling Molecule** (**DSF**), which appears to differ in structure as well as mode of action in regulating virulence functions across closely related *Xanthomonas*. We are using both forward and reverse genetics approach to identify role of DSF like signaling molecules in Xcc, Xoo, Xola and Xac virulence. We have made DSF deficient mutant of Xcc and also in its putative hybrid two component sensor protein RpfC. Using an Xcc ?*rpfF* (marker free deletion mutant), we are trying to make a DSF biosensor which will be used as a tool in the genetic screen for identifying components of DSF dependent cell cell signaling.

Project 2 : Role of secretion system in *Xanthomonas* virulence

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

New activity started last year

Work done in the current reporting year (April 1, 2008 - March 31, 2009)

There are many protein secretion system in *Xanthomonas* such as TypeII and Type III secretion system which are homologous to protein delivery machinery in several animal pathogenic bacteria. We have started systematic knock outs in different protein secretion systems in Xanthomonas in order to address their role in virulence and type of effectors which are secreted by these secretion apparatus.

We have made a knock out of the *rpfF* gene (DSF biosynthetic gene) of Xoo. Analysis of the protein profile indicates that the Xoo *rpfF* mutant secretes extracellular effectors like-cellulase, lipase and xylanase (Type II effectors) and many other proteins which are not present in the wild type extracellular fraction. We have recently made double knockouts of *rpfF* in combination with either Type II or Type III secretion system mutants.

Analysis of the protein profiles of DSF mutant and double mutants indicate that the excess of proteins in the extracellular fraction of DSF mutant are Type II secretion system mediated and not due to leakiness in the membrane.

We are at present trying to understand the reason of this hyper protein secretion/production phenotype mediated by DSF and its possible implication in pathogenesis.

Project 3 : Understanding the mechanism of biofilm formation and motility

Biofilm formation and motility has been implicated in the virulence of Xanthomonas group of plant pathogens. In a screen for virulence deficient mutants, we have isolated a novel transposon induced mutant (SC2), which is proficient in all previously described virulence function.

The transposon insertion in SC2 is in a gene homologous to RHS family of protein.

There is little information about different biological role of Rhs family proteins in bacteria and in particular, plant pathogenic bacteria.

Interestingly the SC2 mutant exhibits a hyper swiming phenotype (Fig1) and is deficient in epiphytic infection, which is the natural mode of entry into rice leaves. We are characterizing the mutant phenotype in detail by obtaining additional transposon insertion and complementation. We have made knockouts in the Rhs homologs (rhsC & rhsD) of Xanthomonas campestris pv. campestris (Xcc, pathogen of crucifers). Interestingly the rhsC and rhsD mutants of Xcc does not exhibits altered motility phenotype, indicating the Xoo rhs gene has unique novel phenotype. Therefore we are proposing a new name for the rhs homolog of Xoo as rhsM (rhs gene involved in Motility).

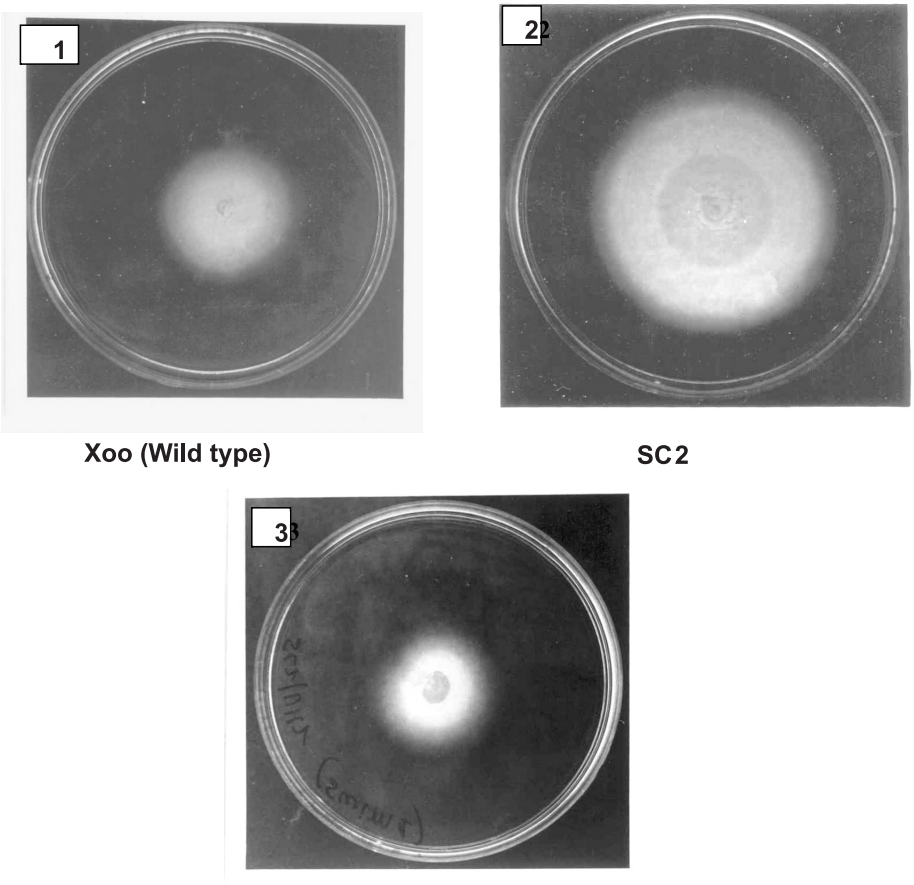

Fig. 1. Swimming assay of different Xoo Strains. 1) Wild type Xoo strain (BXO43; Indian isolate), 2) SC2 (*rhs*::Tn*5*; rhs mutant in the BXO43 background), Sc2/pUH2 (*rhs*::Tn*5*/ Complementing plasmid)

Publications:

1. *Chatterjee S, Almeida RPP and Lindow S (2008) Living in two Worlds:The Plant and Insect Lifestyles of *Xylella Fastidiosa*. ***Annual Review of Phytopathology*** 46:243–71.
2. *Chatterjee S, Newman KL and Lindow SE (2008) Cell-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. ***Molecular Plant-Microbe Interactions*** 21(10): 1309-1315.
3. *Chatterjee S, Wistrom C and Lindow SE (2008) A cell– cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. ***Proceedings of the National Academy of Sciences of the USA*** 105(7): 2670–2675.
4. *Newman KL, Chatterjee S, Kimberly AH and Lindow SE (2008) Virulence of Plant Pathogenic Bacteria Attenuated by Degradation of Fatty Acid Cell-to-Cell Signaling Factors. ***Molecular Plant-Microbe Interactions*** 21(3): 326–334.

* Work done elsewhere

Other Scientific Services / Facilities

National Genomics and Transcriptomics Facility

Principal Investigator	Murali Dharan Bashyam	Staff Scientist
Other Members	Ajay Chaudhary	
C K Reddy	Technical assistant Project assistant (till October 2008)	

Objectives:

To provide services to scientists both within and outside CDFD in the areas of DNA Sequencing, Genotyping, Real Time PCR and Microarrays.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

During the previous reporting year, sequencing data amounting to a total of 7.4 million nucleotides was generated at NGTF. In addition, a total of 8600 genotyping reactions and 80 real time PCR reactions were also performed. Microarrays, the Human Exonic Evidence Based oligonucleotides (HEEBO) were procured from the Stanford Functional Genomics and Transcriptomics Facility and array-based comparative genomic hybridization was carried out for esophagus and colorectal cancer samples.

Details of progress in the current reporting year (April 1, 2008- February 28, 2009)

NGTF continues to provide services to scientists both within and outside CDFD in the areas of DNA sequencing, genotyping, real time PCR and microarrays. A total of 5.5 million nucleotides were sequenced during the reporting period. In addition, a total of 3500 genotyping reactions were performed. We also performed 45 real time PCR reactions for several laboratories within CDFD. Microarrays using the Human Exonic Evidence Based oligonucleotides (HEEBO) were procured from the Stanford Functional Genomics and Transcriptomics Facility (SFGF) and array-based comparative genomic hybridization was carried out for esophagus and colorectal cancer samples. Whole genome transcript profiling was standardized on human microarrays procured from SFGF and also from Agilent technologies.

Publications:

1. Bashyam MD and Ratheesh R (2008) Genetic instability in colorectal cancer. ***The ICFAI Journal of Biotechnology*** 2:13-26.
2. Kwei KA, Bashyam MD, Kao J, Ratheesh R, Reddy EC, Kim YH, Montgomery K, Giacomini CP, Choi Y, Chatterjee S, Karikari CA, Salari K, WangP, Hernandez-Boussard T, Swarnalata G, Rijn MV, Maitra A and Pollack JR (2008) Genomic profiling identifies GATA6 as a candidate oncogene amplified in pancreatobiliary cancer. ***PLOS Genetics*** 4:e100081.

Bioinformatics (Centre of Excellence in Medical Bioinformatics)

Service Co-ordinator	M Kavita Rao	Staff Scientist & In-charge
Other members	M N Pavan	Technical Officer
	R Chandra Mohan	Technical Officer (since Aug '08)
	P Prashanthi	Technical Assistant
Collaboration	European Molecular Biology Network (EMBnet)	
	SUN Microsystems, Inc, California, USA	
	University of Hyderabad, Hyderabad	

Objectives

1. To provide & facilitate carrying out high impact research and training in Bioinformatics
2. To maintain the CDFD website, to provide web based services and e-mail services.
3. To maintain various servers, Workstations, PCs, printers and other peripheral devices.
4. To maintain institute-wide LAN as well as the internet connectivity
5. To develop computer applications for the automation of institute activities
6. To assist in conducting workshops and practical classes for Bioinformatics community and students from other Universities.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008)

Retrofitting the Data Centre set up with additional servers and activities pertaining to planning the shifting of services from Gandipet and Nacharam to Nampally campuses formed a major component. This involved careful site planning studies to ensure minimal downtime of the servers and also elaborate and extensive network planning studies were undertaken to ensure network connectivity between the two campuses with seamless LAN connectivity. Internet connectivity with enhanced bandwidth was implemented. Network infrastructure with high end programmable switches, router and suitable firewall was set up & implemented.

E-mail, Internet, web services were continued to be provided with enhanced functionality. The institute's website was revamped and given a new look and feel with enhanced features such as collecting visitor statistics, internal viewing of institute specific activities and hosting updated online library services. An on-line application for Project Assistants, in addition to the existing Research Scholar Program application was created and hosted on the institute's website. The Symantec anti-virus version was upgraded. Activities related to installation, administration and maintenance of servers which provide various services, databases and computational jobs were undertaken, at both the campuses. A comprehensive PC Annual maintenance contract was awarded to a private vendor.

Details of progress in the current reporting year (April 1, 2008 – 31, March 2009):

Centre of Excellence and its activity in Bioinformatics knowledge sharing:

One of the major mandates of CoE is to enable computational scientific groups to pursue their interest in Bioinformatics Research. A large number of research groups have used the facility and/or the knowledge services to advance their research. This is reflected in rising number of publications that are having bioinformatics component in them. In addition the CoE has been actively involved in knowledge sharing in the field of Bioinformatics.

Sun CoE also participated in University of Hyderabad's M.Tech. Bioinformatics Program and provided lecture and practical training in the field of Bioinformatics to students of M.Tech Bioinformatics program of University of Hyderabad and also to students of Agicultural University, Hyderabad.

The Sun CoE also hosted 6 M.Tech. Bioinformatics students for carrying out M.Tech. Bioinformatics

research leading to MTech thesis. I/c Bioinformatics engaged in talks with CSIR initiative Open Source Drug Discovery Team and gave a presentation about the centre's activities and infrastructure set up, to take the initiative forward.

Facilitation of the Genome Annotation workshop held in our institute conducted by Dr. Lazslo, Purdue University, USA.

IT Services:

Retrofitting the existing Data Center in the new campus with additional servers formed a major component of the activities.

IT services such as E-mail, Internet, web were re-deployed at new campuses with effective global changes to record the new Domain Name server settings.

Internet bandwidth was upgrade from existing 2 Mbps to 4 Mbps in the main campus and a leased circuit of 2 Mbps between the lab building and administrative building was established. The network infrastructure was implemented with improved connectivity including Patch panel, patch chords and high end switches, firewall and router housed in suitable network racks. Installation, administration and maintenance of PCs, Printers and Scanners were also continually done. High-end PCs, workstation, laptops, scanner and printers were procured and installed. A comprehensive PC Annual maintenance contract was awarded to a private vendor. Several copies of new/upgraded software were also installed. These include 96 copies of Adobe Suite CS3 Educational license, Upgraded antivirus from Symantec Corporate Edition 10.2 to Symantec Endpoint security 11.0 in the Anti virus server and 10 Nos. Systat software license was procured and installed accordingly. Few of the existing AMC were also renewed. These include AMC for SGI Fuel machines and Symantec Anti spam for mail gateway system. We have also renewed the agreement for remote monitoring and managed services for Sun servers in the new Data Center set up.

INSTRUMENTATION

Principal Investigator	Raghavendrachar J	Staff Scientist
Ph D Students	Nil	
Other Members	R N Mishra	Technical Officer II
	S Pavan Kumar	Technical Officer I [Till 23rd December 2008]
	M Laxman	Technical Assistant
	Sathyanarayana	Technical Assistant
	N P Sharma	Junior Assistant-II
	K Geeta	[on Contract]

Objectives

To maintain repair and service all the equipment in laboratory. To provide pre-installation requirements for new instruments and to coordinate with the manufacturers / their agents in Installation and warranty service of the new instruments. Also to provide the reports on the newly arrived instruments and to follow up with the suppliers for short shipped items.

Summary of work done until the beginning of this reporting year (April 1, 2007 - March 31, 2008) :

We had performed over 42 new installations of various equipment, like dHPLC, MegaBace 500 DNA Sequencer of Amersham (re-installation), Hybridization Oven, CO_2 Incubators, Gel Documentation Systems, PCR Machines, Water Purification System, Nanodrop Spectro-photometers, Refrigerated Table Top Centrifuges, Shaking Water baths, Orbital Shaker, Tissue Homogenizer, -86^{0}C Freezer, -20^{0}C Freezers, Refrigerators, Micro centrifuges, Electrophoresis Apparatus etc.

We had also completed 395 work orders for repair & maintenance of various laboratory equipments. We were actively involved in reorganizing the Laboratory for additional space and also the newly leased space in the Ground Floor.

Details of progress made in the current reporting year (April 1, 2008 - March 31, 2009)

We have performed over 37 new installations of various equipment, like CO_2 Incubators, Gel Documentation Systems, PCR Machines, Water Purification System, Nanodrop Spectro-photometers, Refrigerated Table Top Centrifuges, Shaking Water baths, Orbital Shaker, Tissue Homogenizer, -86^{0}C Freezer, -20^{0}C Freezers, Refrigerators, Micro centrifuges, Electrophoresis Apparatus etc.

We have also completed 246 work orders for repair & maintenance of various laboratory equipments.

Since we had been entrusted with the additional responsibility of Total maintenance of the CDFD Nacharam Campus, including Electrical, Refrigeration, EPABX & Telephones, UPS Systems, House Keeping, we have successfully monitored these activities till the shifting of the Laboratory to Nampally.

CDFD has been procuring a large number of sophisticated equipment, which requires dedicated trained personnel for their operation and maintenance. Through proper tendering process, we have outsourced the equipment operations through M/s Sandor Proteomics. Similarly, installation, maintenance and repair of the computers and their peripherals required for scientific as well as administrative works are out sourced through M/s Vama Industries.

Since CDFD has been relocated to the Nampally, we were actively involved in the planning for various electrical, telephone, water etc points required for the laboratory. We have got Free EPABX facility with 70 lines installed for the Administrative Office at Gruhakalpa campus and a BSNL Centrex facility with 150 lines for the Tuljaguda Laboratory complex. We have procured and got installed 65 sets of Laboratory tables.

All the Instrumentation staff was involved in the shifting of all the equipments from Nacharam to

Nampally, and have installed them at Nampally campus with minimum downtime. In addition to the installation of all the equipment shifted from Nacharam, we have installed all the newly purchased instruments at Nampally, like -86^{0}C Freezer, -20^{0}C Freezers, Refrigerators, Laminar Flow hoods and CO_2 Incubators in the Tissue culture Laboratory.

In addition we were involved in organizing the audio & visual requirements for presentations in various seminars, lectures and workshops, Foundation day lectures, Distinguished Scientist Lectures held in CDFD both at Nacharam and Gandipet Auditorium. We maintained most of the equipment with maximum uptime in the Laboratory. Most of the Instruments are maintained by our Instrumentation staff, there by saving on the expensive AMCs and with very little downtime of the equipment.

Publications

PATENTS (2008-09)

A. Patents issued:

1 Chandrashekharaiah, Rao CG, Sivaprasad V, Babu M R, Goel AK, Prasad J and Nagaraju J . Superior, Novel quality mulberry silkworm hybrids and a method thereof. Indian Patent 218895, granted on 16 April, 2008.

2 Nagaraju J. Novel FISSR-PCR primers and methods of identifying genotyping diverse genomes of plant and animal systems including rice varieties, a kti thereof. Indian Patent 219765, granted on 13 May, 2008.

3 Gowrishankar J and Harinarayanan R. A method of altering levels of plasmids.

(i) Japanese Patent 4142649, granted on 20 Jun, 2008.

(ii) German Patent DE 60224457 T2, granted on 5 Feb, 2009.

4 Hasnain SE and Prachee C. Peptide antigens which elicit high humoral Immune response and T-cell response. Indian Patent 227179, granted on 5 Jan, 2009.

5 Gowrishankar J and Nandineni M R. A microbial process for arginine production. Indian Patent 230540, granted on 27 Feb, 2009.

B. Patent applications filed:

1 Shamim MTA and Nagarajaram HA. Fold-wise classification of proteins. USA Patent filed on 29 August, 2008.

2 Mukhopadhyay S, Bhat KH and Khan N. A novel protein as potential candidate for development of anti-tuberculosis therapeutics. Indian Patent filed on 24 November, 2008.

3 Mukhopadhyay S and Khan N. A novel candidate protein to diagnose patients with active tuberculosis. USA Patent filed on 19 December, 2008.

Research Papers Published in 2008

1. Achary MS and Nagarajaram HA (2008) Comparative docking studies of CYP1b1 and its PCG associated mutant forms. *Journal of Biosciences* 33:699-713.

2. Ahmed N, Dobrindt U, Hacker J and Hasnain SE (2008) Genomic fluidity and pathogenic bacteria: applications in diagnostics, epidemiology and intervention. *Nature Reviews Microbiology* 6:387-94.

3. Akif M, Khare G, Tyagi AK, Mande SC and Sardesai AA (2008) Functional studies of multiple thioredoxins from *Mycobacterium tuberculosis*. *Journal of Bacteriology* 190: 7087-7095.

4. Arunkumar KP, Kifayathullah L and Nagaraju J (2008) Microsatellite markers for the Indian golden silkmoth, *Antheraea assama* (Saturniidae: Lepidoptera). *Molecular Ecology Resources* 9: 268-270.

5. Arunkumar KP, Tomar A, Daimon T, Shimada T and Nagaraju J (2008) WildSilkbase: An EST database of wild silkmoths. *BMC Genomics* 9: 338.

6. Bashyam MD and Ratheesh R (2008) Genetic instability in colorectal cancer. *The ICFAI Journal of Biotechnology* 2:13-26.

7. *Bhandari R, Juluri KR, Resnick AC and Snyder SH (2008) Gene deletion of inositol hexakisphosphate kinase 1 reveals inositol pyrophosphate regulation of insulin secretion, growth, and spermiogenesis. *Proceedings of the National Academy of Sciences of the USA* 105: 2349-2353.

8. *Chatterjee S, Almeida RPP and Lindow S (2008) Living in two Worlds:The Plant and Insect Lifestyles of *Xylella Fastidiosa*. *Annual Review of Phytopathology* 46:243–71.

9. *Chatterjee S, Newman KL and Lindow SE (2008) Cell-cell signaling in *Xylella fastidiosa* suppresses movement and xylem vessel colonization in grape. *Molecular Plant-Microbe Interactions* 21(10): 1309-1315.

10. *Chatterjee S, Wistrom C and Lindow SE (2008) A cell– cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. *Proceedings of the National Academy of Sciences of the USA* 105(7): 2670–2675.

11. *Draskovic P, Saiardi A, Bhandari R, Burton A, Ilc G, Kovacevic M, Snyder SH and Podobnik M (2008) Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. *Chemistry and Biology* 15: 274-286.

12. Dutta D, Chalissery J and Sen R (2008) Transcription termination factor Rho prefers catalytically active elongation complex for releasing RNA. *Journal of Biological Chemistry* 283(29): 20243-20251.

13. Farhana A, Kumar S, Rathore SS, Ghosh PC, Ehtesham NZ, Tyagi AK and Hasnain SE (2008) Mechanistic insights into a novel exporter-importer system of Mycobacterium tuberculosis unravel its role in trafficking of iron. *PLoS ONE* 7;3 (5):e2087.

14. Hegde S, Manimaran P and Mande SC (2008) Dynamic changes in protein functional linkage networks revealed by integration with gene expression data. *PLoS Computational Biology* 4: e1000237.

15. Hussain MA, Naveed SA, Sechi LA, Ranjan S, Alvi A, Ahmed I, Ranjan A, Mukhopadhyay S, and Ahmed N (2008) Isocitrate dehydrogenase of Helicobacter pylori potentially induces humoral immune response in subjects with peptic ulcer disease and gastritis. *PLoS ONE* 3:e1481.

16. Kanginakudru S, Metta M, Jakati RD and Nagaraju J (2008) Genetic evidence from Indian red jungle fowl corroborates multiple domestication of modern day chicken. *BMC Evolutionary Biology* 8:174.

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** Work done elsewhere*

Research Papers Published in 2009 (Until 31 March)

37 Achary MS and Nagarajaram HA (2009) Effects of Disease Causing Mutations on the Essential Motions in proteins. *Journal of Biomolecular Structure and Dynamics* 26:609-624.

38 Ahmed N, Ehtesham NZ and Hasnain SE (2009) Ancestral Mycobacterium tuberculosis genotypes in India: implications for TB control programmes. *Infection, Genetics and Evolution* 9:142-146.

39 Bashyam MD (2009) Studies on nonsense mediated decay reveal novel therapeutic options for genetic diseases. *Recent patents on DNA & Gene Sequences* 3:7-15.

40 Bose JS, Gangan V, Jain SK and Manna SK (2009) Downregulation of inflammatory responses by novel caffeine acid ester derivative by inhibiting NF-kappaB. *Journal of Clinical Immunology* 29: 90-98.

41 Bose JS, Gangan V, Jain SK and Manna SK (2009) Novel caffeic acid ester derivative induces apoptosis by expressing FasL and downregulating NF-kappaB: Potentiation of cell death mediated by chemotherapeutic agents. *Journal of Cellular Physiology* 218: 653-662.

42 Johny S, Chakraborty S, Gadagkar R and Nagaraju J (2009) Polymorphic microsatellite loci for primitively eusocial wasp Ropalidia marginata. *Molecular Ecology Resources* 9: 2203-2205.

43 Kumar S, Balakrishna K, Agarwal GS, Merwyn S, Rai GP, Batra HV, Sardesai AA and Gowrishankar J (2009) Th1-type immune response to infection by pYV-cured phoP-phoQ null mutant of Yersinia pseudotuberculosis is defective in mouse model. *Antonie Van Leeuwenhoek* 95: 91-100.

44 Kumar S, Farhana A and Hasnain SE (2009) In-vitro helix opening of M. tuberculosis oriC by DnaA occurs at precise location and is inhibited by IciA like protein. *PLoS ONE* 4(1): e4139.

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47 **Rosu V, Ahmed N, Paccagnini D, Gerlach G, Fadda G, Hasnain SE, Zanetti S and Sechi LA (2009) Specific immunoassays confirm association of *Mycobacterium avium* Subsp. paratuberculosis with type-1 but not type-2 diabetes mellitus. *PLoS ONE* 4:e4386.

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**from work performed in CDFD, but institutional affiliation of CDFD not listed.

Research Papers in Press (as on 31 March, 2009)

49 Agarwal S, Tamhankar PM, Kumar R and Dalal A (2009) Clinical and haematological features in a compound heterozygote (HBB:c.92+5G>C /HBB:c.93-2A>C) case of thalassemia major. *International Journal of Laboratory Hematology.*

50 Angalena R, Chaudhary A, Bashyam MD and Dalal A (2009) Hemoglobin D (Iran) masquerading as Hemoglobin E: An interesting case report. *Newsletter of Genetics Chapter of Indian Academy of Pediatrics.*

51 Arunkumar KP, Mita K and Nagaraju J (2009) Silkworm Z chromosome is enriched in testis-specific genes. *Genetics*.

52 Bashyam MD (2009) Nonsensemediated decay: linking a basic cellular process to human disease. *Expert Review of Molecular Diagnostics.*

53 Bhate RH and Ramasarma T (2009) Evidence for H_2O_2 as the product of reduction of oxygen by alternative oxidase in mitochondria from potato tubers. *Archives of Biochemistry and Biophysics*.

54 Bose JS, Gangan V, Prakash R, Jain SK and Manna SK (2009) A dihydrobenzofuran lignan induces cell death by modulating mitochondrial pathway and G2/M cell cycle arrest. *Journal of Medicinal Chemistry.*

55 Dalal A and Mehrotra RN (2009). Hypertrichosis, hyperkeratosis and mental retardation syndrome: further delineation of phenotype. *Clinical Dysmorphology*.

56 Dutta D, Bandyopadhyay K, Datta AB, Sardesai AA and Parrack P (2009) Properties of HflX, an enigmatic protein from Escherichia coli. *Journal of Bacteriology*.

57 Gangadharan C, Thoh M and Manna SK (2009) Inhibition of constitutive activity of nuclear transcription factor kappaB sensitizes Doxorubicin-resistant cells to apoptosis. *Journal of Cellular Biochemistry.*

58 Pani B, Ranjan A and Sen R (2009) Interaction surface of bacteriophage P4 protein Psu required for the complex formation with the transcription terminator Rho. *Journal of Molecular Biology.*

59 Sen R and Muteeb G (2009) Random mutagenesis using mutator strain. *Methods in Molecular Biology, Humana Press.*

Other Publications

1. Gowrishankar J and Divakar P (2008) Scientometrics and modified h-indices. (correspondence) *Current Science* 95: 1656.
2. Nagaraju J (2008) Silkworm Breeding Multiplier Effect. *Biotech News* 3(5): 24-27.
3. Nagaraju J (2008) Silks of India, Grace and Lustre. *Biotech News* 3(5): 4-7.
4. Sardesai AA and Gowrishankar J (2008) Joshua Lederberg – a remembrance. *Journal of Genetics* 87: 311-313.
5. Kaur R (2009) Review of: Lilavati's Daughters: The Women Scientists of India, Edited by Rohini Godbole and Ram Ramaswamy, Indian Academy of Sciences, Bangalore, 2008, 369 pages. *Current Science* (In press).

Human Resource Development

Ph.D. Program

For our Ph.D. program we invite applications from highly motivated candidates willing to take up challenges in modern biology usually in the month of march. Keeping in view the interdisciplinary nature of modern biology, we especially encourage persons from diverse backgrounds to take up challenges in these areas. Those admitted as JRFs are considered for the Ph.D. program of Manipal Academy of Higher Education or University of Hyderabad.

The eligibility for the program is a Masters degree in any branch of Science, Technology, Medicine or Agriculture. Candidates must have cleared the CSIR/UGC/ICMR/ICAR NET for JRF. Those who have appeared, but are awaiting results, are also eligible to apply. Those with independent SRF from CSIR can also apply. As the number of applicants outnumbers the seats available each year by a ratio of 1:10 or more, eligible candidates are invited for a written examination followed by interviews of short-listed candidates.

Currently we have 63 research scholars working for their doctorates in different areas of research. In the reporting year 13 of our Research Scholars have completed Ph.D. and are pursuing careers in science elsewhere in India or abroad.

Postdoctoral Program

In addition to our JRF program, we also carry out training at the post-doctoral level. The post-doctoral fellows are funded through the extramural grants that CDFD receives. Some post-doctoral fellows are also selected competitively by the DST fast track young scientist scheme, or the DBT post-doctoral fellowship program.

Summer Training Program

Every year CDFD receives more than 800 applications for carrying out a 4-6 weeks summer training project. The applications are mostly from students pursuing their M.Sc. in various Universities. Some applications are also from B. Sc. Students. In the past couple of years, CDFD has restricted admission to this program only to those students who are supported by the Indian Academy of Sciences, Bangalore or the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore or the Kishore Vaigyanik Protsahan Yojana, New Delhi. On an average every year 15-20 students receive their summer training at CDFD.

Training for students from BITS, Pilani

CDFD has formalized an agreement with BITS Pilani to provide project training to their M. Sc. Students. Under this programme, the students spend 6 months-1 year at CDFD and work on active projects that are being carried out there. The project work helps the students in gaining hands-on experience in modern biology. Every year 2-3 students avail of this programme.

Lectures, Meetings, Workshops and Important Events

- **Dr Rupak Mitra,** Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, USA, delivered a lecture on "piggyBac: A Smart Transposon" (10.06.2008)
- **Dr Puneeta Arora,** University of North Carolina, USA delivered a lecture on "Persistent Transactivation and Aberrant Trafficking of EGFR in Breast Cancer Cells" (23.06.2008)
- **Dr S R Palli,** Department of Entomology, University of Kentucky, USA, delivered a lecture on "RNAi aided functional characterization of multigene families in *Tribolium castaneum"* (06.08.2008)
- **Dr Monalisa Chatterji,** Biocon Ltd. India, delivered a lecture on "DNA breaks/lesions in normal physiology and cancer" (07.08.2008).
- **Dr Satyajit Rath,** National Institute of Immunology, India delivered a lecture on "Death in the T-Cell lineage: a role of pdcd8" (11.08.08)
- On 11.08.08 a **MoU** was signed between CDFD and Nizam's Institute of Medical Sciences, Hyderabad for establishment of "Medical Genetics Unit" at NIMS campus to provide services, conduct research and training to students in the area of medical genetics.
- **Dr Pankaj V Alone,** University of California - Davis, USA delivered a lecture on "eIF1gamma's role in translation initiation and AUG codon fidelity" (12.08.08)
- **Hindi day** was celebrated in association with IICT and the Chief Guest was the Director, NEERI, 15.09.08
- CDFD hosted "DST Swarnajayanthi Fellowship" presentations at its Gandipet Campus on behalf of Department of Science & Technology" on 17.09.08
- **Dr Vishal Trivedi,** Tufts-New England Medical Centre, Boston, USA delivered a lecture on "Targeting Parasitism: Staying One Step Ahead of Microbes (18.09.2008).
- **Dr Madan Babu,** University of Cambridge, UK delivered lecture on "Evolution of Transcription Regulatory Networks" (25.09.08)
- Workshop was conducted on "Genetic Fidelity Testing of Tissue Culture Raised Micropropagules" co-sponsored by Biotech Consortium of India Limited and department of Biotechnology during October 20-24, 2008
- **Dr Ashwani Kumar,** University of Alabama, USA delivered a lecture on "Mechanism of sensing the protective host signals in *Mycobacterium tuberculosis"* (02.11.2008)
- **Dr Anindya Roy,** Clare Hall laboratories, UK delivered a lecture on "Repair of Transcription-blocking DNA Damage and Hereditary Cancer" (12.11. 2008)
- CDFD hosted Indo-US Workshop on "Low-Cost Diagnostic and Therapeutic Technologies" sponsored by Department of Biotechnology and National Institute of Biomedical Imaging and Bioengineering, USA during 18-20 November, 2008 at Hyderabad, India.
- CDFD hosted an "International Symposium on Glyco-science, Cell-Engineering and Bioinformatics" during the 2nd Department of Biotechnology and National Institute of Advanced Industrial Science & Technology, Japan Bilateral Workshop during 25-26 November, 2008 at Hyderabad, India
- A team of ten German Science journalists lea by Dr Jorg acker, Vice President, DFG visited the Centre and interacted with the faculty on 28.11.08
- Dr Basant Patel, University of Illinois, USA delivered lecture on "Prions: the 'protein-only' infectious agents and conduits of protein conformation-based genetic inheritance" (12.12.08)
- **Dr Thanuja Krishnamoorthy,** The Wistar Institute, Philadelphia, USA delivered a lecture on 'Histone Phosphorylation and Acetylation during Gametogenesis' (16.12.2008)

- **Dr Neeraja Krishnan,** Tata Institute of Fundamental Research, Mumbai, delivered a lecture on "Regulation of Human mitochondrial transfer RNA genes as origins of light strand replication: Disease and Development" (22.12.08)

- On 28.1.09 CDFD celebrated its 12th Foundation Day. On this occasion **Prof. Guy Dodson,** FRS University of York, United Kingdom delivered the foundation day lecture titled "Dorothy Hodgkin's crystallographic discoveries and their relevance to current biology"

- **Dr Murty Madiraju,** Department of Biochemistry, University of Texas Health Center, USA delivered a lecture on *"Mycobacterium tuberculosis:* Replication Initiation and Proliferation" (29.01.2009)

- **Dr Malini R Madiraju,** Department of Biochemistry, University of Texas Health Center, USA delivered a lecture on "Not divide, to conquer: The *Mycobacterium tuberculosis* survival strategy" (29.01.2009)

- **Dr Rajeev Kaul,** University of Pennsylvania, Philadelphia: USA delivered a lecture on "Molecular biology of virus mediated cancer: understandmg the role of Epstem Barr Virus (EBV) latency protein in cancer metastasis" (04.02.09)

- **Dr Amiya Ghosh,** University of Michigan, USA delivered a lecture on "Animal models for genetic and molecular understanding of two human diseases - cancer and ciliopathy' (12.02.2009)

- **Dr Robert Weisberg,** National Institutes of Health, USA delivered a lecture on "Phage Lambda and the Origins of Molecular Biology" (09.03.2009)

- **Dr Molcolm Buckle,** Ecole Normale Superieure de Cochan, France delivered the lecture titled "Bacterial Chromatic Organization, Regulation and the Dynamics of Macromolecular Complexes" (02.03.09)

- **Prof Kazuei Mita,** National Institute of Agrobiological Sciences, Japan delivered a lecture on "Recent developments in Bombyx Genome Analysis" (06.03.09)

- **Dr Robert Weisberg,** National Institutes of Health, USA, delivered a lecture on "Inhibition of a Transcriptional pause by RNA anchoring to RNA polymerase" 10.3.2009

- **Prof Loszlo Csonko**, Professor, Department of Biological Sciences, Purdue University, USA visited CDFD from 16.3.09 to 27.3.09 as an American Society of Microbiology Visiting Professor to conduct a seminar course and computer assignment.

- A team of 17 faculty from National Defence College, New Delhi led by Major General Abhijit Guha and Lt.Gen. Prakash Menon visited the Centre as part of faculty tour on "Economy and Science & Technology" (17.03.09)

- **Dr Mohit Prasad,** Johns Hopkins University, USA delivered a lecture on "Border Cell Migration: Coupling Genetics, Live-Imaging and Quantitative Analyses" (17.03.09)

- **Dr Nisheeth Agarwal,** Johns Hopkins School of Medicine, USA delivered a lecture on 'Intoxication of Host Macrophages by Mycobacterium tuberculosis Adenylate Cyclase' (17.03.2009)

- **Prof Loszlo Csonko**, Professor, Dept. of Biological Sciences, Purdue University, USA delivered lecture titled "The surprising role of mg2+ transport in the regulation of thermotolerance" (18.3.09)

Senior Staff and Officers of CDFD

Scientific Staff

Dr J Gowrishankar, Director

Dr E A Siddiq, Adjunct Scientist

Prof T Ramasarma, Adjunct Scientist

Dr J Nagaraju, Staff Scientist

Dr Shekhar C Mande, Staff Scientist

Dr S Mahalingam, Adjunct Faculty

Dr Murali D Bashyam, Staff Scientist

Dr Sunil Kumar Manna, Staff Scientist

Dr H A Nagarajaram, Staff Scientist

Dr Akash Ranjan, Staff Scientist

Dr Sangita Mukhopadhyay, Staff Scientist

Dr Gayatri Ramakrishna, Staff Scientist

Dr G V Rao, Staff Scientist (under suspension)

Dr Sanjeev Khosla, Staff Scientist

Dr Ranjan Sen, Staff Scientist

Dr Rupinder Kaur, Staff Scientist

Dr Madhusudan R Nandineni, Staff Scientist

Dr Niyaz Ahmed, Staff Scientist (until December, 2008)

Dr Ashwin Dalal, Staff Scientist

Dr Abhijit A Sardesai, Staff Scientist

Dr Rashna Bhandari, Staff Scientist

Dr P Janila, Staff Scientist (Science Communication)

Dr R Harinarayanan

Dr Subhadeep Chatterjee

Dr Ashok Khar, Staff Scientist (Extramural activities)

Administrative Staff

Mr K Ananda Rao, Staff Scientist (Engg.)

Mr Raghavendrachar J, Staff Scientist (Instrumentation)

Mr J Sanjeev Rao, Head - Administration

Deputations abroad of CDFD Personnel

STUDENTS

Mr Yusuf Akhtar (Germany) Under DAAD fellowship from 04.06.07 - 30.09.09

Mr Jayendra Nath Shukla (USA) To attend CSHL hostel conference entitled "Systems Biology : Global Regulation of Gene Expression" from 27.03.08 - 30.03.08

Mr C M Santosh Kumar (USA) To attend a meeting on 'Molecular chaperones and stress responses" from 30.04.08 - 04.05.08

Mr G Gokul (Massachusetts) to attend the AACR Special Conference "Cancer epi genetics" from 28.05.08 - 31.05.08

Mr Pankaj Kumar (Canada) to attend the 16th International Conference on "Intelligent Systems for Molecular Biology (ISMB 2008)" from 19.07.08 - 23.07.09

Ms P Uma Devi (Canada) to attend the 16th International Conference on "Intelligent Systems for Molecular Biology (ISMB 2008)" from 19.07.08 - 23.07.09

Ms Jyoti Singh (Japan) To visit the Prof.Toru Shimada's laboratory, Tokyo University, Japan under SPS collaborative program from 18.01.09 - 01.02.09

Ms Shiny Nair (Canada) To attend the Key stone symposia for the session titled "Pattern Recognition Molecules and Immune Sensors of Pathogenes" from 29.03.09 - 03.04.09

Committees of the institute

(As on 31.03.2009)

MEMBERS OF CDFD SOCIETY

Shri Kapil Sibal Hon'ble Minister for S&T and of Earth Sciences	-	President
Prof M K Bhan Secretary, DBT, New Delhi	-	Member
Prof P Balaram Director, IISc, Bangalore	-	Member
Director General, CSIR, New Delhi	-	Member
Prof V S Chauhan Director, ICGEB, New Delhi	-	Member
Dr Siddhartha Roy Director, IICB	-	Member
Joint Secretary (PM), MHA, New Delhi	-	Member
Joint Secretary & Legal Adviser, Ministry of Law, New Delhi	-	Member
Joint Secretary & Financial Advisor, DBT, New Delhi	-	Member
Director General, Bureau of Police Research Development	-	Member
Dr Alka Sharma Jt.Director, DBT, New Delhi	-	Member
Dr J Gowrishankar Director, CDFD	-	Member - Secretary

MEMBERS OF CDFD GOVERNING COUNCIL

Prof M K Bhan Secretary, DBT, New Delhi	-	Chairperson
Director General, CSIR, New Delhi	-	Member
Prof V S Chauhan Director, ICGEB, New Delhi	-	Member
Prof P Balaram Director, IISc, Bangalore	-	Member
Dr Siddhartha Roy Director, IICB, New Delhi	-	Member
Joint Secretary, Ministry of Law, New Delhi	-	Member
Joint Secretary (PM), MHA, New Delhi	-	Member
Joint Secretary & Financial Advisor, DBT, New Delhi	-	Member
Director General Bureau of Police Research & Development, New Delhi	_	Member
Dr Alka Sharma Jt.Director, DBT, New Delhi	-	Member
Dr J Gowrishankar Director, CDFD	-	Member-Secretary

MEMBERS OF CDFD FINANCE COMMITTEE

Dr V S Chauhan Director, ICGEB, New Delhi	-	Chairman
Dr Siddhartha Roy Director, IICB, New Delhi	-	Member
Dr J Gowrishankar Director, CDFD	-	Member
Joint Secretary & Financial Advisor, DBT, New Delhi	-	Member
Shri Virendra Kapoor Deputy Secretary, DBT, New Delhi	-	Member
Joint Secretary, MHA, New Delhi	-	Member
Shri A K Prakash (up to November, 2008) Head Finance & Accounts, CDFD	-	Member Secretary

MEMBERS OF CDFD BUILDING COMMITTEE

Prof V S Chauhan Director, ICGEB, New Delhi	-	Chairman
Dr. J. Gowrishankar Director, CDFD	-	Member
Shri Samant Joint Secretary, DBT, New Delhi	-	Member
Shri Virendra Kapoor Deputy Secretary, DBT, New Delhi	-	Member
Shri B Bose Management Consultant, NII, New Delhi	-	Member
Shri J Sanjeev Rao Head Administration, CDFD	-	Member
Shri A K Prakash (up to November, 2008) Head Finance & Accounts, CDFD	-	Member
Shri K Ananda Rao Staff Scientist (Engg), CDFD	-	Member & Convenor

Budget and Finance

CENTRE FOR DNA FINGERPRINTING AND DIAGNOSTICS
HYDERABAD

Budget & Finance 2008 - 09

Sources of Funds

The Financial resources of the Centre are the Core Plan Grant-in Aid provided by the Department of Biotechnology, Government of India as against Annual Budgetary projections made by the Institute. Other resources are in the form of Research Grants provided by various National and International agencies and also from Services rendered by CDFD. The components of the core grants are Plan (Recurring) essentially for meeting expenditures on salaries, Operating expenses etc., and Plan (Non-Recurring) for meeting expenses on account of Equipments, Infrastructure and Furnishing etc.,

Receipts during the year 2008-09

Particulars	Amount in Lakhs	Percentage - %
Plan Grant in Aid	2590.00	81.16
Sponsored Projects	568.93	17.83
CDFD Services	29.92	0.94
Miscellaneous Receipts	2.50	0.07
Total	**3191.35**	**100.00**

I. Application of Funds during 2008-09 (Plan Grant in Aid)

S No	Particulars	Amount in Lakhs	Percentage- %
1	**Recurring**		
	Salaries & Wages	505.19	19.21
	Operating Expenses	1146.05	43.59
	Total	**1651.24**	**62.80**
2	**Non-Recurring**		
	Equipments, Infrastructure & Furnishing	978.10	37.20
	Total	**978.10**	**37.20**
	Grand Total	**2629.34**	**100.00**

II. Application of Funds during 2008-09 (Extra Mural Projects)

S No	Particulars	Amount in Lakhs	Percentage- %
1	**Recurring**		
	Salaries & Wages	132.75	20.37
	Operating Exp	355.92	54.63
	Total	**488.67**	**75.00**
2	**Non-Recurring**		
	Equipments	162.88	25.00
	Total	**162.88**	**25.00**
	Grand Total	**651.56**	**100.00**

BAPUJI & VENKAT

Chartered Accountants

AUDITOR'S REPORT

Date: 31-08-2009

The Director,
Centre for DNA Fingerprinting and Diagnostics,
Nampally, Hyderabad – 500 001

We have audited the attached Balance Sheet of CENTRE FOR DNA FINGERPRINTING AND DIAGNOSTICS, Hyderabad, as at 31st March 2009 and also the Income & Expenditure Account for the year ended on that date annexed there to. These financial statements are the responsibility of the organization management. Our responsibility is to express an opinion on these financial statements based on our audit.

We report that:

1. We have obtained all the information and explanations, which are to the best of our knowledge and belief, were necessary for the purpose of our audit.
2. In our opinion, the organization has kept proper books of account as required by law so far, as appears from our examination of those books.
3. The Balance sheet and Income & Expenditure account dealt with by this report in agreement with the books of account.
4. (a) The centre has maintained accounts on Cash basis.

   (b) The Centre receives extra mural grants from various National & International agencies for specific research activities. The Centre has a policy of allocating the overheads and transfer of expenditure of CDFD to different projects at the end of the financial year on adhoc basis after taking into account the amount of maximum permissible limit of overheads and also based on the approved budget estimates of the respective projects during the financial year.
5. In our opinion and to the best of our information and according to the explanations given to us, the said Balance sheet and the Income & Expenditure account read together with the notes thereon gives the information required in the manner so required and give a true and fair view.

   a) In so far it relates to the Balance sheet of the state of the organization as at 31st March 2009 and

   b) In so far as it relates to the Income & Expenditure account of the surplus of the organization for the year ended on 31st March 2009.

for **BAPUJI & VENKAT**
Chartered Accountants
[K VENKATACHARYULU]

Place: Hyderabad

Date: 31/08/09

Schedule 24: Significant Accounting Policies and Schedule 25: Contingent Liabilities & Notes on Account for the period ended 31/03/09

Schedule 24: Significant Accounting Policies & Schedule 25: Contingent Liabilities & Notes on Account for the period ended 31/03/09

1. Method of Accounting:

a. The accounting system adopted by the organization is on "Cash basis".

b. The organization has been allocating plan grant-in-aid under the "Non-recurring" & "Recurring" heads.

2. Revenue recognition:

Income comprises of Grant-in-Aid, Internal Resources through services and interest from short term deposits. Income accounted on the basis of the Cash/DD/Cheques/Cr notes received.

3. Fixed Assets:

(a) Fixed assets are stated at cost. Cost includes freight, duties, and taxes etc.,

(b) Depreciation: No depreciation on the Fixed Assets is charged and as such no ageing of fixed assets are being done.

(c) Capital work in progress has been entered to the extent of the last running account bills paid.

(d) Realization on sale of obsolete/surplus fixed assets which is not required for the purpose of research activities are adjusted against capital cost.

4. Inventories:

All purchases of chemicals, glassware and other consumables have been charged to consumption at the time of purchase.

5. Foreign Currency transactions:

Foreign Currency transactions are recognized in the books at the exchange rates prevailing on the date of transaction.

6. Investments:

Investments in STDR's are stated at book values.

7. Advances:

It is observed from the objection book register that advances to suppliers for consumables & Equipments are to be reconciled and adjustment entries are to be passed in the books of accounts.

8. The previous year balances have been regrouped/rearranged, wherever necessary.

Director, CDFD | Head Finance & Accounts | for **Bapuji & Venkat**
Chartered Accountants
[K VENKATACHARYULU]

Place: Hyderabad
Date: 31/08/091.

CENTRE FOR DNA FINGERPRINTING AND DIAGNOSTICS HYDERABAD

CLARIFICATION ON NOTES ON ACCOUNTS: 2008-09

- Notes on Accounts 1 to 6 & 8: Method of Accounting/ Revenue recognition/Fixed Asset/Inventories/ Foreign Currency transactions/Investments:

  These are all only informatory items.

- Notes on Accounts 7: Advances:

  The observation of the audit has been noted. The action has already been initiated to reconcile the objection book register.

E V RAO
Head, Finance & Accounts,
CDFD