IDENTIFICATION OF GENE SYNTENY USING SOMATIC CELL HYBRIDS IN RIVER BUFFALO (*Bubalus bubalis*)

RANK DHARAMSHIBHAI NATHABHAI
(ID NO. DPV-92001)

Thesis submitted in part fulfilment of the requirement for the degree of

DOCTOR OF PHILOSOPHY
in
ANIMAL GENETICS AND BREEDING
to
TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY

TNV 885

DEPARTMENT OF ANIMAL GENETICS
MADRAS VETERINARY COLLEGE
TAMIL NADU VETERINARY AND ANIMAL SCIENCES UNIVERSITY
MADRAS

1996

CERTIFICATE

This is to certify that this thesis entitled **"IDENTIFICATION OF GENE SYNTENY USING SOMATIC CELL HYBRIDS IN RIVER BUFFALO (Bubalus bubalis)"** submitted in part fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL GENETICS AND BREEDING to the Tamil Nadu Veterinary and Animal Sciences University, Madras - 600 007, is a record of bonafide research work carried out by **RANK DHARAMSHIBHAI NATHABHAI** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma or fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journals or magazines.

Date : 2.2.96

Place: Madras - 600 007.

(P.KANAKARAJ)
Chairman

APPROVED

Chairman : Dr.P.Kanakaraj

Members : 1. Dr.A.R.Krishnan

2. Dr.V.Thiagarajan

3. Dr.K.Kumanan

Date : 12.04.96

External Examiner

(V.R. Chendsa Reddy)

CURRICULUM VITAE

Name of the Candidate	:	**RANK DHARAMSHIBHAI NATHABHAI**
Date of Birth	:	01.06.1962
Place of Birth	:	Rabarika Dist- Amreli (Gujarat)
Major Field of Specialization	:	Animal Genetics and Breeding
Educational Status	:	B.V.Sc. and A.H. in 1985 from College of Veterinary Science and Animal Husbandry, Anand M.V.Sc. (Animal Genetics and Breeding) in 1989 from College of Veterinary Science and Animal Husbandry, Anand Joined Ph.D. (Animal Genetics and Breeding) study in December 1992 at Madras Veterinary College, Madras.
Professional Experience	:	Worked as Senior Research Assistant during 10.08.1985 to 21.09.1989 at College of Veterinary Science and Animal Husbandry, Anand. Working as Assistant Research Scientist Assistant Professor since 22.09.1989 at College of Veterinary Science and Animal Husbandry, Anand.
Marital Status	:	Married
Permanent Address	:	At and P.O. Rabarika Via-Dedan Dist. Amreli (Gujarat) Pin - 364550
Publications	:	Research articles - 08 Popular article - 01
Awards	:	Merit Cum Means Scholarship, ICAR - 1980 Senior Research Fellowship, CSIR - 1992.
Membership of Professional Society	:	The Indian Society for Animal Production and Management Association for the Promotion of DNA Fingerprinting and Other DNA Technologies.

Dedicated

to

My Beloved Parents

ACKNOWLEDGEMENT

I express my deep sense of gratitude to my guide Dr.P.Kanakaraj for giving me whole-hearted support and full freedom to work in the tissue culture laboratory anytime every time. He always admired my success and encouraged me in succeeding the momentary failures during the difficult stages of this work.

I acknowledge my personal indebtedness to Dr.A.R.Krishnan, who has been more than just a member of my doctoral advisory committee. he has taught me to approach scientific problems judiciously. His fatherly attitude gave me the feeling of homely environment. I am fortunate at having had the opportunity to work in the department under his leadership.

My profound thanks are due to Drs.V.Thiagarajan, K.Kumanan and N.Daniel Joy Chandran for giving me valuable suggestions at critical junctures and generously sparing lab facilities and chemicals during emergency needs.

I am immensely thankful to my colleague Joshi, for his inestimable help, without which a major portion of this work could not have been carried out.

Working in the tissue culture and cytogenetics laboratories has been a wonderful experience for me. I shall always cherish the memories of moments with all my past and present colleagues, Tank, Kharadi, Brahmkshtri, Thilak, Balasubramaniam, Kannan, Murali, Lydia, Jayashree, Haribaskar, Ravikumar and Vivekanandan who made the working in the department so enjoyable and pleasant all the time.

I owe my special thanks to Dr.Ramesh Babu for improving my manuscript. I am thankful Dr.Archibald David and Dr.Kirubakaran for their help in photodocumentation of results, to Dr.R.Ramasamy and Dr.Ajith Kumar for co-operation in collection of blood samples and to Mr.Yashwanth for giving practical tips in chromosome banding. My thanks are also due to Velmurugan, Senthilvèl and Shaija for assisting in culture work.

I shall always remember the interest shown by Dr.B.P.Chowdhary, Swedish University of Agricultural Sciences, Sweden in progress of my work.

I convey my heartfelt thanks to Dr.P.Thangaraju A.M.Nainar, K.S.Raman, K.Venkoba Rao, S.Jayarajan, M.John Edwin, J.Kalatharan, P.S.Rahumathulla, S.N.Sivaselvam, S.Subramanian, P.Kumarasamy and all the staff members of Department of Animal Genetics who always considered me one among themselves and rendered help and co-operation during the tenure of my work.

I thankfully acknowledge the financial support offered by Council of Scientific and Industrial Research by awarding Senior Research Fellowship. I am also thankful to Gujarat Agricultural University for deputing me to Madras Veterinary College for doctoral study.

I have no words to express my gratitude to my parents without whose blessings, I would not have succeeded in my assignment.

ABSTRACT

IDENTIFICATION OF GENE SYNTENY USING SOMATIC CELL HYBRIDS IN RIVER BUFFALO (*Bubalus bubalis*)

Name	:	**RANK DHARAMSHIBHAI NATHABHAI**
Degree for which Submitted	:	Ph.D. in Animal Genetics and Breeding
Chairman Advisory Committee	:	**Dr.P.Kanakaraj** Professor, Dept. of Animal Genetics
Department	:	Animal Genetics
Place	:	Madras Veterinary College, Madras.
University	:	Tamil Nadu Veterinary and Animal Sciences University
Year	:	1996

Somatic cell hybrids between buffalo leucocytes and mouse adenocarcinoma cell line, RAG were developed by suspension fusion technique using polyethylene glycol (PEG). Hybridization frequency was influenced by duration of PEG exposure, concentration and grade of PEG and parent cell density. Exposure for 90 seconds to PEG-1450 gave the maximum hybridization frequency. The density of 1 x 10^5 PEG treated parent cells per well in 96-well microtitre plate was found to be optimum.

The buffalo-mouse hybrid cells were isolated in HAT medium and fast growing 12 clones were selected for evaluation of their chromosome composition and enzyme analysis. Parent cells were also examined karyotypically and electrophoretically. Buffalo karyotype was prepared from GTG-banded metaphase chromosomes. It consisted of five pairs of metacentric and twenty pairs of acrocentric chromosomes including the sex chromosomes. RAG cells were heteroploid in nature with modal chromosome number 66. The hybrid clones exhibited wide variation in their chromosome content, with modal numbers ranging from 66 to 164.

The buffalo-mouse hybrid clones segregated the chromosomes on propagation. Segregation was progressive and the chromosomes of both the parental origin were lost. Three out of six clones retained buffalo chromosome 4.

These hybrid clones were characterized electrophoretically for the presence of buffalo LDHB, PEPB and TPI isozymes. These isozymes were concordantly present or absent in all the clones. Hence, the synteny between the genes encoding these enzymes is proposed. Also, the expression of these isozymes was concordant with the buffalo chromosome 4. Hence, their chromosomal localization is further proposed on buffalo chromosome 4.

CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
	LIST OF TABLES	
	LIST OF PLATES	
	LIST OF FIGURES	
1	INTRODUCTION	1-3
2	REVIEW OF LITERATURE	4-3[illegible]
	2.1 METHODS EMPLOYED IN MAPPING GENES	5
	2.1.1 Linkage analysis	5
	2.1.2 *In situ* Hybridization and Fluorescence *In situ* Hybridization (FISH)	6
	2.1.3 Comparative mapping	7
	2.1.4 Somatic cell hybridization	8
	2.2 SOMATIC CELL HYBRIDIZATION-HISTORY	11
	2.2.1 Discovery	11
	2.2.2 Hybrid cell selection	12
	2.2.3 Use of fusion agents	14
	2.3 HYBRIDIZATION TECHNIQUE	15
	2.3.1 Monolayer fusion technique	15
	2.3.2 Suspension fusion technique	16
	2.3.3 Pancake fusion technique	17
	2.4 FACTORS AFFECTING HYBRIDIZATION FREQUENCY	17
	2.4.1 Time of exposure to PEG	17
	2.4.2 PEG concentration	18
	2.4.3 Grade (molecular weight) of PEG	19

CHAPTER NO.	TITLE	PAGE NO.

	2.4.4Cell density	20
2.5	PARENT CELLS	21
2.6	CHROMOSOME SEGREGATION FROM SOMATIC CELL HYBRIDS	22
	2.6.1 Direction of chromosome segregation	23
	2.6.2 Mechanism of chromosome segregation	24
2.7	SOMATIC CELL HYBRIDIZATION AND GEN MAPPING	24
	2.7.1 Gene mapping in bovidae	25
	2.7.2 Gene mapping in buffalo	28
2.8	CHROMOSOME ANALYSIS	29
	2.8.1 Karyotypic characterization of buffalo	29
	2.8.2 Karyotypic characterization of RAG	30
	2.8.3 Karyotypic characterization of hybrid cells	31
2.9	ISOZYME ANALYSIS	31
	2.9.1 Cell lysate preparation	32
	2.9.2 Buffer system	33
	2.9.3 Histochemical staining	33
	2.9.4 Zymogram evaluation	34
2.10	SYNTENIC IDENTIFICATION AND CHROMOSOMAL LOCALIZATION OF LDHB, PEPB AND TPI	36
	MATERIALS AND METHODS	38-54
3.1	PREPARATION OF MEDIA AND SOLUTIONS	38
	3.1.1 Dulbecco's Modified Eagle's Medium (DMEM)	38

CHAPTER	TITLE	PAGE NO.

	3.1.2 Rosewel Park Memorial Institute 1640 (RPMI-1640) medium	39
	3.1.3 Hypoxanthine, Aminopterin, Thymidine (HAT) medium	39
	3.1.4 8-Azaguanine medium	39
	3.1.5 Trypsin-Versene-Glucose (TVG) solution	40
	3.1.6 Polyethylene Glycol (PEG) solution	40
	3.1.7 Colchicine solution	41
	3.1.8 Phosphate bufferred saline (PBS)	41
	3.1.9 Phosphate buffer	41
	3.1.10 RBC lysing buffer	42
	3.1.11 Cell lysis buffer	42
	3.1.12 Giemsa stain	42
3.2	SETTING UP LEUCOCYTE CULTURE	43
3.3	CULTURE OF RAG CELL LINE	44
3.4	SOMATIC CELL HYBRIDIZATION	45
	3.4.1 Pancake fusion	45
	3.4.2 Suspension fusion	45
3.5	HYBRID CLONES	46
	3.5.1 Selection and cloning of hybrid cells	46
	3.5.2 Maintenance of hybrid clones	47
3.6	FREEZING AND THAWING OF CELLS	47
3.7	CHROMOSOME ANALYSIS	48
	3.7.1 Chromosome preparation	48
	3.7.2 Chromosome banding	49
	3.7.3 Chromosome evaluation	49
3.8	ISOZYME ANALYSIS	50
	3.8.1 Preparation of cell lysates	50
	3.8.2 Preparation of cellulose acetate gel	50
	3.8.3 Electrophoresis	50
	3.8.4 Enzyme specific staining	51

CHAPTER	TITLE	PAGE NO.

	3.8.4.1 Lactate dehydrogenase (LDH) EC 1.1.1.27	51
	3.8.4.2 Peptidase B (PEPB) EC 3.4.11 or 13	52
	3.8.4.3 Triose phosphate isomerase (TPI) EC 5.3.1.1	53
	3.8.5 Isozyme evaluation	54
	3.9 STATISTICAL ANALYSIS	54
4	**RESULTS**	55-57
	4.1 SOMATIC CELL HYBRIDIZATION	55
	4.1.1 Pancake fusion	55
	4.1.2 Suspension fusion	55
	4.1.2.1 Effect of duration of PEG exposure	55
	4.1.2.2 Effect of concentration and grade of PEG	57
	4.1.2.3 Effect of parent cell density	58
	4.2 KARYOTYPIC CHARACTERIZATION OF PARENTAL CELL	59
	4.2.1 Buffalo	59
	4.2.2 RAG	59
	4.3 KARYOTYPIC CHARACTERIZATION OF BUFFALO-MOUSE HYBRIDS	60
	4.3.1 Uncloned hybrids	61
	4.3.2 Hybrid panel	61
	4.4 CHROMOSOME SEGREGATION	63
	4.5 ISOZYME ANALYSIS	74
	4.5.1 Zymogram pattern of LDHB	75
	4.5.2 Zymogram pattern of *PEPB*	75
	4.5.3 Zymogram pattern of TPI	76
	4.6 SYNTENY IDENTIFICATION AND CHROMOSOMAL ASSIGNMENT	76

CHAPTER	TITLE	PAGE NO.
5	**DISCUSSION**	78-94
	5.1 SOMATIC CELL HYBRIDIZATION	78
	5.1.1 Selection of parental cell line	78
	5.1.2 Hybridization technique	78
	5.1.2.1 Pancake fusion	78
	5.1.2.2 Suspension fusion	79
	5.1.3 Factors affecting hybridization frequency	79
	5.1.3.1 Effect of duration of PEG exposure	79
	5.1.3.2 Effect of concentration and grade of PEG	81
	5.1.3.3 Effect of cell density	82
	5.2 KARYOTYPIC CHARACTERIZATION OF PARENTAL CELLS	84
	5.2.1 Buffalo	84
	5.2.2 RAG	85
	5.3 KARYOTYPIC CHARACTERIZATION OF BUFFALO-MOUSE HYBRIDS	86
	5.3.1 Uncloned hybrids	86
	5.3.2 Hybrid panel	86
	5.4 CHROMOSOME SEGREGATION	87
	5.5 ZYMOGRAM ANALYSIS	90
	5.5.1 LDHB	90
	5.5.2 PEPB	91
	5.5.3 TPI	92
	5.6 SYNTENY IDENTIFICATION AND CHROMOSOMAL ASSIGNMENT	93
6	**SUMMARY**	95-97
7	**REFERENCES**	98-111

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
2.1	Current status of physical gene maps in various mammalian species	5
4.1	Effect of duration of PEG exposure to parental cells on hybridization frequency	56
4.2	Effect of PEG concentration and grade on hybridization frequency	57
4.3	Effect of cell density on growth of hybrid cell colonies	58
4.4	Frequency distribution of chromosomes in RAG	60
4.5	Frequency distribution of chromosomes in uncloned hybrids	62
4.6	Karyotypic characteristics of hybrid clones	64
4.7	Total and biarmed chromosomes per cell in different clones	65
4.8	Buffalo chromosomes retained in the hybrid clones	72
4.9	Expression of buffalo isozymes and occurrence of buffalo chromosome 4 in the hybrid clones	77

LIST OF PLATES

PLATE NO.	TITLE	BETWEEN PAGES
4.1	Hybrid cell colony on day-7 (100 X)	56-57
4.2	Hybrid cell colony on day-10 (100 X)	56-57
4.3	Hybrid cell colony on day-13 (100 X)	56-57
4.4	GTG-banded buffalo metaphase complement (1250 X)	59-60
4.5	GTG-banded karyotype of buffalo	59-60
4.6	A representative Giemsa stained RAG metaphase complement (1250 X)	59-60
4.7	GTG-banded RAG metaphase complement (1250 X)	59-60
4.8	A representative GTG-banded hybrid complement (Clone A9, Partial) (1250 X)	63-64
4.9	Zymogram pattern of LDHB in parental cells and representative hybrid clones	75-76
4.10	Zymogram pattern of PEPB in parental cells and representative hybrid clones	76-77
4.11	Zymogram pattern of TPI in parental cells and representative hybrid clones	76-77

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
4.1	Distribution of total and biarmed chromosomes in clone A9	66
4.2	Distribution of total and biarmed chromosomes in clone D12	67
4.3	Distribution of total and biarmed chromosomes in clone G3	68
4.4	Distribution of total and biarmed chromosomes in clone A5	69
4.5	Distribution of total and biarmed chromosomes in clone B10	70
4.6	Distribution of total and biarmed chromosomes in clone C3	71

Introduction

INTRODUCTION

Animal breeding in the last few decades has witnessed tremendous improvement in production. The success lies in selective breeding of livestock with the aim of increasing the population frequency of favourable alleles governing the production traits. The success of breeding to augment any particular trait depends on heritability of the trait. Most economic traits are quantitative in nature, governed by large number of genes and exhibit low to moderate heritability. Hence, the progress in improving these traits is obviously slow. Identification of genes affecting such traits could be the first step towards more successful manipulation of them for a potential improvement. In this regard, genome analysis with respect to loci affecting these traits may be achieved by gene mapping. It is now technically feasible to construct a foundation map atleast in bovine that will make it possible to map any simply inherited traits or even any quantitative trait loci relative to one or more polymorphic markers (Soller, 1990, 1991). Marker assisted selection for productivity and disease resistance is a realistic goal awaiting the development of a gene map sufficiently concentrated with polymorphic markers (Kashi *et al.*, 1990).

A recent summary on the status of mammalian gene map (O'Brien and Marshall-Graves, 1992) has demonstrated that the progress in livestock genome mapping compared to that in human is far from satisfactory. Although, cattle is the best characterized livestock species, only 350 genes have been mapped so far. Buffalo is probably the least explored species. Only six genes have been localized on buffalo chromosomes (Iannuzzi *et al.*, 1994b). The main

handicap being that buffalo population is concentrated mainly in Asia and Africa, and research approaches in this regard have not taken the impetus that is needed.

Among various approaches for gene mapping, somatic cell hybridization is comparatively simpler and easily adoptable approach (Womack, 1990). Somatic cell hybridization combined with molecular genetics has served as the most important technique for physical mapping of genes. Among 350 genes mapped in cattle, 269 have been mapped through this approach (Fries *et al.*, 1993). These technologies have produced the human gene map with powerful diagnostic and prognostic capabilities for inherited diseases. The same technologies can be applied to the genome of livestock species to map Mendelian genes or quantitative trait loci relative to one or more polymorphic markers and to practice marker assisted selection.

India has the largest buffalo population in the world. Buffaloes contribute to over 48 per cent of the total milk production in India (FAO, 1993), though they are numerically far less than cows. Thus, the importance of buffaloes as the prime milk producer in the country needs little emphasis. India being the leading country in buffalo production, gene mapping initiatives are urgently warranted.

Given this scenario, present study was undertaken with the following objectives.

i) To standardize the technique for interspecific somatic cell hybridization.

ii) To standardize the technique for isozyme evaluation from hybrid panels by electrophoresis

iii) To identify the synteny, if exist, among genes encoding enzymes viz., LDHB, PEPB and TPI

iv) To assign the synteny to a particular buffalo chromosome.

Review of Literature

REVIEW OF LITERATURE

For years, gene mapping in mammals was considered an esoteric enterprise, practiced only by a few mouse geneticists studying the linkage among genes based on the frequency of recombination between parental combination. Even in late 1970's, after the discovery of extensive biochemical and immunological polymorphisms which pushed the number of mapped genes in the mouse to over 300, only a few livestock geneticists appreciated the application of gene mapping for potential improvement of livestock. Meanwhile, a revolution in medical genetics had precipitated on the advent of somatic cell genetics and its application to human gene mapping. This technology eliminated the need for extensive pedigree or large breeding experiments and thus opened up the avenue for gene mapping in virtually any animal species. The recombinant DNA era of the 1980's quickly legitimized human gene mapping as a medical endeavour. The potential value of new biotechnologies to animal genetics has suddenly become obvious. It is now possible to map any simply inherited trait or even quantitative trait loci (QTL) relative to one or more polymorphic markers.

A summary on the status of mammalian gene map (Table - 2.1) demonstrated that relatively few of the estimated 100,000 genes have been localized on mammalian genome.

Table 2.1: Current status of physical gene maps in various mammalian species (O'Brien and Marshall-Graves, 1992)

	Haploid Chromosome No.	No. of Syntenic Groups	No. of Genes Mapped
Human	23	23	2500
Mouse	20	20	2200
Cattle	30	29	250
Sheep	27	19	50
Pig	19	17	80

2.1 METHODS EMPLOYED IN MAPPING GENES

There are number of complementary approaches to establish genetic and physical gene maps. Genetic maps are constructed by studying the meiotic linkage relationship of gene loci (linkage analysis) and provide information about the distance of genes as a function of the frequency of the meiotic crossovers occurring along the chromosomes. The construction of physical maps is based on methods that make use of gene transfer between mammalian somatic cells from different species (somatic cell hybridization), methods that involve the direct assignment of genes by molecular hybridization of specific DNA probes to fixed chromosomes (*in situ* hybridization) and comparative investigations that allow provisional mapping based on the evolutionary conserved location of genes in different genomes (comparative mapping).

2.1.1 Linkage Analysis

To determine whether two genetic loci are on the same chromosome, the meiotic recombination frequency between these loci is measured in

informative families. If the recombination frequency (θ) is less than 0.5 and if the assumption of random segregation at the loci is correct, the two loci are likely to be on the same chromosome. As a statistical test to determine whether the hypothesis of linkage can be accepted or must be rejected the "lod" (logarithms of odds) score test (Morton, 1955) is widely used. The map distance is measured in crossover units or centimorgans (cM). The frequency of recombination between homologous chromosomes, a normal event in meiosis, is generally proportional to the distance between two linked loci and can therefore be used as an unit of chromosomal length. One centimorgan corresponds to the distance separating two loci exhibiting one per cent recombination rate.

With the availability of DNA level markers, which are highly polymorphic and frequent, hence highly informative in linkage analysis, it is now possible to map even quantitative trait loci affecting traits of economic importance (Beckmann and Soller, 1987, 1988).

2.1.2 *In situ* Hybridization and Fluorescence *in situ* Hybridization (FISH)

In situ hybridization is a direct mapping technique involving the hybridization of radiolabelled DNA probes to fixed metaphase chromosomes and the subsequent visualization of the signal as silver grain after autoradiography. Dark spots or silver grains are produced on the film indicating the position of DNA hybridization. The grain distribution over the chromosome is determined and interpreted with respect to the banding pattern of same preparation photographed prior to hybridization. This technique was

first developed by Gall and Pardue (1969) and John *et al.* (1969) and later improved by Harper and Saunders (1981).

FISH involves use of non-radioactive or fluorescent (biotin or digoxigenin)-labelled probes (Trask, 1991). FISH allows a more precise localization of the probe signal on the chromosomes and therefore an increased mapping resolution. A distinct advantage of FISH is the possibility to amplify the signal using immunological techniques. However, in both the procedures an essential step is the unambiguous identification of chromosomes.

2.1.3 Comparative Mapping

Comparative gene mapping involves mapping of homologous gene loci in multiple species based on the observation that the location of genes on specific chromosome regions is conserved in different species. This has been proved to be very powerful means of extrapolating mapping data from one species to another, based on the establishment of conserved chromosomal segment.

Extensive gene and chromosomal homologies have been identified between cattle and human (Womack and Moll, 1986; Womack *et al.,* 1987, 1989, 1991). Extensive chromosomal homologies have also been observed between closely related livestock species at cytogenetic (Hediger *et al.,* 1989, 1991; Hayes *et al.,* 1991, 1993) and genetics level (Chowdhary *et al.*, 1991; Hediger *et al.,* 1991). The river buffalo represents the best characterized example of chromosome conservation among family Bovidae. Direct comparisons of G- and R- banding patterns have documented extensive

chromosome homology between cattle and river buffalo (Di Berardino *et al.*, 1981; Iannuzzi and Di Berardino, 1985; Iannuzzi *et al.*, 1989, 1990a). It is now possible to interpolate and extrapolate the position of genes between these species. Thus, on the basis of established banding homology, it is possible to make indirect assignments of bovine syntenies to homologous chromosomes in the related species, provided atleast one gene included in the syntenic group is sublocalized in specific chromosome.

2.1.4 Somatic Cell Hybridization

Somatic cell hybridization is the fusion of somatic cells of different types to form a hybrid cell that contains genetic elements of both parental cells. Somatic cell hybridization provides a method for combining mammalian genomes *in vitro*, completely bypassing the route of sexual reproduction. Somatic cells from virtually any species can be fused to produce viable hybrid cells by using inactivated Sendai virus (Harris and Watkins, 1965) or polyethylene glycol (Pontecorvo, 1975) as fusion agents. The use of mutant cell lines that lack hypoxanthine guanine phosphoribosyl transferase ($HPRT^-$), an enzyme in the purine salvage pathway or thymidine kinase (TK^-), an enzyme in the pyrimidine salvage pathway as one fusion partner and the cells that are $HPRT^+$ or TK^+ as the other, with subsequent culture in medium that contains hypoxanthine, aminopterin and thymidine (HAT) allows the efficient selection of hybrid cells from parent cells (Littlefield, 1964). Generally, it involves the fusion of leucocytes or fibroblasts from human or any livestock species (donor) with a rodent or hamster cell line (recipient) under culture conditions selective for complementing hybrid cells. As a general rule, the chromosomes from

primary cell progenitor will be preferentially lost when hybridized to transformed cell line. Thus, in such hybrids there is progressive and preferential loss of non-rodent or non-hamster chromosomes (Weiss and Green, 1967). This generates panel of cloned hybrids each retaining different combinations of chromosomes from donor parent. In the absence of rearrangements of the non-rodent chromosomes, the assignment of a gene or other genetic marker to a specific chromosome is straight forward; if the presence/absence of a marker is concordant with one chromosome and discordant with every other chromosome in a hybrid cell panel, the relevant marker is assigned to the concordantly segregating chromosome (Ruddle, 1973; Wijnen *et al.*, 1977).

Until recently, the method of choice in gene assignment was the correlation of particular gene product, i.e. an enzyme with an identifiable donor chromosome or fragment. The presence or absence of donor enzyme can be detected under electrophoretic conditions that separate the donor and recipient electromorphs. The presence of specific donor chromosome can be determined by karyological screening of hybrid complement. This allows identification of group of genes that are generally lost or retained together. Such genes are said to be syntenic and assumed to be on the same chromosome. Concordance of segregation or retention of a specific chromosome with a group of syntenic genes is sufficient to assign the syntenic group to that chromosome. A considerable number of syntenic relationships and gene assignments have been established in mammalian species (O'Brien, 1993). Especially, in human and cattle a significant number of genes have been assigned by using somatic cell hybrids.

The regional localization of specific genes on chromosome or linear order of syntenic genes on a chromosome can only be determined in laborious experiments involving broken or rearranged chromosomes. The accurate karyotypic definition of hybrid cell panels remains an enigma to somatic cell mapping of livestock. Many of the smaller chromosomes particularly acrocentrics are difficult to distinguish one from the other especially, in a partial donor karyotype against a background of rodent or hamster chromosomes. Consequently at this time the assignment of genes to chromosomes lags behind the definition of syntenic groups. In cattle, synteny groups representing 28 autosomes and X-chromosome are identified. These are designated U1-U24, U26-U29 and X. However, only 19 syntenic groups have been assigned to the specific chromosomes (Fries *et al.*, 1993).

The growing number of cloned DNA probes available for gene mapping gives an important new dimension to somatic cell genetics. Mapping is no longer restricted to genes whose products are expressed in cell culture, but any DNA sequences for which a cloned probe is available can now be mapped in a panel of hybrid cells. Restriction fragment length polymorphism (RFLP) analysis and probe hybridization to hybrid cell DNA allows synteny identification and chromosomal mapping by establishing the concordance between DNA fragments carrying the probed gene and the marker for an already assigned synteny (Adkison *et al.*, 1988a,b).

2.2 SOMATIC CELL HYBRIDIZATION - HISTORY

2.2.1 Discovery

Somatic cell genetics is relatively an young field of study. Its origin dates back to little more than three decades. The phenomena of somatic cell hybridization was first observed by Barski and his colleagues in 1960, accidently while they were studying the mouse cancer cell lines. They had mixed cultures of two cancer cell lines that could be distinguished by differences in cell morphology and in shape of some of their chromosomes. After a few months, they unexpectedly observed that a few cells of new type had appeared containing in a single nucleus the chromosomes of both the parents. The hybrid cells had a chromosome number approximately equal to the sum of chromosome numbers of parental cells. The hybrid cells had arisen spontaneously by the fusion of two different types of cells. Barski and his colleagues were successful in producing and growing the pure cultures of these hybrid cells.

The occurrence of somatic cell hybrids was confirmed by Sorieul and Ephrussi (1961), wherein hybrids resulted from fusion of closely related cells. Subsequent experiments, however, showed that the isolation of viable hybrids was not limited to crosses between closely related ones and that hybrids could be formed between unrelated cells. Since the markers used to identify hybrids were the novel chromosomes occurring in the cells of permanent lines but not present in normal diploid cells, these were not used in the early hybridization experiments.

In later studies, normal cells cultured from a strain of mouse carrying a cytological marker were hybridized with mouse cells of a permanent line (Scaletta and Ephrussi, 1965). The results clearly demonstrated that the normal diploid cells could fuse with other cells to produce viable hybrids, atleast when the other parental cells belonged to a permanent line. The hybrids that were isolated, had an unlimited capacity for *in vitro* proliferation much like the permanent cell line.

In Barski's experiment, the hybrids turned out to have a selective advantage over the parent cells and grew more rapidly so that they soon constituted a large enough fraction of the total cell population to be isolated by cloning. Since hybrids were rare, it took long time for enough of them to accumulate so that they could be isolated. Further some crosses were almost surely failing since the hybrids had no selective advantage, it was clear that a method conferring a decisive selective advantage on hybrid cells could be extremely valuable.

2.2.2 Hybrid Cell Selection

In 1964, Littlefield devised a system for the selective isolation of hybrid cells. He cultured in a medium containing hypoxanthine, aminopterin and thymidine (HAT) two mouse cell lines, one HPRT$^-$ and another TK$^-$. Aminopterin blocks the "de novo" biosynthetic pathway of DNA synthesis from nucleotide precursor. However, DNA synthesis continues via "salvage" pathway in the presence of HPRT and TK enzymes. Because of their enzyme deficiencies neither cell line could survive in HAT medium but hybrid cells were able to grow in HAT medium by virtue of mutual complementation; each parent

supplied the gene for the enzyme the other parent lacked. Littlefield's selective medium therefore kills cells of both parental lines while allowing the survival and unhampered growth of hybrid cells.

Davidson and Ephrussi (1965), then modified Littlefield's method to device "half selective" system, in which only one parental cell line lacks one of the enzymes necessary for growth in Littlefield's medium. The other parent can be a normal line carrying no selective marker, provided that it grows slowly or is inoculated in small number. In the selective medium the majority parent degenerates, leaving discrete colonies of the minority parent and of hybrid cells. The hybrids can usually be recognized by their shape and their hybrid nature is confirmed by examination of the chromosomes.

The half selective system became a standard procedure for the isolation of hybrids between mouse cells and freshly explanted human peripheral white blood cells, which have no ability at all to grow in the culture. Such hybrids have played a major role in the mapping of human genome.

Kao and Puck (1970) proposed another system of selection. They fused Chinese hamster cell line auxotrophic for certain nutrients viz., inositol, proline and glycine, with normal human fibroblasts and grown in media lacking specific auxotrophic requirement of parental mutant cells. Hybrid cells could be isolated in the deficient medium due to the auxotrophic markers.

Baker *et al.* (1974) proposed yet another selection system in combination with HAT system based on cells' resistance to ouabain. Somatic cell mutants resistant to ouabain were isolated from mouse L and Chinese

hamster ovary (CHO) cells. These cells were fused with ouabain sensitive partner (fibroblasts) and hybrid cells were isolated in HAT medium containing ouabain.

2.2.3 Use of Fusion Agents

These hybrid cell selection systems were however, suboptimal. The frequency of spontaneously occurring hybrid cells was so negligible that inspite of selective advantage, isolation of hybrid cells from overwhelming population of parent cells was extremely difficult. The first envisaged method for increasing cell fusion frequency involved the use of virus as a fusing agent.

In 1958, Okada observed that the myxovirus known as the haemagglutinating virus of Japan, now commonly known as Sendai virus, causes animal cells in suspension to clump together and that many of the clumped cells undergo multiple fusions. With this observation in mind, Harris and Watkins (1965) brought about the fusion of human and mouse cells using Sendai virus that had been rendered non-infections by UV radiation. This fusion resulted in the multinucleate interspecific heterokaryons which were, however unable to proliferate. In 1966, Yerganian and Nell, successfully isolated virus induced viable hybrids capable of multiplication. However, with the observation that ß-propiolactone, an alkylating chemical agent completely destroys the virus infectivity leaving the cell fusing property unaffected, the use of UV for virus inactivation was soon replaced by ß-propiolactone.

An important advancement in the somatic cell genetics was experienced with the discovery of PEG as a reliable chemical fusogen for

mammalian cell fusion. Initially, Kao and Michayluk (1974) observed that PEG could efficiently agglutinate plant protoplasts and that these protoplasts subsequently were fused at high frequency. It soon became clear that PEG induced cell fusion was not cell specific and that PEG was an efficient fusogen for mammalian cells too. With the successful isolation of somatic cell hybrids between mouse and hamster cell line (Pontecorvo, 1975) the use of Sendai virus was superseded by PEG.

A totally new approach to *in vitro* cell fusion was offered by electrofusion. Zimmermann (1986) and Zimmermann and Urnovitz (1987) had shown that cell fusion was possible due to combined action of dielectrophoresis and transient change in membrane permeability obtained by electric pulses. This approach however, has not been accepted in mammalian cell fusion, beyond its experimental verification.

2.3 HYBRIDIZATION TECHNIQUE

Soon after the demonstration by Pontecorvo in 1975, that PEG could induce the fusion among mammalian cells and the resultant hybrid cells having unlimited potential for multiplication, PEG was recognized as a standard fusogen for fusion of variety of cells, either in monolayer or in suspension.

2.3.1 Monolayer Fusion Technique

Pontecorvo (1975) developed a method of fusion of cells capable of attaching to the substrate. In his pioneering experiment, he described the

fusion of mouse and hamster cell lines by treating the cell monolayer of mixed culture with PEG and their subsequent growth in HAT medium. Although this technique was successful in isolating viable hybrids, it was constrained by a considerable cell death.

Davidson and Gerald (1976) and Davidson *et al.* (1976) refined the protocol by optimizing the concentration, grade and time of exposure to PEG. They also suggested modification in removal and dilution of PEG. The technique was found to be effective in fusing virtually any type of adherent cells from several species viz., cattle (Heuertz and Hors - Cayla, 1978), sheep (Saidi-Mehtar *et al.*, 1981, 1991), pig (Leong *et al.*, 1983), buffalo (Joshi, 1995) with mouse or hamster cell lines.

2.3.2 Suspension Fusion Technique

The first hybridomas were produced using Sendai virus by treating the cells in suspension (Kohler and Milstein, 1975). However, PEG-induced fusion of cells in suspension was first described by Davidson and Gerald (1976). The cells in suspension (mouse/hamster cell line and human lymphocytes) were exposed to PEG and hybrids were isolated in HAT medium. Similar technique is now routinely being used for production of hybridomas, following first demonstration of PEG-induced fusion of myeloma cells by Galfre *et al.* (1977). Although, there have been a number of minor modifications described since then, the procedure has not been changed in its essentials.

2.3.3 Pancake Fusion Technique

O'Malley and Davidson (1977) developed a novel technique called 'Pancake' fusion technique which is based on centrifugation of cells on to a coverslip and exposure of cells to PEG as if they were attached in a monolayer.

Pancake technique is the method of choice for the fusion of leucocytes with mouse or hamster cell lines. Later, many workers reported this method to derive somatic cell hybrids for gene mapping studies (Adkison *et al.,* 1988a,b; Gallagher *et al.,* 1991, 1992 a,b; Ryan and Womack, 1993).

2.4 FACTORS AFFECTING HYBRIDIZATION FREQUENCY

2.4.1 Time of Exposure to PEG

The first viable somatic cell hybrids were obtained by Pontecorvo (1975) at a prolonged exposure to 50 per cent PEG i.e. 5-15 minutes in the monolayer fusion. However, he gave a shorter exposure time of 3-5 minutes for the fusion of lymphocytes with the cells in monolayer.

In further experiments, efforts were devoted to find out the optimum time of exposure to PEG solution to give the maximum yield of hybrids without affecting cell viability. Davidson and Gerald (1976) evaluated the effect of variable time of exposure ranging from the shortest possible of 15 seconds to 5 minutes. They observed increased frequency of hybridization when the exposure time was cut down from 5 minutes to 1 minute. Further reduction in time, however reduced the yield of hybrids. For the fusion of cells in suspension exposure for two minutes was found to be optimum.

Davidson *et al.* (1976) also recommended one minute of exposure for monolayer fusion. O'Malley and Davidson (1977) exposed the cells either in monolayer or in suspension to PEG for one minute. However, the same cells in pancake fusion technique required two minutes of exposure. In the recent trial involving buffalo fibroblasts and mouse cell lines, Joshi *et al.* (1995) and Rank *et al.* (1996) observed better hybridization frequency when the cells in monolayer were exposed to PEG for 60 seconds than for 30 or 90 seconds.

2.4.2 PEG Concentration

After the first successful attempt by Pontecorvo (1975) to isolate the viable hybrids, efforts were made to define the optimum PEG concentration for maximizing hybridization frequency. Davidson and Gerald (1976) tested a set of PEG concentrations ranging from 40 to 70 per cent for fusion of cells in monolayer. They observed a sharp peak of hybridization inducing activity around 50 per cent. Small change in PEG concentration in either direction drastically decreased the yield of hybrids. When the concentration of PEG increased to 55 per cent and more, there was a marked cell damage resulting in lower hybridization frequency. At PEG concentration lower than 50 per cent, however, cell viability was not affected but rate of fusion decreased.

Gefter *et al.*, (1977) evaluated the efficiency of variable PEG concentrations on rate of fusion. They observed that increase in PEG concentration, although enhanced the rate of fusion, it also increased the cell toxicity. The cells could tolerate longer exposure of up to seven minutes to PEG at 35-40 per cent concentration, but there was a marked cell damage beyond two minutes of exposure to PEG at 50 per cent concentration.

There seems to be no comparative study on the effect of variable PEG concentration in suspension fusion. O'Malley and Davidson (1977) in the conventional method and in the improved method of pancake technique used sole 50 per cent concentration.

Very recently, Rank *et al.* (1996) attempted fusion between buffalo fibroblasts and mouse cell line, LM (TK⁻). They obtained better hybridization frequency at 45% PEG/10% DMSO than at 50% PEG or 50% PEG/10% DMSO. However, in a similar study on hybridization between buffalo fibroblasts and mouse cell line RAG, Joshi *et al.* (1995) obtained satisfactory fusion frequency, by using 50 per cent PEG concentration alone.

The beneficial effect of incorporation of dimethyl sulfoxide (DMSO) in the PEG solution was first described by Norwood *et al.* (1976). They included DMSO at the 10 per cent level in the PEG solution and observed improvement in hybridization frequency.

2.4.3 Grade (Molecular Weight) of PEG

The first hybridization experiment was performed using PEG-6000 (MW 6000-7500). Later Davidson and Gerald (1976) also used this grade for fusion of several mouse/hamster cell lines and human leucocytes.

Davidson *et al.* (1976) made a comparative study on effect of varying grades of PEG on hybridization frequency. They observed that although all the grades of PEG, ranging from PEG-200 (MW 190-210) to PEG-6000 (MW 6000-7500) induced fusion, the highest hybridization frequency resulted at

PEG-1000 (MW 950-1050) used at 50 per cent concentration. Further, they reported that when the PEG concentration was increased from 50 to 55 per cent, the peak of hybridization frequency was shifted towards lower molecular weight.

Once it was known that PEG at 50 per cent concentration in a wide range of molecular weight can induce successful hybridization, several workers reported usage of PEG of different grades, viz., PEG-1000 (O'Malley and Davidson, 1977; Adkison *et al.,* 1988a,b; Hallerman *et al.,* 1988), PEG-3350 (Joshi *et al.,* 1995, 1996; Rank *et al.,* 1996), PEG-6000 (Ryttman *et al.,* 1986).

2.4.4 Cell Density

In the past, many workers reported different concentrations of cells of various species fused by adopting diverse techniques.

Davidson *et al.* (1976) described different cell concentrations of mouse and hamster cell lines to give relatively sparse to confluent monolayers. They observed two to three fold increase in the frequency of hybridization when the cell concentration was decreased from 2.5×10^6 cells to 5.0×10^5 cells. In the recent past, while working with mouse cell line and buffalo fibroblasts, Joshi *et al.* (1995) reported that parent cells in 10^5 - 10^6 concentration yielded maximum hybridization frequency. Further, they also observed that too scanty or too densely populated cells did not yield appreciable hybridization.

Very scanty literature is available pertaining to density of PEG treated cells and growth of hybrid cell colonies. In this regard, Olsson and Kaplan

(1983) observed highly cell density - dependent nature of human hybridomas. They reported growth in maximum wells at 2 x 10^5 cells/well seeding rate. Lower or higher than this, resulted in poor viability and thus lower hybrid cell output.

2.5 PARENT CELLS

The use of mutant cell lines that lack HPRT or TK enzyme activities, as parents in the somatic cell hybridization with subsequent culture in HAT medium allows efficient selection of hybrid cells from parental cells. (Littlefield, 1964). Hence, $HPRT^-$ or TK^- hamster and mouse cell lines have been used in somatic cell hybridization.

In majority of attempts, $HPRT^-$hamster cell lines, Wg3h and E-36, $HPRT^-$mouse cell line, RAG and TK^-mouse cell line, LM (TK^-) have been used in somatic cell hybridization for gene mapping in various livestock species viz., cattle (Heuertz and Hors-Cayla, 1981; Gallagher *et al.*, 1991, 1992a,b; Monteagudo *et al.,* 1991, 1992; Threadgill and Womack 1991a,b), buffalo (Othman *et al.*, 1994; Joshi, 1995), sheep (Saidi-Mehtar *et al.*, 1981, 1991; Imam-Ghali *et al.*, 1987, 1992), and pig (Leong *et al.*, 1983; Zijlstra *et al.*, 1994).

Besides $HPRT^-$and TK^-cell lines, auxotrophic Chinese hamster cell lines have been fused with normal cells of donor parent and grown in media lacking nutritional supplement required by mutant parental cells (Kao and Puck, 1970; Broad *et al.*, 1984; Puck and Kao, 1982; Jones *et al.,* 1985; Womack *et al.*, 1986, 1987).

The donor parental cells include primary (fibroblast) or terminal (separated lymphocyte or leucocyte) cells. The cells from human donor carrying a defined translocation have specifically been used in human - mouse and human - hamster somatic cell hybridization (Bruns *et al.,* 1978a,b; Sparkes *et al.*, 1978) for subchromosomal localization of genes.

2.6 CHROMOSOME SEGREGATION FROM SOMATIC CELL HYBRIDS

With the advent of somatic cell hybrids it was soon revealed that there was an accidental loss of chromosomes from hybrid genome. The rate and extent of loss varied in different hybrids, but it was generally greater in interspecific than intraspecific hybrids. The chromosomal constitution of intraspecific (mouse-mouse) somatic cell hybrids was rather stable, since the loss usually did not exceed 10-20 per cent of the number of chromosomes in original fused cells (Weiss and Ephrussi, 1965). Analysis of the number and kinds of chromosomes in successive generations of rat-mouse hybrids showed that although the decrease in the total number of chromosomes was not much greater than it was in mouse-mouse hybrids, the loss was slightly preferential, significantly more of rat chromosomes than that of mouse disappeared (Weiss and Ephrussi, 1966). With human-mouse hybrids, Weiss and Green (1967) observed that these hybrids presented an extreme case of preferential chromosome loss. These hybrids had lost most of their human chromosomes soon after being formed. Hybrids between human and CHO cells also lost human chromosomes in some cases even more rapidly than human-mouse hybrids (Kao and Puck, 1967, 1968). Kao and Puck (1970) combined this

extremely rapid segregation of human chromosomes with the use of auxotrophic markers for linkage determination between selected human genes.

2.6.1 Direction of Chromosome Segregation

Methods have also been developed to control whether the chromosomes of one parental cell or the other will be lost in the interspecific hybrids. Pontecorvo (1975) tried to induce the chromosomal elimination from the hybrid cells by irradiating one of the parental cells just before fusion. One of the parental cell line was mouse cell line and the other, Chinese hamster line. In a series of experiments, either the Chinese hamster or the mouse cells were irradiated just prior to fusion with non-irradiated cells of other species. The hybrid clones showed extensive loss of chromosomes which was dependent upon the irradiation dose. In a similar experiment carried out on human-mouse hybrids, it was observed that when the mouse cells were irradiated before fusion with human diploid cells, the number of hybrids formed was greatly reduced. However, those hybrids that did grow segregated human chromosomes, as did hybrids with non-irradiated cells.

Unilateral segregation of non-rodent or non-hamster chromosomes from interspecific somatic cell hybrids is observed to be an universal phenomena. Such segregation may be non-random. This was observed atleast in pig hybrids. The number and type of pig chromosomes segregated was found to be related to the cell line used as fusion partner (Leong *et al.,* 1983; Echard *et al.,* 1984; Forster and Hecht, 1986; Ryttman *et al.,* 1986; Zijlstra *et al.,* 1994.).

2.6.2 Mechanism of Chromosome Segregation

Although chromosome segregation from mammalian cell hybrids is a crucial element of somatic cell genetic analysis, its mechanism still remains obscure. Chromosome segregation seems to follow some empirical rules. Most notably, the direction of segregation depends largely on the species of origin of parental cells and is rather difficult to reverse. However, reversal of the direction has been reported in hybrids in which established human cells were fused with diploid rodent cells, suggesting an overriding effect of cell transformation (Minna and Coon, 1974; Croce, 1976). Reversal of the direction of loss was also accomplished by varying the input ratio of parental components in experiments with polyploid series of mouse and hamster cells (Graves, 1984). Graves and Barbieri (1992) in an experiment involving, human and Chinese hamster established cell lines, developed human-hamster cell hybrids and human cell-hamster karyoplast hybrids. The cell hybrids retained one or two sets of hamster chromosomes and lost most of the human chromosomes. The karyoplast hybrids, however, retained a complete set of human chromosomes and lost most of the Chinese hamster chromosomes indicating the cytoplasmic factors as major determinants of the direction of chromosome segregation.

2.7 SOMATIC CELL HYBRIDIZATION AND GENE MAPPING

Following successful hybridization between TK$^-$mouse cell line and human cells, Migeon and Miller (1968) observed that very few human chromosomes were retained in the hybrids. Attempts were made to correlate the presence of specific human chromosome with the expression of TK activity,

but the techniques for chromosome identification at that time were not adequate to permit the identification of specific human chromosome carrying the TK gene. But this study established that human-mouse hybrids could be the important tool for mapping the human genome. Meanwhile, Bodmor *et al.* (1969) isolated hybrids between HPRT$^-$ mouse cell line and human leucocytes in HAT medium and correlated the expression of HPRT with the retention of human chromosome. This led them to assign HPRT to X-chromosome, this being the first gene assignment.

Initially the gene mapping studies were based on the detection of the protein products of the genes to be mapped. Enzyme electrophoresis was used to determine the presence or absence of several dozens of gene products. Thus only genes that are expressed in the hybrid cells would be mapped. However, the availability of cloned DNA probes during 1980s made it possible to map the genes which are not expressed.

2.7.1 Gene Mapping in Bovidae

Gene mapping in livestock was in infantile stage when the human gene mapping was being revolutionized by recombinant DNA technology. The first report on gene mapping in livestock species appeared only in 1978, when Heuertz and Hors-Cayla, using cattle-hamster hybrids identified synteny among enzyme genes G6PD, HPRT, α-GLA and PGK. Based on the concordance studies, they assigned this synteny to X-chromosome. In 1981, they reported three more syntenic groups comprising of eight loci, LDHB-PEPB-TPI, PGM3-ME1-SOD2, PGD-ENO1 using cattle-hamster hybrid panel. Shimizu *et al.* (1981) confirmed the synteny and X-linkage of G6PD, PGK and

α-GLA in cattle using cattle-mouse hybrids. Dain *et al.* (1984), however, made the first autosomal gene assignment in cattle by mapping LDHB, PEPB and TPI on chromosome number 19, PEPC tentatively on chromosome number 5 and possibly SOD1 on chromosome number 13.

Bovine gene mapping was greatly benefitted and boosted by the work of Womack and Moll (1986). Using cattle-hamster hybrid panel, they defined 21 syntenic groups comprising 28 enzyme loci. This hybrid panel served as basis for syntenic assignment of large number of genes in the subsequent studies. Later, eight more syntenic groups were identified to take the total tally of syntenic groups to 29, 28 autosomal and an X (Womack *et al.,* 1989, 1991).

With the availability of large number of human probes and the information regarding genetic homology, the pace of livestock gene mapping, particularly of bovine increased dramatically. Adkison *et al.* (1988b) for the first time used human probes for synteny identification in cattle. They analysed DNA from cattle-hamster and cattle-mouse somatic cell hybrids segregating bovine chromosomes by Southern blotting and hybridized with human fibronectin and gamma crystallin probes. Concordance of retention of these bovine genes (FN1 and CRYG) was compared to cattle isozyme loci representing previously described syntenic groups. FN1 and CRYG fragments were concordant with each other and with isocitrate dehydrogenase (IDH1) representing the bovine syntenic group U17. This allowed them to assign FN1 and CRY to U17.

Later, from studies using cattle-hamster and cattle-mouse hybrids many new members were added to the growing list of loci belonging to various syntenic groups by establishing the concordance of retention of genes or gene products with the marker for established syntenic groups (Hallerman *et al.*, 1988; Threadgill *et al.*, 1990; Arruga *et al.*, 1992; Dietz *et al.*, 1992a,b; Gallagher *et al.*, 1991, 1992a,b).

Several members of these syntenies were assigned to specific chromosome or regionally localized to the subchromosomal regions by *in situ* hybridization (Zneimer and Womack, 1989; Hediger *et al.*, 1990, Chowdhary *et al.*, 1991; Fries *et al.*, 1991; Gallagher *et al.*, 1993; Johnson *et al.*, 1993). This led to the assignment of these syntenies to the specific chromosomes, to which any single representative member of those syntenies were sublocalised.

The recent report on bovine gene map (Fries *et al.*, 1993) enlists 353 bovine loci belonging to 29 syntenic groups. However, the chromosomal assignment is lagging behind the syntenic identification. The report reveals only 19 syntenic groups assigned to the specific chromosomes. A vast majority of these assignments have been made from analysis of somatic cell hybrid panels, 269 out of 353.

The next well characterized species is sheep. The first report on sheep gene mapping appeared in 1981 from Saidi-Mehtar and his group. They identified four syntenic groups consisting of 10 ovine loci using sheep - hamster hybrids segregating sheep chromosomes. However, these syntenic groups were not assigned to any specific sheep chromosome. Meanwhile, a synteny was established among SHMT, LDHB and TPI using the auxotrophic markers by

Jones *et al.* (1985). The synteny was assigned to sheep chromosome M3. This was probably the first chromosomal assignment in sheep.

Sheep gene mapping was greatly benefitted by rapidly growing bovine gene map. With the availability of heterologous probes, several syntenies were identified using the sheep-hamster and sheep-mouse hybrid panels. Major contribution came from Saidi-Mehtar and his group (Imam-Ghali *et al.,* 1987; 1992; Saidi-Mehtar *et al.*, 1991).

Presently, there are only six genes regionally mapped on to the sheep chromosomes (Hediger *et al.*, 1990, 1991; Chowdhary *et al.*, 1991; Johnson *et al.*, 1993). Hence, equivalent number of syntenies are assigned to the specific sheep chromosomes. Recent sheep gene map consists of 50 genes belonging to 19 syntenic groups (O'Brien and Marshall-Graves, 1992).

2.7.2 Gene Mapping in Buffalo

Buffalo is one of the least explored species with respect to gene mapping studies. The first report on buffalo gene mapping appeared very recently. de Hondt *et al.* (1991), using buffalo-hamster hybrid cell panel, analysed the syntenic relationship among eight genes and proposed two syntenic groups GAPD-TPI1-LDHB and ME1-SOD2. Other three genes were found to be asyntenic. However, the first chromosomal assignment of buffalo genes was made by El-Nahas *et al.* (1993) who mapped GAPD-TPI1-LDHB synteny to buffalo chromosome.

Presently only six loci have been mapped to different river buffalo chromosomes using *in situ* hybridization (Iannuzzi *et al.,* 1993a,b,c, 1994a,b; Hassanane *et al.,* 1993), but since these loci marked the cattle syntenic groups U3, U4, U10, U18, U20, U29 and were assigned to homologous chromosomes of cattle, the entire syntenic groups consisting of approximately 133 loci are tentatively assigned to river buffalo chromosomes (Iannuzzi *et al.*, 1994a).

Recently, five more syntenic groups comprising 10 genes have been reported. These genes form the part of cattle syntenic groups U2, U10, U11, U22 and U24 (Othman *et al.,* 1994).

2.8 CHROMOSOME ANALYSIS

2.8.1 Karyotypic Characterization of Buffalo

Gustavsson and Rockborn (1964) reported first Robertsonian like translocation, 1;29 translocation in cattle which subsequently was found to be related to infertility. This stimulated interest in livestock cytogenetics and many laboratories were established worldwide for the study of chromosome disorders in domestic animals. Considerable effort was also devoted to the accumulation and application of knowledge of the normal and abnormal chromosomes of livestock. In early seventies, the application of banding methods on domestic animal chromosomes produced different systems of cytogenetic nomenclature of the various species that resulted in confusion and indicated a need for standardization. By 1976, trypsin G-banding (GTG) of chromosomes prepared in mid metaphase was achieved. This was presented at the Reading Conference with the objective to describe G-banding patterns in sufficient detail to permit the identification of individual chromosomes. This

led to the development of first internationally accepted nomenclature and karyotypes of these species (Ford *et al.,* 1980). Since then several other chromosome banding techniques were developed. The second conference for the standardization of domestic animal karyotypes was held in 1989 with the objective to update and standardize the karyotypes with different banding techniques (ISCNDA, 1989). Although, number of publications were available (Di Berardino and Iannuzzi, 1981, 1982, 1984; Bongso and Hilmi, 1982; Iannuzzi and Di Berardino, 1985; Thiagarajan, 1987; Basrur *et al.,* 1988), buffalo karyotype was not dealt with. Subsequent reports suggesing further improvement in buffalo chromosome characterization (Iannuzzi *et al.,* 1989, 1990a,b) encouraged to constitute, during 10th European Colloquium on Cytogenetics of Domestic Animals, an international committee for the standardization of river buffalo chromosomes (Iannuzzi, 1992). The report of the committee (Iannuzzi, 1994) established the standard banded karyotype of river buffalo. With the availability of standard karyotype, it is now possible to identify individual chromosomes unambiguously.

2.8.2 Karyotypic Characterization of RAG

Use of RAG in somatic cell hybridization as a fusion partner is not uncommon. However, the workers have seldom reported the RAG complement. A stray report (Chen, 1987) available on karyotypic characterization of RAG, describes GTG banded karyotype with modal number of chromosomes as 65/66. A few reports describe different than, but close to this number (Hashmi *et al.,* 1974; Joshi *et al.,* 1995).

2.8.3 Karyotypic Characterization of Hybrid Cells

In prebanding period, techniques like G-11 staining which differentially stain the parental chromosomes have been employed to assess the retention (and segregation) of donor chromosomes in human-hamster hybrids (Bruns *et al.,* 1978a,b). With the standardization of GTG banding, it is known that bovine and mouse chromosomes show differential banding expression at centromeric region. All bovid chromosomes show G-negative (Di Berardino *et al.,* 1991; Iannuzzi, 1994), while, the mouse chromosomes G-positive (Chen, 1987) band at centromeric region. Very recently, Joshi *et.al.* (1996) utilized this feature for identifying the parental origin of the chromosomes in buffalo-mouse hybrids.

Modern molecular cytogenetic techniques like chromosome painting (El-Nahta *et al.,* 1994) and FISH (Zijlstra *et al.,* 1994) have been employed for critical analysis of hybrid complement.

2.9 ISOZYME ANALYSIS

Isozymes (or isoenzymes) are multiple molecular forms of an enzyme occurring either in a single individual or in different members of same species. They catalyze the same reaction. Isozyme separation is essential to know the source of isozyme and thus the gene in the hybrid cell. Isozymes are extracted by cell lysis.

2.9.1 Cell Lysate Preparation

Enzymes can be liberated from the cells or tissues either by homogenization, sonication, freezing-thawing or combination of these. Siciliano and Shaw (1976) suggested washing of cells in isotonic solution followed by mechanical disruption by rigorous grinding in homogenizing medium. Homogenates were then cleared by centrifugation at 12,000 x g for 30 minutes. Harris and Hopkinson (1976) also recommended tissue homogenization in appropriate buffer followed by centrifugation at 20,000 x g for 60 minutes to produce clear supernatant.

Meera Khan (1971) recommended sonication of cells. The cells were washed thrice in isotonic saline before being sonicated in lysis buffer. The sonicate was mixed thoroughly with 0.1 ml of carbon tetrachloride and centrifuged at 27,000 x g for 30 minutes. Harris and Hopkinson (1976) suggested sonication for WBCs and cultured cells. The cells were washed thrice with 0.15 M (0.9%) NaCl and disrupted by the addition of equal volume of distilled water and finally sonicated in the same medium. The sonicate was directly used for electrophoresis or centrifuged at 20,000 x g for 60 minutes to produce a clear supernatant.

Successive freezing and thawing has been recommended by Harris and Hopkinson (1976) for lysis of RBCs. RBCs after separation on centrifugation were washed thrice with 0.15 M (0.9%) NaCl and lysed by freezing - thawing.

2.9.2 Buffer System

A variety of buffer systems have been employed for electrophoretic separation of isozymes. Lactate dehydrogenase (LDH) isozymes have been electrophoretically separated using phosphate-citrate (Meera Khan, 1971), phosphate (Harris and Hopkinson, 1976), tris-citrate (Siciliano and Shaw, 1976), versenate (Heuertz and Hors-Cayla, 1981), tris-maleate-EDTA (Dain *et al.,* 1984), citrate-phosphate (Womack and Moll, 1986) and tris-borate-EDTA (de Hondt *et al.,* 1991) buffer systems.

Peptidase B (PEPB) has been characterized using tris-versene-borate (Siciliano and Shaw, 1976), tris - phosphate (Harris and Hopkinson, 1976) and tris-citrate (Womack and Moll, 1986) buffer systems.

Triose phosphate isomerase (TPI) isozymes have been resolved employing tris-EDTA (Harris and Hopkinson, 1976), phosphate (Womack and Moll, 1986), and tris-borate-EDTA (de Hondt *et al.,* 1991) buffer systems.

2.9.3 Histochemical Staining

Since its first demonstration by Hunter and Markert in 1957, histochemical staining protocol for large number of enzymes has been developed and used for visualization of the enzyme on the gel. The visual display of the enzyme on the gel is termed as zymogram. Meera Khan (1971) developed recipe and protocol for histochemical staining for visualization of several enzymes on cellulose acetate gel. Siciliano and Shaw (1976) have described the staining procedure for number of enzymes on starch gel. Most

exhaustive information comes from Harris and Hopkinson (1976) who have given huge compilation of histochemical staining procedures and electrophoretic protocols for large number of enzymes. Although these protocols differ among each other, they utilize essentially the same co-enzyme system for visualization of specific enzyme.

The zymogram patterns of LDH in the somatic cell hybrids were studied by Meera Khan (1971), Chen *et al.* (1973), Heuertz and Hors-Cayla (1981), Dain *et al.* (1984) Forster and Hecht (1986), de Hondt *et al.* (1991) Monteagudo *et al.* (1991), Saidi-Mehtar *et al.* (1991) and El-Nahas *et al.* (1993) using the specific histochemical staining.

The resolution and visualization of isozymes by histochemical staining for PEPB in somatic cell hybrids was described by Heuertz and Hors-Cayla (1981), Saidi-Mehtar *et al.* (1981), Ryttman *et al.* (1986) and Womack and Moll (1986).

Histochemical staining for visualization of TPI isozymes in somatic cell hybrids has been described by Heuertz and Hors-Cayla (1981), Saidi-Mehtar *et al.* (1981), Womack and Moll (1986) and de Hondt *et al.* (1991).

2.9.4 Zymogram Evaluation

Zymogram patterns of LDHB, PEPB and TPI have been studied in human, animals and somatic cell hybrids by several workers. Number and intensity of bands depended on the type of tissues used as source of these enzymes. Nabholz *et al.* (1969) have described four anodal bands of LDH in a

human cell line, Hela and five anodal bands in a mouse cell line. Human isozymes had higher mobility than mouse isozymes and migrated more anodal to the mouse isozymes. Meera Khan (1971) observed two anodal and a cathodal band of LDH in human, only one anodal band in Chinese hamster and three to eight bands in human - Chinese hamster hybrids. Human LDH was electrophoretically indistinguishable to mouse and Chinese hamster LDH. Chen *et al.* (1973) also observed identical electrophoretic mobility of LDH isozyme in human and mouse. Dain *et al.* (1984) reported five isozyme bands of LDH in cattle, four anodal and a cathodal and three anodal bands in mouse cell line, NSO but electrophoretically slower than in cattle, while the hybridomas displayed variable number of bands. Heuertz and Hors-Cayla (1981) observed zymogram pattern of LDH in cattle-hamster hybrids similar to that in human hamster hybrids. Saidi-Mehtar *et al.* (1981) studied the zymogram patterns of LDH in sheep, Chinese hamster cell line, Wg3h and their somatic cell hybrids. Sheep fibroblasts exhibited five anodal bands, Wg3h cell line only one anodal band and the hybrid clones one to six anodal bands. Forster and Hecht (1986) reported five anodal bands both in pig and mouse but with differential mobility. Ryttman *et al.* (1986) have described five anodal bands in pig lymphocytes, two anodal bands in RAG cell line and two to four anodal bands in pig-mouse hybrids. de Hondt *et al.* (1991) studied the zymogram patterns of LDH in buffalo-hamster hybrids. They observed four anodal and a cathodal bands in buffalo, one anodal band in Chinese hamster cell line, Wg3h and one to ten bands in their hybrids.

Zymogram patterns of PEPB in cattle and Chinese hamster have been studied by Heuertz and Hors-Cayla (1981). Both the species showed one anodal

band, but with differential electrophoretic migration. Saidi-Mehtar *et al.* (1981) also found one anodal band, both in sheep and Chinese hamster. However, Sheep PEPB band was more anodal. Pig and mouse cell line RAG also expressed one anodal band, mouse one being slower and less anodal (Saidi-Mehtar *et al.*, 1981) while, man and Chinese hamster PEPB were found to be identical in electrophoretic mobility (Chen *et al.,* 1973).

TPI was evaluated electrophoretically in cattle and Wg3h cell line by Heuertz and Hors-Cayla (1981). Both the species displayed one anodal band. However, bovine TPI was more anodal. Saidi-Mehtar *et al.* (1981) observed a cathodal band in sheep and an anodal band in Chinese hamster cell line, Wg3h.

2.10 SYNTENIC IDENTIFICATION AND CHROMOSOMAL LOCALIZATION OF LDHB, PEPB AND TPI

In human synteny among LDHB, PEPB and TPI was reported by Chen *et al.* (1973). The synteny was confirmed later and assigned to human chromosome 12 (HSA 12) (Jongsma *et al.* 1973, 1974). Kao *et al.* (1976) confirmed the assignment of this synteny.

In bovine, Heuertz and Hors-Cayla (1981) established the synteny among these genes using cattle - hamster hybrids. This was the first report on syntenic identification of autosomal genes in cattle. Dain *et al.* (1984) confirmed this synteny and reported the chromosomal localization of LDHB on bovine chromosome 19 (BTA 19). Womack and Moll (1986) tested the panel of cattle - hamster hybrid clones and organized the 28 enzyme loci into 21

syntenic groups, LDHB - PEPB - TPI constituted syntenic group U3. U3 was later assigned to bovine chromosome 5 (Fries *et al.,* 1991), correcting the erratic assignment made by Dain and his colleagues. Earlier the synteny between LDHB and TPI had also been reported by Womack and Cummins (1984).

These genes were also found to be syntenic in sheep. The synteny was reported by Saidi-Mehtar *et al.* (1981) using sheep-hamster hybrids. Jones *et al.* (1985) confirmed this synteny using auxotrophic marker in sheep-hamster hybrid panel and assigned to ovine chromosome 3.

The synteny among these genes has also been found to be conserved in buffalo. de Hondt *et al.* (1991) reported the synteny between LDHB and TPI in Egyptian buffalo using buffalo-hamster hybrids. This was the first report on gene mapping in buffalo. The synteny was later assigned to buffalo chromosome 4 (El-Nahas *et al.*, 1993).

In swine, initially, the synteny between LDHB and PEPB was not observed, instead these were found to be localized on different chromosomes, 5 and 11 respectively (Forster and Hecht, 1984, 1986). However, the synteny was established by Ryttman *et al.* (1986) and correct localization was done on chromosome 5.

These gene loci are also found to be syntenic in several species of monkeys, cat and minks (Lalley and McKusick, 1985). However, these genes are localized on different chromosomes in rabbit (Echard *et al.*, 1982). Asynteny is also reported in mouse (Lalley and McKusick, 1985).

Materials and Methods

MATERIALS AND METHODS

In vitro culture of cells was an important component of present study, hence strict asepsis was maintained throughout its entire laboratory procedure. Various labware used were either presterilized or prepared and sterilized as per the standard protocols. Triple glass distilled water was used to reconstitute reagents and solutions. The chemicals and media used were of ExcelaR grade from Qualigens or BDH unless specifically mentioned.

3.1 PREPARATION OF MEDIA AND SOLUTIONS

3.1.1 Dulbecco's Modified Eagle's Medium (DMEM)

The medium was reconstituted as per the instruction of the manufacturer. The content of one vial (13.40g) of powdered DMEM (Sigma, D-6655) was dissolved in approximately 900ml of distilled water. Sodium bicarbonate 3.7 g and L-glutamine (Sigma G-5763) 570 mg were added and the pH of the medium was adjusted to 7.0 - 7.2 by 1N HCl, about 0.2 unit less than desired. The final volume of one litre was made up by adding little more distilled water. The medium was then filtered through 0.22 µm pore sized cellulose acetate or cellulose nitrate membrane (Sartorius). Upon filtration the pH usually rises by approximately 0.2 units to attain 7.2 - 7.4. The medium was distributed in storage bottles and kept at 4°C until used. Sterility of medium was checked by incubating an aliquot of medium for 48 hours.

3.1.2 Rosewel Park Memorial Institute 1640 (RPMI-1640) Medium

The medium was reconstituted as per the procedure followed for DMEM by dissolving the content of one vial (10.40g) of powdered RPMI-1640 (Sigma, R-6504), 2.0 g sodium bicarbonate and 200 mg L-glutamine in one litre distilled water and adjusting the pH with 1N HCl. The medium was membrane filtered and kept in storage bottles at 4°C. Sterility of the medium was checked by incubating an aliquot of the medium at 37°C for 48 hrs.

Antibiotic antimycotic (100X) (Sigma, A-4668) was included in both the media in the initial experiments but excluded in subsequent experiments as it was not found to be obligatory. HEPES was also not incorporated.

3.1.3 Hypoxanthine, Aminopterin, Thymidine (HAT) Medium

HAT medium was used to selectively culture hybrid cells. HAT medium was reconstituted by dissolving the contents of one vial of hypoxanthine - thymidine (HT, 50X) (Sigma, H-0137) and one vial of aminopterin (50X) (Sigma, A-5159) each in 10 ml distilled water, filtered through 0.22 µm disposable filter (Minisart, Sartorius) and mixed in 480 ml of DMEM. The final concentration achieved was hypoxanthine $1x10^{-4}$ M, aminopterin $4X10^{-7}$ M and thymidine 1.6×10^{-5} M.

3.1.4 8-Azaguanine Medium

8-Azaguanine medium was used to counter-select the hypoxanthine guanine phosphoribosyl transferase deficient ($HPRT^-$) cells. The medium was reconstituted by dissolving the contents of one vial of 8-azaguanine (50X)

(Sigma, A-5284) in 10 ml of distilled water, filtered through Minisart and mixed in 490ml of DMEM. The final concentration of 8-azaguanine derived was 1.32×10^{-4} M.

3.1.5 Trypsin - Versene - Glucose (TVG) Solution

TVG solution was prepared using the following ingredients.

Trypsin (Difco, 0152-13-1 or Sigma, T-0646)	-	100 mg
Sodium EDTA (Sigma, ED2SS)	-	20 mg
D-Glucose	-	50 mg
Sodium chloride	-	800 mg
Potassium chloride	-	20 mg
Disodium hydrogen orthophosphate	-	190 mg
Sodium dihydrogen orthophosphate	-	20 mg
Sodium bicarbonate	-	58 mg

The above substances were dissolved in 100ml of distilled water, filtered through Minisart and stored at 4°C.

3.1.6 Polyethylene Glycol (PEG) Solution

PEG - 1450 (MW 1300-1600, av. MW 1450) (Sigma, P-7777) and PEG-3350 (MW 3000-3700, av. MW 3350) (Sigma, P--2906) were used to reconstitute fusogen. Autoclaved, five g PEG in warm liquified state was mixed with five ml DMEM to prepare 50% w/v PEG solution. Similarly, 4.5 g PEG was mixed with one ml of dimethyl sulfoxide (DMSO) (Sigma, D-2650) and 4.5 ml DMEM to prepare 45% PEG/10% DMSO solution. The pH of 7.2-7.4 was achieved

using 1N NaOH. The desirable pH was judged by slight purple colour of the solution. The PEG solution, was prepared just prior to use.

3.1.7 Colchicine Solution

Stock solution (0.1%) was prepared by dissolving 10 mg colchicine (Sigma, C-3915) powder in 10 ml distilled water. Working solution (0.001%) was prepared by mixing 100 µl of stock solution in 10 ml distilled water. Both solutions were membrane filtered and stored at 4°C.

3.1.8 Phosphate Buffered Saline (PBS)

PBS was prepared by dissolving 9.55g of Dulbecco's PBS (Ca^{++}, Mg^{++} free) (HiMedia) powder in one litre of distilled water. The solution was autoclaved and stored in storage bottles at 4°C.

3.1.9 Phosphate Buffer

Solution A : 0.2 M Sodium dihydrogen orthophosphate solution was prepared by dissolving 3.12 g of NaH_2PO_4, $2H_2O$ in 100 ml of distilled water and stored at 4°C.

Solution B : 0.2 M disodium hydrogen orthophosphate solution was prepared by dissolving 2.84 g of Na_2HPO_4 in 100 ml of distilled water and stored at 4°C.

Working solution was prepared by mixing 51ml of solution-A and 49 ml of solution-B to give a pH 6.8.

3.1.10 RBC Lysing Buffer

RBC lysing buffer was prepared by dissolving 840mg of ammonium chloride in 100 ml of 0.01M tris-HCl buffer, pH 7.5. The solution was membrane filtered and stored at room temperature until used.

Depending on availability, ready to use RBC lysing buffer (Sigma, R-7757) was employed.

3.1.11 Cell Lysis Buffer

Cell lysis buffer was prepared as suggested by Meera Khan (1971) with some modifications by dissolving 37.2 mg sodium EDTA, 7.8 mg ß-mercaptoethanol (Sigma, M-7522) and 1.5 mg NADP (Boehringer, 236659) in 100 ml phosphate buffer to attain the final concentrations as follow :

5×10^{-3} M phosphate buffer, pH 6.4

1×10^{-3} M sodium EDTA

1×10^{-3} M ß-mercaptoethanol and

2×10^{-5} M NADP

3.1.12 Giemsa Stain

Stock solution was prepared by dissolving one g of Giemsa (Merck) powder in 54 ml of glycerol by grinding in a mortar with pestle and adding warm glycerol slowly. The solution was cooled, mixed in 84 ml of methanol, filtered through Whatman paper and stored at 4°C.

Working solutions (4% and 10%) were prepared by diluting stock solution with phosphate buffer. Working solution was prepared freshly every time.

3.2 SETTING UP LEUCOCYTE CULTURE

Leucocytes separated from buffalo blood were cultured in RPMI-1640 medium for chromosome harvest. Approximately 10 ml blood was collected aseptically from jugular vein in a sterile heparinized centrifuge tube. Blood was allowed to stand for 30 minutes. Plasma fraction rich in leucocytes was withdrawn and inoculated directly in the culture medium. Alternatively, this plasma fraction was centrifuged at 700-800 rpm for 10 minutes, clear plasma discarded and cell sediment suspended slowly in five ml RBC lysing buffer and allowed to stand for about 20 minutes at room temperature. Leucocytes, after centrifugation were washed twice in five ml PBS or culture medium and finally suspended in 10 ml culture medium. Occasionally, lymphocytes were separated over Histopaque (Sigma, 1077-1). For this, freshly collected blood diluted with PBS was layered over equal volume of Histopaque in a centrifuge tube and centrifuged at 1500 rpm for 15-20 minutes. The interface between Histopaque and plasma, which contains only lymphocytes was withdrawn carefully and washed twice with PBS or culture medium and finally suspended in 10ml medium.

Cultures were set by inoculating one ml of leucocyte/lymphocyte suspension in eight ml of RPMI-1640. The cultures were supplemented with one ml of foetal bovine serum (FBS) (Sigma F-4010 or Gibco, 16140) and 0.2 ml (0.2 mg) pokeweed mitogen (PWM) (Sigma, L-9379 or Gibco, 670-5360 AC).

Cultures were incubated at 37°C in CO_2 incubator (Nuaire) under five per cent CO_2 tension and shaken periodically. Cultures were harvested after 72 hours for chromosome preparations.

3.3 CULTURE OF RAG CELL LINE

RAG, mouse renal adenocarcinoma cell line was obtained from National Facility for Animal Tissue and Cell Culture (NFATCC), Pune. The cell line was cultured and maintained in DMEM supplemented with 10 per cent FBS in 25-sq.cm tissue culture flasks (TC flasks, Nunc) under five per cent CO_2 tension. On confluence, the culture medium was discarded, the monolayer washed with fresh prewarmed medium and trypsinized with TVG. For trypsinization the monolayer was treated with two ml prewarmed TVG and monitored under inverted microscope (Meopta). Once the cells showed rounding off, TVG was discarded and TC flask incubated for two to five minutes. The monolayer was then flushed with two ml fresh culture medium and the cell suspension distributed among three or four TC flasks. Fresh 4.5 ml culture medium was added in each TC flask and supplemented with 10 per cent FBS. Cultures were refed on second or third day and passaged on fourth or fifth day on monolayer formation.

RAG, an HPRT deficient mutant cell line isolated from a mouse transplantable renal adenocarcinoma is non-revertant to wild type (ATCC, 1988) but it was, in any case propagated for three to four passages in 8-azaguanine medium which is lethal to wild type cells, to select against the revertants, which might at all have generated at very low frequency.

3.4 SOMATIC CELL HYBRIDIZATION

Somatic cell hybridization between buffalo leucocytes and RAG cells was mediated using PEG as fusogen. Fusion was carried out using following techniques:

3.4.1 Pancake Fusion

The technique derived by O'Malley and Davidson (1977) with modification (Womack and Moll, 1986) was followed. In brief, freshly separated leucocytes and RAG cells trypsinised at log phase of growth were washed thrice in DMEM. Five ml of cell suspension carrying both the parent cells in appropriate ratio were centrifuged at 2000 x g for 20 minutes in a 50 ml capacity centrifuge tube (Corning) fitted at bottom with a number 4 autoclavable rubber stopper and a round coverslip (25 mm dia) over it. The supernatant was aspirated off and the rubber stopper with coverslip was pushed to the top by long needle heated at top pierced from the bottom of the centrifuge tube. The coverslip was lifted with the help of forceps and dipped in prewarmed PEG solution followed by two successive dips in the culture medium.

3.4.2 Suspension Fusion

The technique developed by Davidson and Gerald (1976) was followed. Freshly separated leucocytes and RAG cells at log phase of growth were washed twice with DMEM and mixed and suspended in desired ratio in DMEM. The parent cells were pelleted in round bottom test tube (Nunc), the

medium completely removed and the cells suspended in one ml PEG solution prewarmed at 37°C. PEG solution was poured drop by drop and the cells were mixed gently by continuous agitation. After specific period of exposure, cells were diluted rapidly in 10 ml DMEM and washed twice. The cells were again washed once with growth medium and plated in 96-well microtitre plates (Nunc).

3.5 HYBRID CLONES

3.5.1 Selection and Cloning of Hybrid Cells

PEG treated cells were plated by multichannel pipette (Brinkmann) in 96-well microtitre plate @ 200µl cell suspension per well. The plates were sealed with adhesive tape after swabbing all sides by denatured spirit. Wells were renewed with fresh HAT medium supplemented with 10 per cent FBS on every fourth day. Growth of hybrid cells in terms of expanding colonies was recorded by screening the wells under inverted microscope. The wells carrying single colony were trypsinized on confluence and the cells transferred to 24-well plates (Nunc). The wells were inoculated with one ml of fresh HAT medium supplemented with 10 per cent FBS. The wells which grew confluent within 16 days of inoculation were only transferred, however, other wells were screened for growth of colonies for maximum of upto four weeks. The hybrid cells in 24-well plates were refed every third day and on confluence, these were trypsinized and transferred to 6-well plate (Nunc). Finally these were expanded to TC flasks. Hybrid clones from each well of 96-well microtitre plate were expanded independently. Twelve fast growing clones from first hybridization trial were selected for karyotypic and isozymic analysis.

3.5.2 Maintenance of Hybrid Clones

The hybrid clones after successful isolation in HAT medium were maintained on growth medium at 37°C under five per cent CO_2 tension. On monolayer formation, usually four to five days after seeding, the clones were subcultured in 1:4 split ratio and refed every third day after each passaging. Three clones (G_3, D_{12} and A_9) were propagated upto 20-22 passages, the other three (C_3, B_{10} and A_8) upto five - six passages and the remaining six (C_5, E_2, E_9, E_{11}, F_6 and H_4) only once. Utmost care was taken to avoid any cross contamination among clones and cell lines.

3.6 FREEZING AND THAWING OF CELLS

RAG and hybrid clones were frozen periodically. For this, the cells were disaggregated from the substrate by trypsinization, washed with culture medium and suspended in freezing medium. Freezing medium consisted of 80% DMEM, 10% FBS and 10% DMSO. The cell concentration was adjusted to 5-10x10^5 cells/ml of freezing medium and transferred to 1.8 ml cryovials (Nunc). Usually three cryovials could be prepared from the harvest of one TC flask. The cryovials were capped tightly, labelled and frozen immediately using 10-16 * MR-3 * Controlled Rate Freezer (Planner Biomed) at following cooling rate:

From ambient to - 40°C @ -1°C/minute

From -40°C to -90°C @ -10°C/minute

The cryovials were held at -90°C for 10 minutes and then plunged into liquid nitrogen (LN_2).

On requirement, the frozen cells were revived by quick thawing at 37°C in waterbath. The cell suspension was transferred to TC flask and diluted slowly with growth medium and incubated at 37°C in CO_2 incubator. The medium was renewed the following day.

3.7 CHROMOSOME ANALYSIS

3.7.1 Chromosome Preparation

Buffalo leucocyte cultures were harvested 72 hours after setting the culture. One to three hours prior to harvest, colchicine was added in the culture at a rate of 0.01-0.02 µg/ml to arrest the mitotic cells at metaphase. The culture was centrifuged and supernatant discarded. The cell sediment was suspended in about 0.1 ml residual medium by tapping the bottom of centrifuge tube and exposed to hypotonic by diluting in five ml 0.075 M (0.56%) prewarmed potassium chloride and incubated for 15-20 minutes. One ml Carnoy's fixative (methanol-acetic acid 3:1) was then added in the hypotonic, mixed and centrifuged immediately. Supernatant was discarded and the cells were fixed in same fixative for one hour at 4°C. Fixative was then renewed and subsequent two washes were given after overnight refrigeration. After final wash, the cells were resuspended in one ml fixative and the suspension dropped onto the prechilled clean glass slides (Special Super Deluxe, Blue Star). The slides were air dried for further analysis.

RAG and hybrid cells were harvested in log phase of growth, usually 36-48 hours after subculturing. Colchicine was added at the rate of 0.01-0.02 µg/ml for one to three hours. Medium from TC flask was discarded and cell monolayer was washed with two ml fresh medium and the cells were

trypsinized. Cells were exposed to hypotonic for 15-20 minutes and given three washes with fixative in the same way as for leucocytes. The cell suspension was dropped onto the prechilled glass slides, the slides were air dried.

3.7.2 Chromosome Banding

Initially a few slides were prepared from the cell suspension and stained with 10% Giemsa in phosphate buffer (pH 6.8) for 10 minutes. Slides were then rinsed with distilled water, dried and screened under microscope (Carl-Zeiss Photomicroscope-III). If the slides were found to be suitable for banding, additional slides were prepared and GTG banded.

For GTG banding, slides either freshly prepared or stored at -20°C, were aged for 3-10 days at room temperature and banded as per the method developed by Seabright (1971) with modification. The slides were immersed in couplin jar containing chilled TVG and exposed for 45-75 seconds. The slides were then quickly washed under running tap water followed by rinsing with distilled water. The slides after drying were stained with four per cent Giemsa in phosphate buffer (pH 6.8) for 7-10 minutes, and screened under microscope. Good preparations were photographed.

3.7.3 Chromosome Evaluation

Hybrid clones were evaluated for presence of buffalo chromosomes. Buffalo chromosomes were identified in hybrid complement by their banding pattern. Particular buffalo chromosome was considered 'present', if observed in atleast 20 per cent cells.

3.8 ISOZYME ANALYSIS

3.8.1 Preparation of Cell Lysates

About 10^6 - 10^7 RAG and hybrid cells and about 10^8 - 10^9 freshly separated leucocytes were suspended in the culture medium, washed thrice with isotonic saline and suspended in 0.15 ml lysis buffer. The cells in lysis buffer were transferred in 1.8 ml cryovials (Nunc), labelled and stored in LN_2 until used. Whenever required, the cells were removed from LN_2 and subjected to three cycles of freezing-thawing (-196°C to 5°C). The cell lysates extracted were cleared by centrifugation at 12,000 x g for 30 minutes at 4°C in a high speed refrigerated centrifuge (Hettich). Clear supernatant was concentrated using Centrisart (Sartorius) by centrifuging at 2500 x g for five minutes at 4°C.

3.8.2 Preparation of Cellulose Acetate Gel

Cellulose acetate gels (Helena) were used for electrophoresis. These are precast gel plates available in ready-to-use form. The gel plates were cut in 14"x9" size and soaked in appropriate electrophoresis buffer for 10 minutes and blotted between two sheets of fine grained filter paper or tissue paper. Care had been exercised not to allow the formation of white patches in the gel, due to drying, throughout the entire procedure.

3.8.3 Electrophoresis

An electrophoresis apparatus (Biotech) with shoulder gap of nine cm was used for isozyme separation. The buffer impregnated gel was blotted and positioned in the tank with its penetrable surface facing upward and

connected with buffer using filter wicks. The initial voltage was set to 200 V and the system was equilibrated for 10 minutes.

The current was broken and one to two µl aliquot of each sample was applied with a variable volume micropipette, (Labsystems) using gel loading tips at about two cm away from and parallel to the cathodic shoulder. On complete adsorption of samples applied on the gel, the current was resumed and the run was allowed for specific period.

3.8.4 Enzyme Specific Staining

A required amount of appropriate reaction mixture was freshly prepared at the end of each run. The current was broken, the starting end was marked and the porous side of the gel was brought into contact with the reaction mixture spread on a glass plate for about 30 seconds. The gel was blotted and incubated on a clean dry glass plate in a moist chamber (CO_2 incubator). Once the bands had developed satisfactorily, the reaction was stopped by immersing the gel in 40% formaldehyde for five minutes. The gel was washed under tap water and preserved in distilled water at 4°C until photographed.

The specific stain recipe and buffer system employed for the visualization of each enzyme were prepared as given below :

3.8.4.1 Lactate Dehydrogenase (LDH) EC 1.1.1.27

The staining mixture was prepared as suggested by Meera Khan (1971) with some modifications by mixing following ingredients in order:

(i)	Tris-HCl, pH 8.6 Sodium EDTA	1.0 M 0.004 M	1.0 ml
(ii)	Sodium lactate (Sigma, L-1375)	0.4 M	1.0 ml
(iii)	NAD (ß-Nicotinamide adenine dinucleotide, free acid, Boehringer, 621 650)	10.0 mg/ml	0.4 ml
(iv)	MTT (3- 4,5 - dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide, Sigma, M-2128)	2.0 mg/ml	0.4 ml
(v)	PMS (Phenazine methosulfate, Sigma, P-9625)	0.4 mg/ml	0.4 ml

Buffer : 0.01 M phosphate citrate buffer, pH 7.0

Na_2HPO_4 2.42 g dissolved in one litre distilled water. pH 7.0 was adjusted with five per cent citric acid.

Electrophoresis run time : 45 minutes

3.8.4.2 Peptidase B (PEPB) EC 3.4.11 or 13

The staining mixture was prepared as described by Siciliano and Shaw (1976) with some modification by dissolving the following ingredients:

(i)	Tris-Hcl, pH 8.0,	0.2 M	2.5 ml
(ii)	Distilled water		2.5 ml
(iii)	Magnesium chloride		5.0 mg
(iv)	O-Dianisidine dihydrochloride (Sigma, D-3252)		5.0 mg
(v)	Peroxidase (Sigma, P-8375)		150 units

(vi)	Snake venom (*Crotalus adamanteus*) (Sigma, V-6875)	1.0 mg
	The substrate was added after complete dissolution	
(vii)	L-Leucyl-l-glycyl-glycine (Sigma, L-9750)	8.0 mg

Buffer: 0.5 M tris/0.016 M versene/0.65 M borate, pH 8.0.

Tris 60.0 g, boric acid 40.0 g and sodium EDTA 6.0 g dissolved in one litre distilled water. pH was adjusted with concentrated HCl.

Electrophoresis run time: 45 minutes.

Reaction mixture was applied to gel and incubated overnight.

3.8.4.3 Triose Phosphate Isomerase (TPI) EC 5.3.1.1

The reaction mixture was prepared by modification of Harris and Hopkinson (1976)'s recipe by mixing following ingredients in order:

(i)	Tris-HCl pH 8.0 adjusted with 1:1 HCl	0.02 M	20 ml
(ii)	α-Glycerophosphate (Sigma, G-2138)		650 mg
(iii)	Sodium pyruvate (Sigma, P-2256)		220 mg
(iv)	NAD (Boehringer, 621 650)		20 mg
(v)	α-Glycerophosphate-dehydrogenase (Sigma,P-2256)	80 units/ml	20 µl
(vi)	Lactate dehydrogenase (SRL,124045)	2750 units/ml	20 µl

The solution was incubated at 37°C for two hours. The reaction was stopped by drop wise addition of concentrated HCl until pH 2.0 was attained. The pH was immediately readjusted to 8.0 with concentrated NaOH

(vii)	NAD (Boehringer, 621 650)		20 mg
(viii)	Glyceraldehyde -3-phosphate dehydrogenase (Sigma, G-9013)	800 units/ml	50 µl
(ix)	Sodium arsenate (Sigma, A-6756)		50 mg
(x)	MTT (Sigma, M-2128)	5 mg/ml	1.2 ml
(xi)	PMs (Sigma, P-9625)	5 mg/ml	100 µl

Buffer: 0.11 M Tris/0.04M EDTA was used as buffer.

Tris 13.32 g and sodium EDTA 13.32 g dissolved in one litre distilled water, pH adjusted with 1 N HCl

Electrophoresis run time : 60 minutes

3.8.5 Isozyme Evaluation

Cell lysates from parents and hybrid clones were subjected to electrophoresis simultaneously and stained for visualization of specific enzyme. Particular hybrid clone was judged 'positive' for the buffalo isozyme, if it exhibited all the bands corresponding to those in buffalo cell lysate.

3.9 STATISTICAL ANALYSIS

Statistical analysis was carried out as per the standard procedure laid down by Snedecor and Cochran (1980).

Results

RESULTS

4.1 SOMATIC CELL HYBRIDIZATION

Somatic cell hybridization between buffalo leucocytes and mouse adenocarcinoma was attempted by following two techniques:

4.1.1 Pancake Fusion

Two grades of PEG (PEG-1450 and PEG-3350) at 50 and 45 per cent (45% PEG/10% DMSO) concentration were tried over a range of parental cell concentrations to isolate the hybrid cells. However, all the attempts failed and no hybrid cell colony could be obtained.

4.1.2 Suspension Fusion

Hybridization between buffalo leucocytes and RAG was successfully mediated using PEG by suspension technique.

While attempting hybridization following parameters were studied:

4.1.2.1 Effect of Duration of PEG Exposure

Initially attempts were made to identify the optimum duration of PEG exposure. Parental cells in 5 x 10^6 concentration from either species were exposed to 50 per cent PEG-3350 for variable time. Effect of duration of PEG

exposure was evaluated in terms of number of wells showing growing hybrid cell colonies and total number of colonies in 96-well microtitre plate.

The hybrid cell colonies started appearing between day-4 and day-7, and continued to increase till day-28. However, majority of colonies appeared by day-16 and there was only a marginal increase in the number of hybrid cell colonies after day-22. Expanding hybrid cell colony is shown in plates 4.1, 4.2, and 4.3.

Separate identity of individual colonies could not be maintained beyond day-16 as the expanding colonies coalesced. Hence, only number of wells showing the hybrid cell colonies were recorded beyond day-16. In some wells, colonies coalesced before day-16. In such cases, number of colonies observed on preceding count were considered to be the count on day-16. Results are presented in table 4.1.

Table 4.1: Effect of duration of PEG exposure to parental cells on hybridization frequency

Exposure time	Number of wells showing hybrid cell growth						
	Day 4	Day 7	Day 10	Day 13	Day 16	Day 22	Day 28
30 Sec	0 (0)	1 (1)	3 (3)	4 (4)	6 (6)	6	6
60 Sec	1 (1)	8 (10)	19 (28)	30 (44)	40 (65)	41	41
90 Sec	0 (0)	9 (10)	32 (45)	45 (86)	64 (122)	67	68
120 Sec	0 (0)	8 (8)	14 (15)	18 (20)	21 (24)	22	22

Figures in parentheses indicate the total number of hybrid cell colonies.

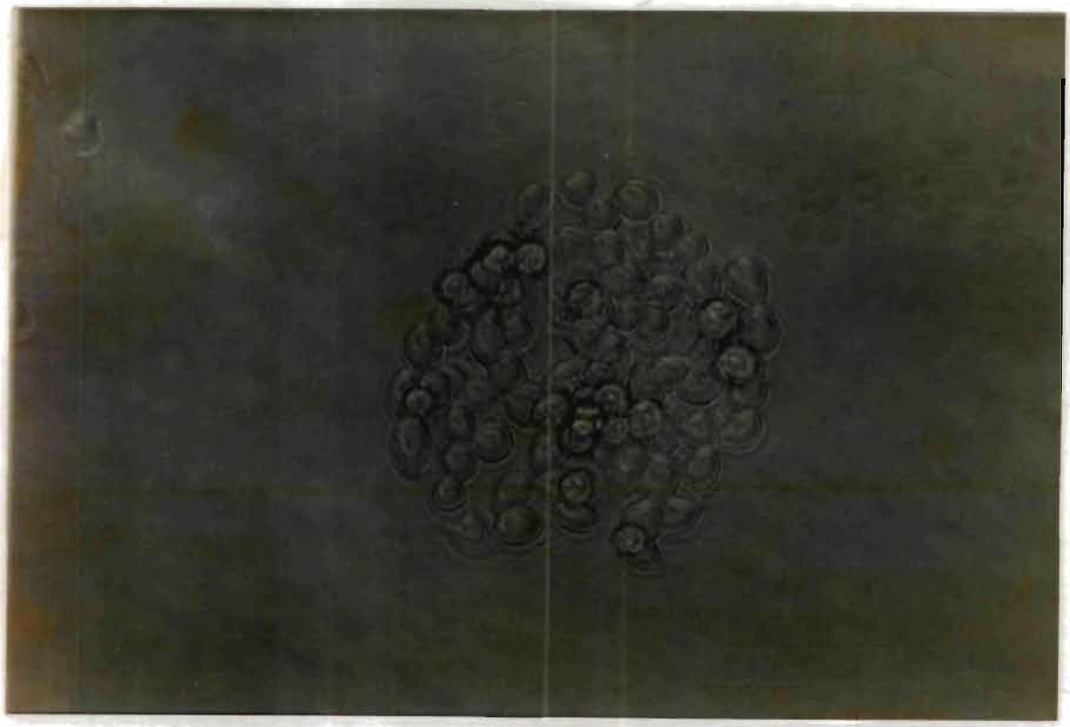


Plate 4.1 Hybrid cell colony on day-7 (100 X)

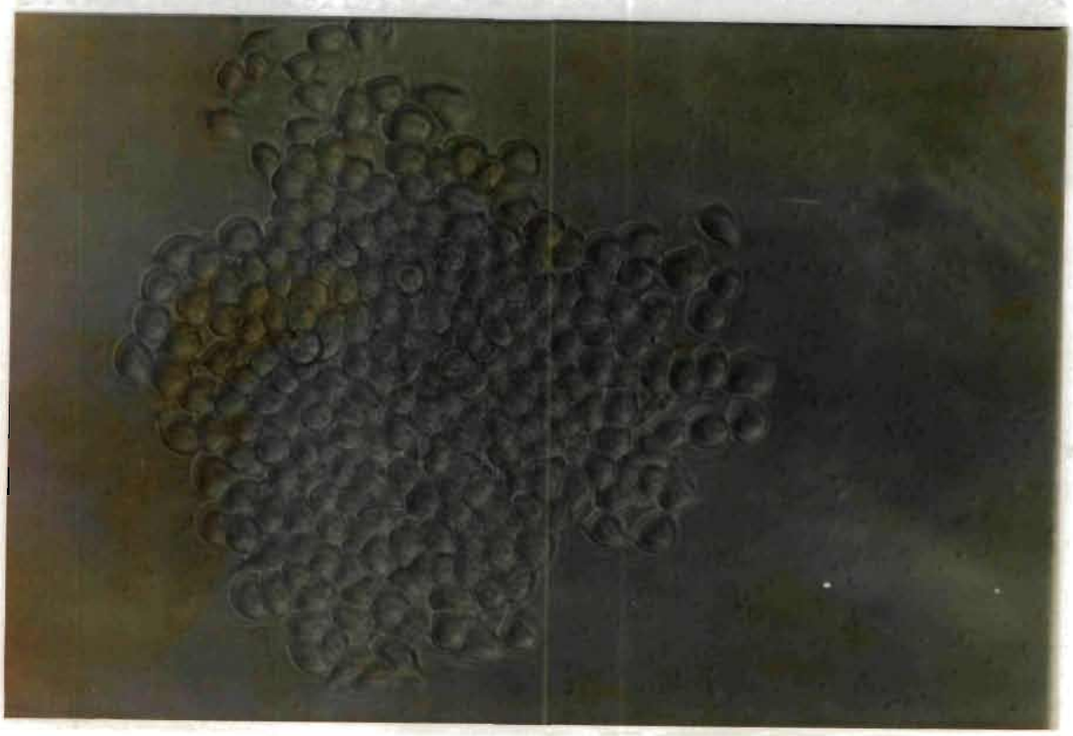


Plate 4.2 Hybrid cell colony on day-10 (100 X)

Plate 4.3 Hybrid cell colony on day-13 (100 X)

Very few colonies appeared on PEG exposure for 30 seconds. Maximum colonies (122) as well as wells showing the hybrid cell colonies (64) were observed on PEG exposure for 90 seconds, while PEG exposure for 60 or 120 seconds gave intermediate output.

4.1.2.2 Effect of Concentration and Grade of PEG

The optimum duration of PEG exposure, having been established, efforts were made to determine the optimum concentration and grade of PEG to maximize the hybridization frequency. Parental cells at 5 x 10^6 density were exposed to PEG for 90 seconds. Two grades, PEG-3350 and PEG-1450 at 45 per cent (45% PEG/10% DMSO) and 50 per cent concentration were evaluated. Hybridization frequency was scored in terms of number of wells showing the hybrid cell colonies on day - 10 and day - 16. Results are presented in table 4.2.

Table 4.2: Effect of PEG concentration and grade on hybridization frequency

PEG Concentration/ Grade	No. of wells with expanding hybrid cell colonies					
	50% PEG		45% PEG/10% DMSO		Mean	
	Day 10	Day 16	Day 10	Day 16	Day 10	Day 16
PEG-3350	33	46	41	56	37	51
PEG-1450	47	64	43	60	45	62
Mean	40	55	42	58		

Hybrid cell colonies obtained at 45 per cent PEG concentration were higher than obtained at 50 per cent. Also, the output of hybrid cell colonies

was higher at PEG-1450 than at PEG-3350. Among the four concentration - grade combinations, PEG-1450 at 50 per cent concentration gave the highest hybridization frequency.

4.1.2.3 Effect of Parent Cell Density

Parental cells in equal proportion were treated with PEG - 1450 at 50 per cent concentration for 90 seconds and these PEG-treated cells were dispensed either in one or five 96-well microtitre plates. Hybridization frequency was judged in terms of number of wells carrying hybrid cell colonies on day - 10. Results are presented in table 4.3.

Table 4.3: Effect of cell density on growth of hybrid cell colonies

Cell population per well	No. of wells showing hybrid cell colonies on day - 10
2×10^4	0, 2, 1, 0, 4 (1.4)*
1×10^5	66
4×10^5	28, 12, 40, 31, 34 (27)*
2×10^6	12

* Average of five microtitre plates

Hybrid cell colonies were observed in maximum number of wells when the cells inoculated at 1×10^5 per well seeding rate, and in least number of wells when the cells inoculated at 2×10^4 per well seeding rate. Other seeding rates gave intermediate output.

4.2 KARYOTYPIC CHARACTERIZATION OF PARENTAL CELLS

4.2.1 Buffalo

Metaphase chromosomes from buffalo were GTG-banded (Plate 4.4) and their karyotype was prepared (Plate 4.5). It consisted of five pairs of submetacentric and twenty pairs of acrocentric chromosomes including a pair of acrocentric sex chromosomes. X-chromosome was the largest acrocentric, while Y-chromosome was similar in size to chromosome 22. All chromosomes present G-negative centromeric region.

4.2.2 RAG

The modal chromosome number in RAG cell line was ascertained by analyzing sixty seven metaphase plates. A representative RAG complement is shown in Plate 4.6. Frequency distribution of chromosomes per cell is presented in table 4.4. Total chromosomes per cell ranged from 61 to 72 with modal number 66. Biarmed chromosomes per cell ranged from 3 to 7 with modal number 5. A few diploid complements were encountered which were excluded from analysis. Extreme values were also excluded while estimating total and the number of biarmed chromosomes. The chromosome distribution appeared almost symmetric and modes and means were nearly equal. Since it was often difficult to distinguish submetacentric chromosomes from metacentric, the chromosomes were differentiated as acrocentric and biarmed chromosomes only.

RAG chromosomes were GTG-banded (Plate 4.7). It revealed that the cell line did not possess diploid configuration. Some chromosomes were present

Plate 4.4 GTG-banded buffalo metaphase complement (1250 X)

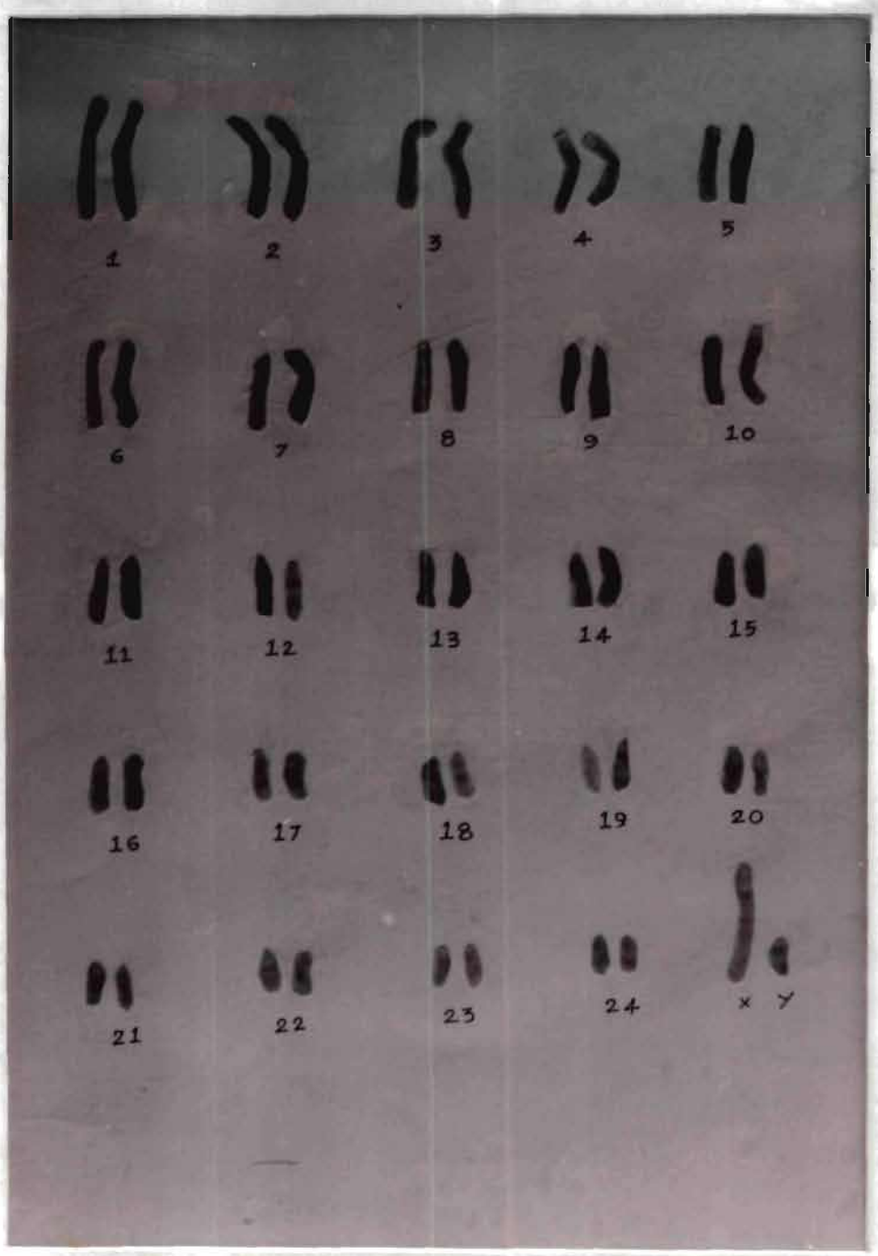


Plate 4.5 GTG-banded karyotype of buffalo

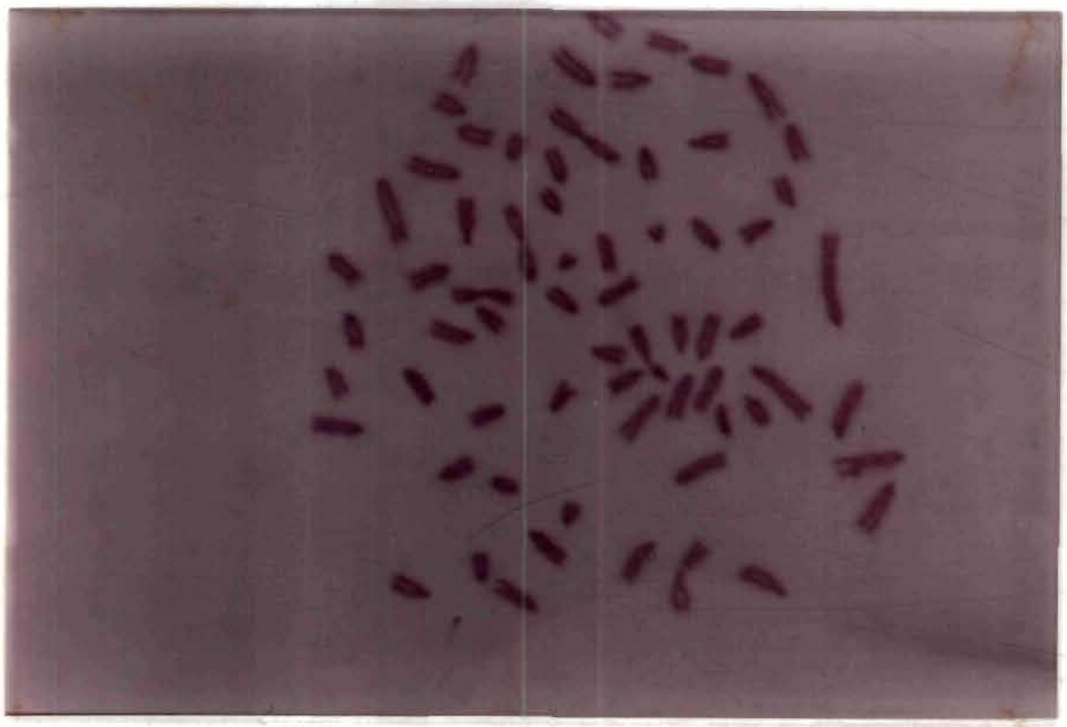


Plate 4.6 A representative Giemsa stained RAG metaphase complement (1250 X)

Plate 4.7 GTG-banded RAG metaphase complement (1250 X)

Table 4.4: Frequency distribution of chromosomes in RAG

	Total chromosomes												Mean	SD	Mode	PC*
Chr. No.	61	62	63	64	65	66	67	68	69	70	71	72	65.96	2.09	66	20.90
No.of cells	1	2	3	10	12	14	13	5	3	2	1	1				
	Biarmed chromosomes															
Chr.No.	3		4		5		6		7				5.07	0.66	5	74.63
No.of cells	1		6		50		7		3							

* Per cent cells having modal number of chromosomes

in single copy while few in multiple copies. Atleast two biarmed chromosomes were found to be dicentric. A feature common to all RAG chromosomes was their G-positive centromeric region.

4.3 KARYOTYPIC CHARACTERIZATION OF BUFFALO-MOUSE HYBRIDS

4.3.1 Uncloned Hybrids

Eighty five metaphase plates of uncloned hybrids were analyzed to determine the modal chromosome number. The distribution of chromosomes showed a wide range. Hence their frequency distribution is presented in discrete classes (Table.4.5). It ranged from 76 to 146 with two peaks of modal number, 94 and 122. Biarmed chromosomes per cell were also counted. It ranged from 5 to 15 with modal value 9.

4.3.2 Hybrid Panel

Twelve clones isolated in HAT medium were subsequently maintained in growth medium. Clones A9, D12 and G3 were propagated atleast upto 20 passages; clones A5, B10 and C3 propagated upto five passages and the remaining six clones passaged only once. These clones were characterized karyotypically by analyzing Giemsa stained metaphase spreads. Atleast 40 spreads were analyzed in all clones except C5, E9 and F6. Twenty to twenty five spreads were analyzed for C5, E9 and F6. First six clones were also characterized by GTG-banding.

Table 4.5 Frequency distribution of chromosomes in uncloned hybrids

Parameter	Total Chromosomes								
Chr. No	<81	81-90	91-100	101-110	111-120	121-130	131-140	>140	
No.of cells	3	8	19	10	15	18	7	5	
Modal No.	94 (9)* and 122 (9)*								
Per cent cell with modal No.	10.59								
	Biarmed chromosomes								
Chr. No.	7	8	9	10	11	12	13	14	15
No.of cells	5	9	12	17	14	10	8	6	4
Modal No.	10(17)*								
Per cent cells with modal No	20.00								

* No. of cells bearing the modal chromosomes are shown in parentheses.

The karyotypic characteristics of these clones are summarized in Table 4.6. More than half of the clones presented modal number exceeding sum of that of both the parents. The chromosomes were distributed over a wide range, particularly in those clones having the higher modal number. A representative hybrid complement is shown in Plate 4.8. The modal chromosome number in these clones varied from 66 (in A9) to 164 (in H4). Per cent cells having modal chromosome number ranged from 15.91 (in E9) to 26.67 (in E11).(Table 4.6)

The biarmed chromosomes per cell varied from 5 (in A9, G3 and E11) to 10 (in F6 and H4). Per cent cells having modal biarmed chromosomes was higher than that of total chromosomes in all the clones. It was extraordinarily higher (63.41 per cent) in A9. Excluding this extreme value per cent cells having modal biarmed chromosomes ranged from 18.18 (in E9) to 30.77 (in F6).

4.4 CHROMOSOME SEGREGATION

The clones maintained beyond first passage were evaluated on every fourth or fifth passage to ascertain their total and biarmed chromosome content at different passage level. Loss of chromosomes from these clones was assessed from the change in modal number and distribution of chromosomes per cell estimated at different passages. Table 4.7 and figures 4.1 to 4.6 visualize the results pertaining to the total and biarmed chromosomes counted per cell in six clones. The loss estimated from the modal value accounted for only a few chromosomes ranging from 0 (in A9 and A5) to 2 (in D12, B10 and C3) at fifth passage and from 1 (in A9) to 9 (in D12) at twentieth passage. The histograms representing the total chromosomes of the same clone have become

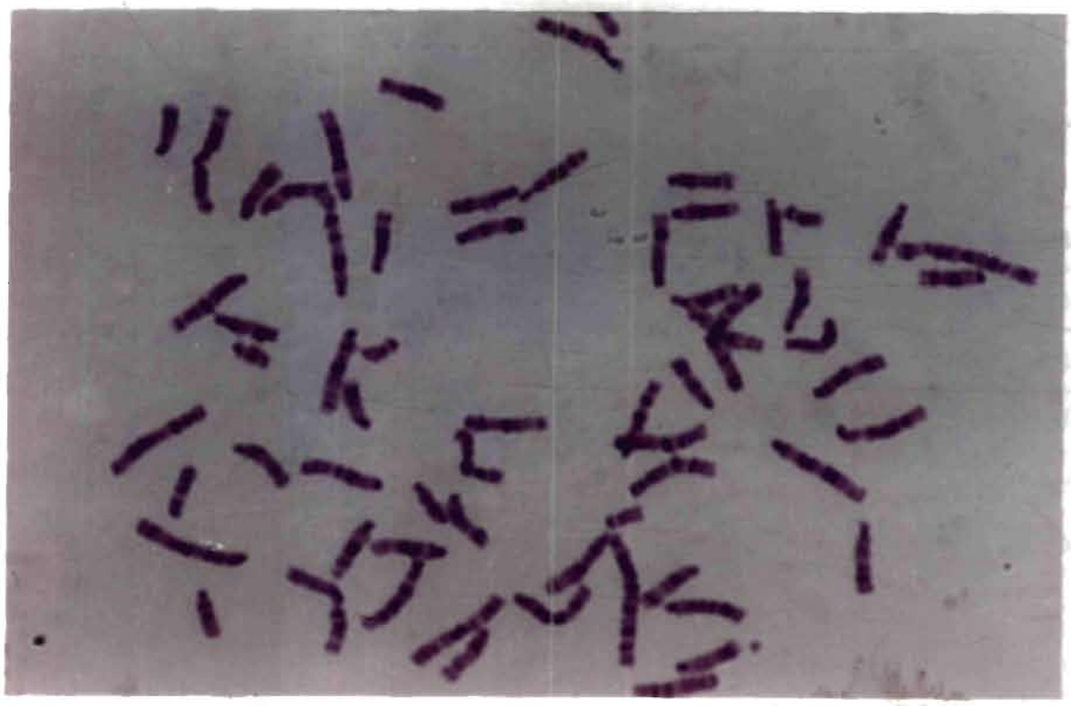


Plate 4.8 **A representative GTG-banded hybrid complement (Clone A9, Partial) (1250 X)**

Table 4.6: Karyotypic characteristics of hybrid clones

Clones	No. of cells analyzed	No. of chromosomes per cell (range)	Mean	SD	Mode	PC*
A9	41	58-79 (3-8)**	66.93 (5.29)	4.30 (0.95)	66 (5)	19.51 (63.41)
D12	41	131-156 (5-14)	143.68 (9.15)	5.60 (2.22)	144 (9)	19.51 (21.95)
G3	44	68-85 (3-10)	77.57 (5.98)	3.60 (1.85)	78 (5)	20.45 (20.45)
A5	42	104-127 (5-12)	112.86 (7.88)	5.14 (1.90)	112 (8)	16.67 (21.43)
B10	41	91-105 (3-12)	97.12 (6.49)	2.98 (2.34)	98 (6)	19.51 (24.39)
C3	45	115-140 (5-13)	124.22 (9.00)	5.40 (2.20)	124 (9)	17.78 (20.00)
C5	28	118-145 (6-12)	131.57 (8.39)	5.73 (1.34)	132 (8/9)	21.43 (28.57)
E2	46	98-118 (3-12)	106.52 (6.50)	4.32 (1.82)	105/107 (6)	17.39 (26.09)
E9	44	106-131 (4-14)	119.05 (8.57)	5.02 (1.99)	120 (8)	15.91 (18.18)
E11	45	75-95 (3-10)	82.53 (5.73)	4.11 (1.71)	82 (5/6)	26.67 (24.44)
F6	26	134-162 (5-14)	146.96 (9.73)	5.92 (1.99)	148 (10)	23.08 (30.77)
H4	25	150-175 (6-14)	162.12 (10.36)	6.95 (1.91)	164 (10)	20.00 (24.00)

* Per cent cells having modal chromosome number

** Estimates for biarmed chromosomes in parentheses.

Table 4.7 Total and biarmed chromosomes per cell in different clones

Clone. passage No.	No. of cells analysis	Total chromosomes		Biarmed chromosomes	
		Range	Mode	Range	Mode
A9.1	41	58 - 79	66	3 - 8	5
.5	41	60 - 75	66	3 - 8	5
.10	42	60 - 75	65	3 - 8	5
.15	46	60 - 74	65/66	3 - 7	5
.20	40	60 - 72	65	3 - 8	5
D12.1	41	131 - 156	144	5 - 14	9
.6	44	132 - 153	142	4 - 13	9
.10	45	128 - 148	140	4 - 12	7/8
.16	40	125 - 144	136	5 - 14	7
.21	42	121 - 139	135	4 - 12	7
G3.1	44	68 - 85	78	3 - 10	5
.5	41	70 - 89	75	3 10	5
.11	40	68 - 82	76	3 - 10	5
.15	43	68 - 78	73	3 - 10	5
.22	40	66 - 78	73/74	3 - 10	5
A5.1	42	104 - 127	112	5 - 12	8
.5	43	101 - 121	112	5 - 12	8
B10.1	41	91 - 105	98	3 - 12	6
.5	44	90 - 102	96	3 - 11	6
C3.1	45	115 - 140	124	5 - 13	9
.5	43	115 - 134	122	5 - 13	9

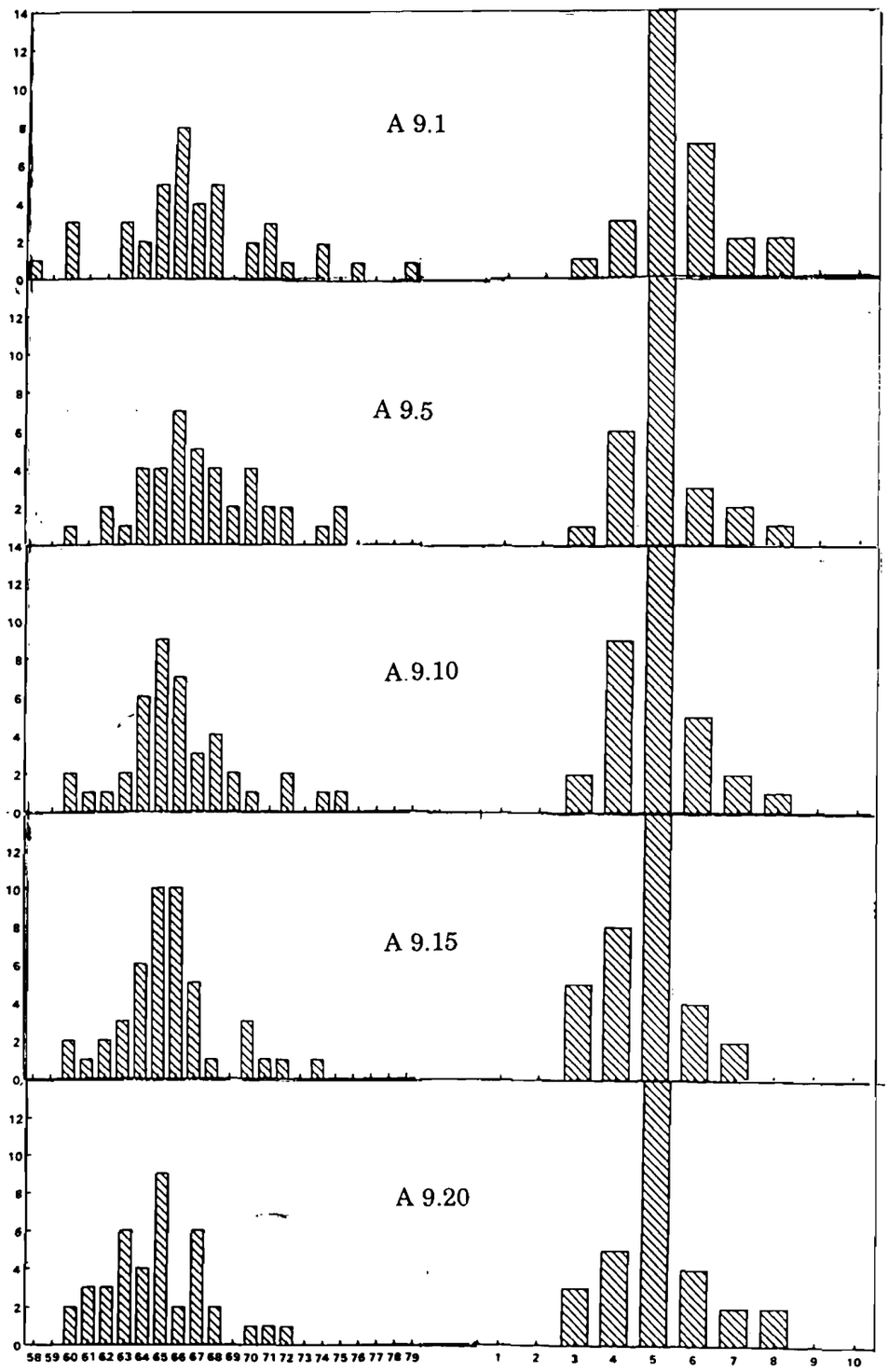


Fig.4.1 **Distribution of total and biarmed chromosomes in clone A9**

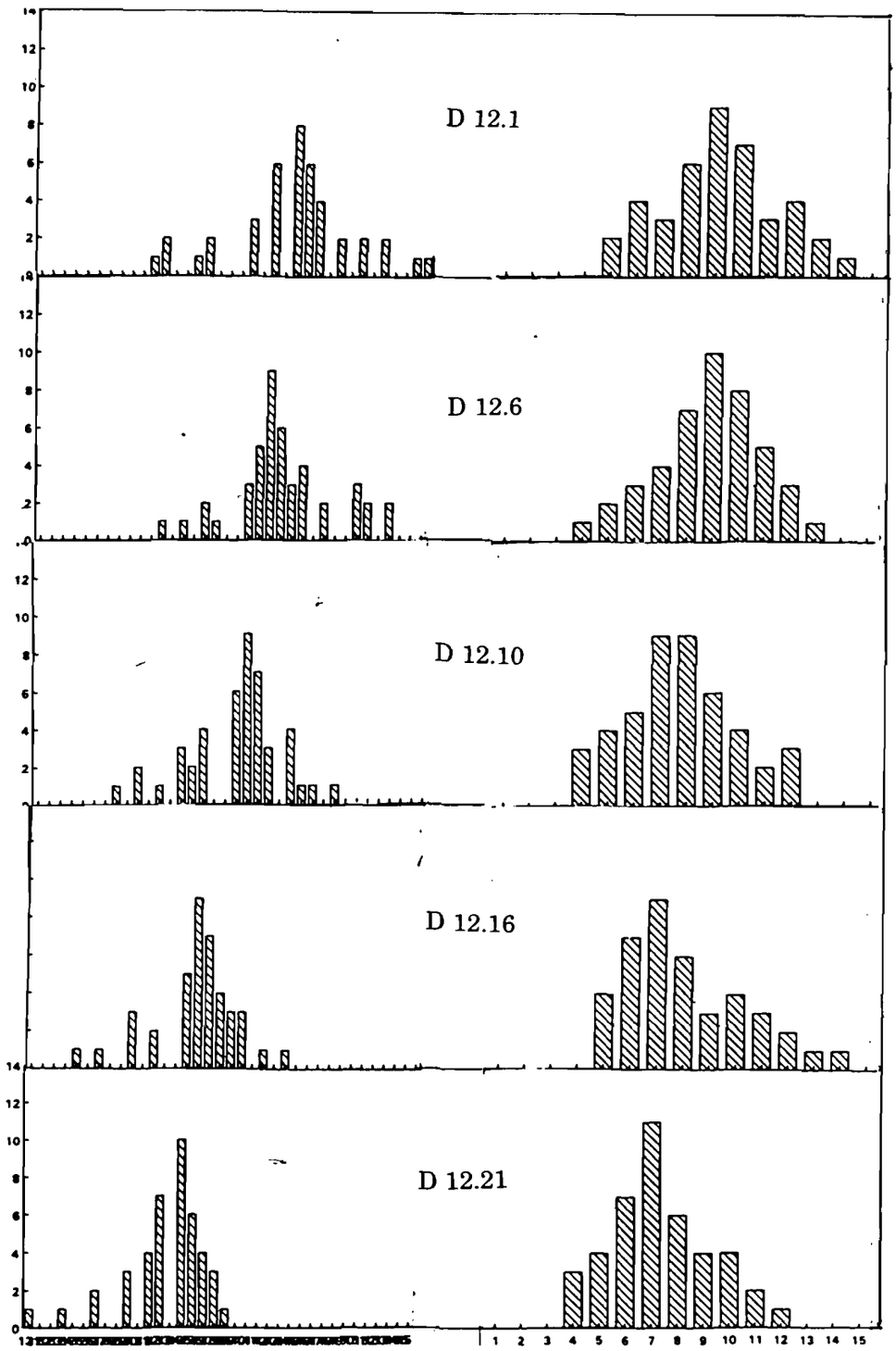


Fig.4.2 **Distribution of total and biarmed chromosomes in clone D12**

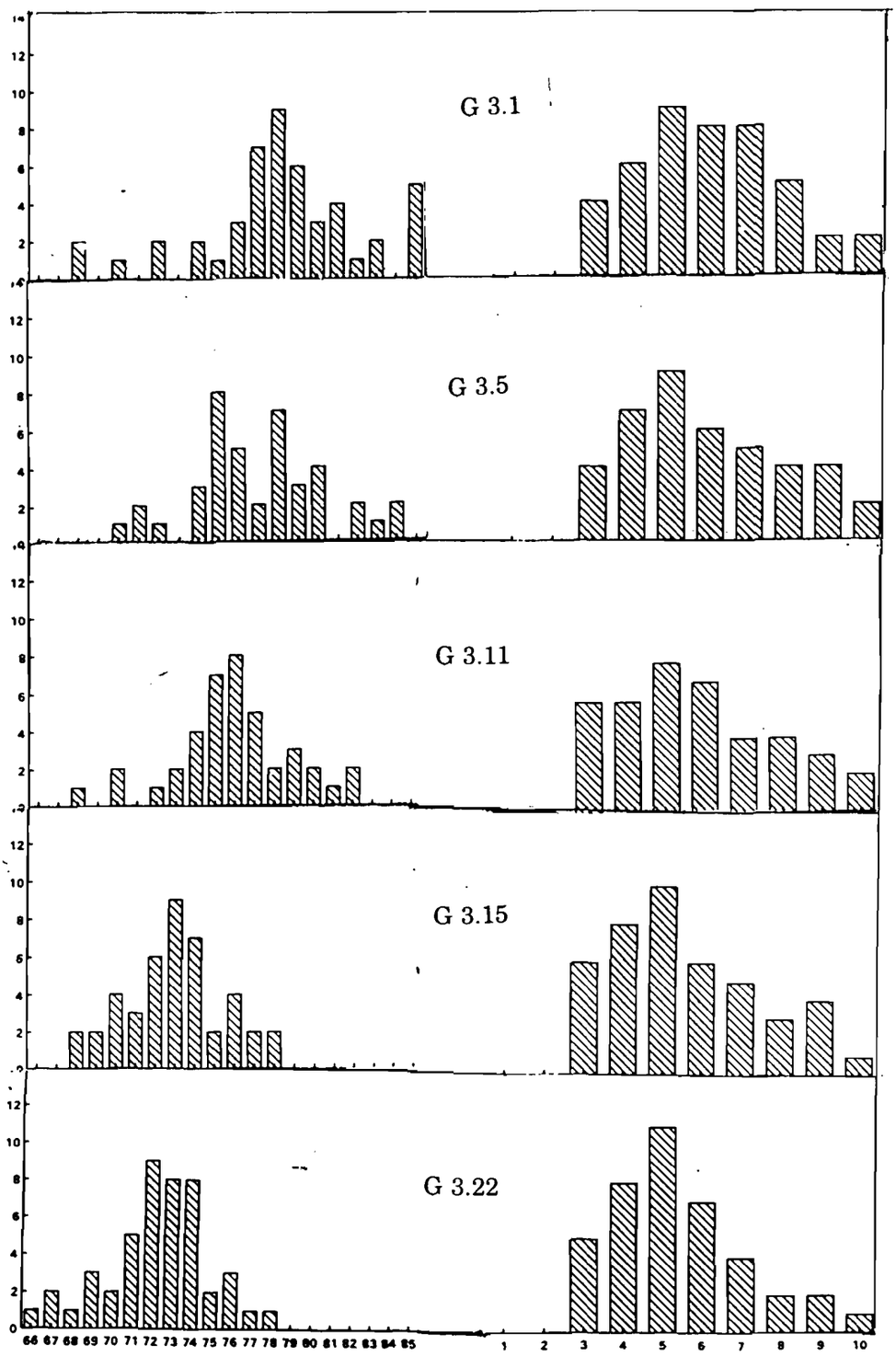


Fig.4.3 **Distribution of total and biarmed chromosomes in clone G3**

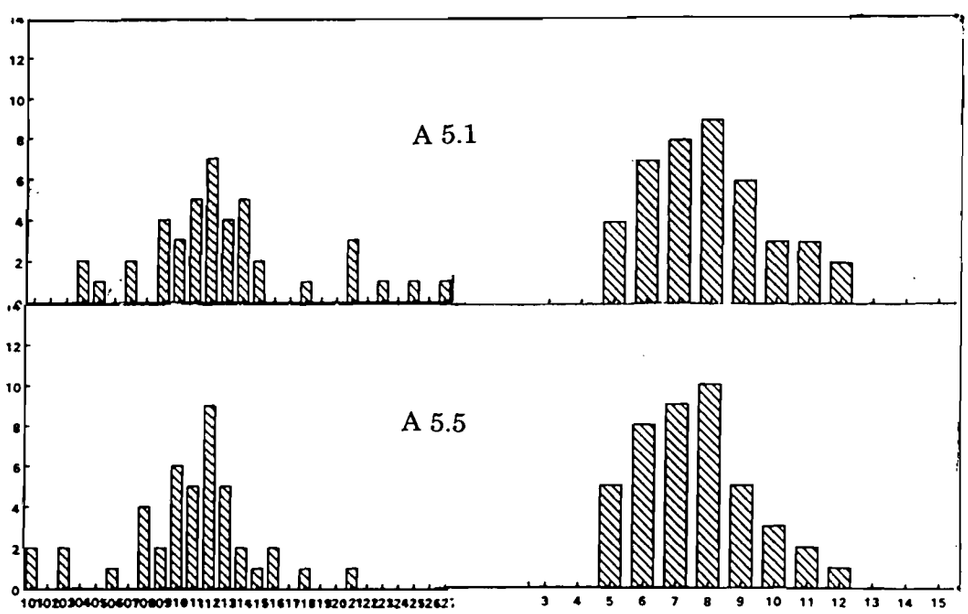


Fig.4.4 **Distribution of total and biarmed chromosomes in clone A5**

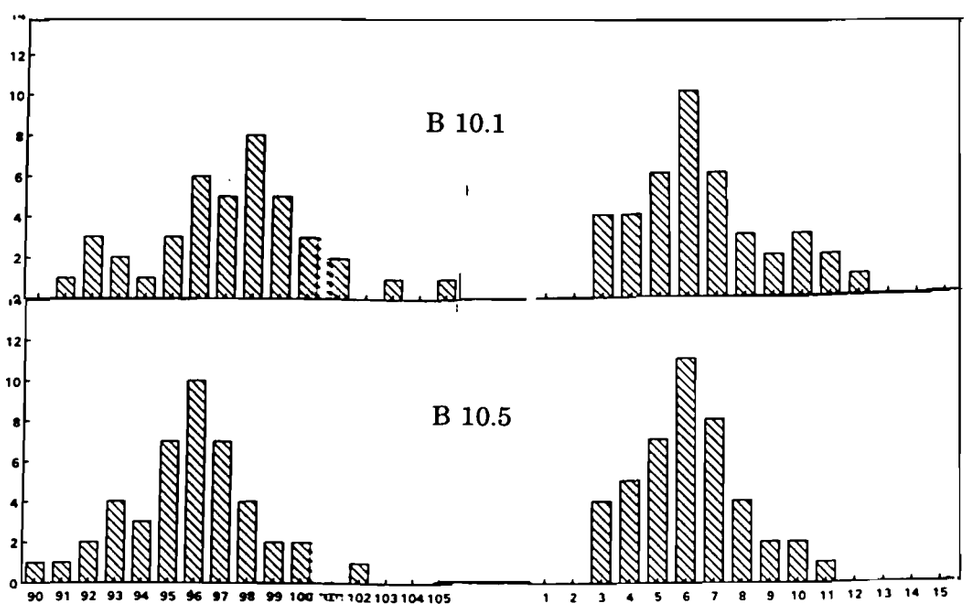


Fig.4.5 **Distribution of total and biarmed chromosomes in clone B10**

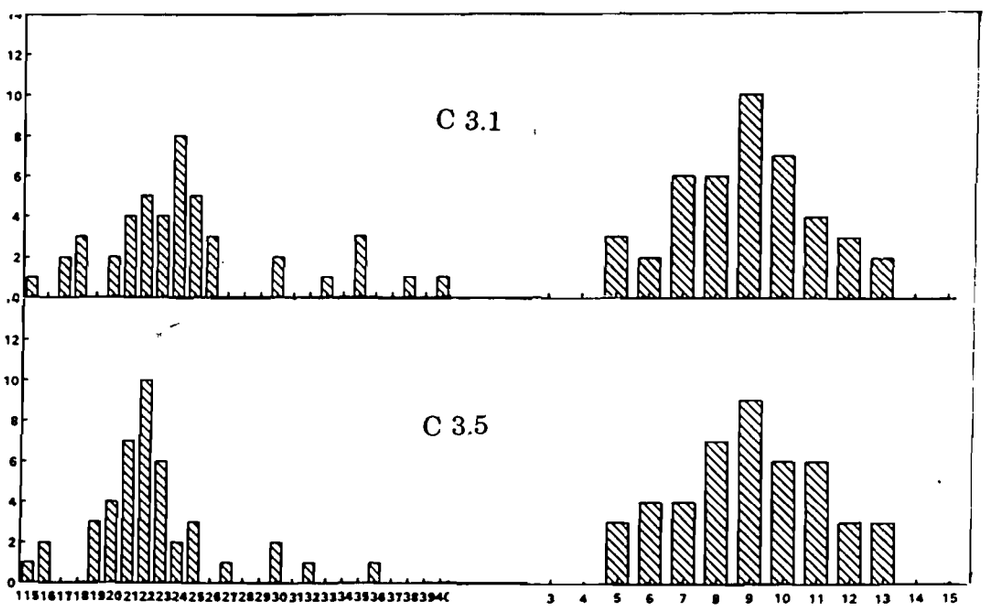


Fig.4.6 **Distribution of total and biarmed chromosomes in clone C3**

narrower, also the left shift was observed in these histograms. The histograms representing biarmed chromosomes in the hybrid clones remained unchanged.

To ascertain the number of buffalo chromosomes present in the hybrid clone and extent of their loss over passaging, GTG-banded metaphase spreads of clones A9, D12, G3, A5, B10 and C3 at different passage levels were analyzed. Table 4.8 summarizes the results pertaining retention/segregation of buffalo chromosomes in the various hybrid clones.

Table 4.8 illustrates that very few buffalo chromosomes were present in the hybrids when analyzed. Average number of buffalo chromosomes retained per cell ranged from 1.85 (in A9) to 8.25 (in C3) at fifth passage. Further segregation of buffalo chromosomes was observed in the clones propagated beyond fifth passage. At twentieth passage, average numbers of buffalo chromosomes retained per cell were 1.83, 4.92 and 1.83 in the clones A9, D12 and G3 respectively. Decline in total chromosome number from fifth to twentieth passage in these clones was 2.15, 9.99 and 5.19 respectively.

Heterogeneity in the chromosome content of hybrid clones was assessed by comparing the variability in total and buffalo chromosomes counted per cell at fifth passage. The total chromosomes counted per cell in the clones analyzed by banding ranged from 59 to 76 (difference 17), 132 to 156 (difference 16), 70 to 85 (difference 15), 105 to 121 (difference 16), 90 to 105 (difference 15) and 116 to 133 (deference 17) in A9, D12, G3, A5, B10 and C3 respectively. Correspondingly, buffalo chromosomes counted in these clones ranged from 0 to 4 (difference 4), 5 to 10 (difference 5), 1 to 5 (difference 4), 3 to 6 (difference 3), 2 to 6 (difference 4) and 6 to 11 (difference 5) respectively.

Table 4.8 Buffalo chromosomes retained in the hybrid clones.

Clone. passage No.	Metaphase plates analysed	No. of buffalo chromosomes per cell means (range)	Buffalo chromosomes 4			Proportion of cells carrying buffalo chr. 4	Buffalo chr. 4 retained (+) or segregated (-)
			Present	Ambiguous	Absent		
A9.5	13	1.85 (0-4)	0	1	12	0	-
A9.10	15	1.93 (1-4)	0	2	13	0	-
A9.15	15	1.60 (1-3)	0	2	13	0	-
A9.20	14	1.83 (1-3)	0	1	13	0	-
D12.6	12	6.92 (5-10)	8	2	2	66.67	+
D12.16	14	5.29 (3-8)	2	4	8	14.29	-
D12.21	12	4.92 (3-6)	2	3	7	16.67	-
G3.5	15	3.87 (2-6)	0	3	12	0	-
G3.15	12	1.92 (1-4)	0	2	10	0	-
G3.22	12	1.83 (1-4)	0	1	11	0	-
A5.5	10	4.90 (3-6)	6	2	2	60.00	+
B10.5	12	3.58 (2-6)	0	2	10	0	-
C3.5	8	8.25 (6-11)	3	3	2	37.50	+

Presence of buffalo chromosomes in the hybrid complement was proportional to the total chromosome content of the clone. Usually, the clones having higher modal number carried more buffalo chromosomes. However, clone C3 having modal number (124) lesser than D12 (144) had the highest number of buffalo chromosomes in its complement. (8-25).

Clones A9, G3 and B10 did not possess buffalo chromosome 4. However, there was some ambiguity in identification of this chromosome, but in fewer than 20 per cent of cells. Hence, these clones were declared "negative" for buffalo chromosome 4. Clones A5, and C3 contained buffalo chromosome 4 in more than 20 per cent of cells, hence judged "positive" for buffalo chromosome 4. Clone D12 possessed buffalo chromosome 4 in more than 20 per cent of cells when analyzed at 6th passage, but in less than 20 per cent of cells when analyzed at 16th and 20th passage. Hence clone D12 was declared "positive" at 6th passage and "negative" in subsequent analysis. However, there were few ambiguities in the identification of this chromosome.

4.5 ISOZYME ANALYSIS

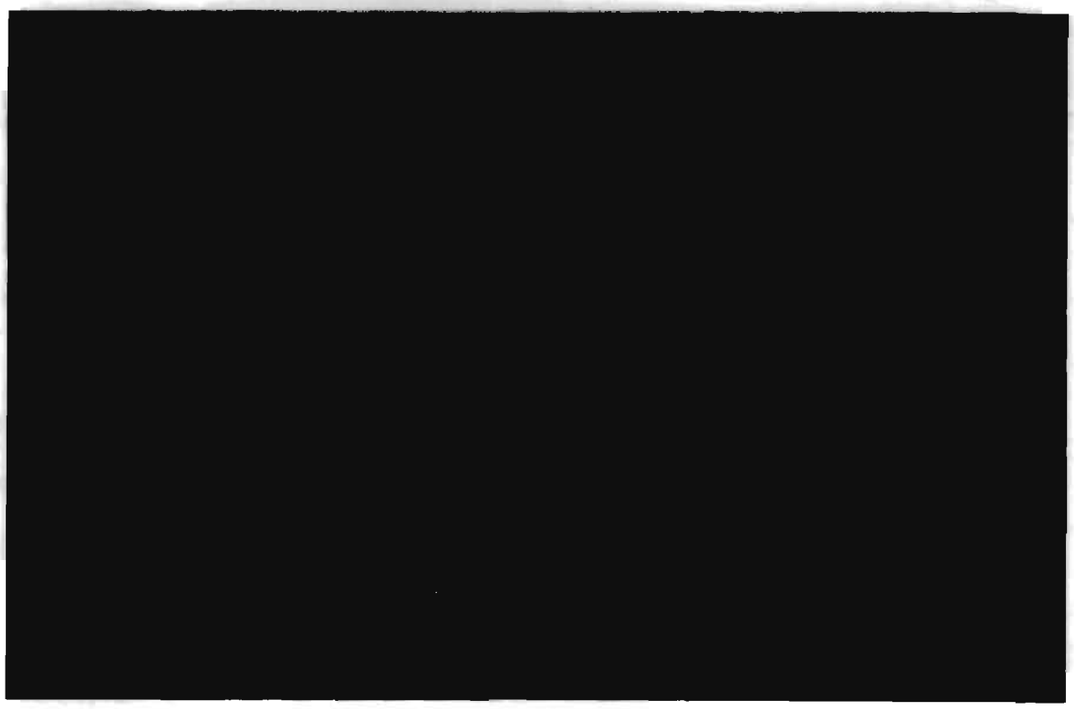
Cell lysates from parental cells and hybrid clones were electrophoresed on cellulose acetate gel plates. Clones A9, D12, G3, B10, C3 and A5 were sampled and homogenates prepared at first and last passage. On each plate five to seven samples were applied. Electrophoresis of samples, followed by specific histochemical staining was used to characterize the buffalo isozymes expressed in the hybrid clones. Electrophoretic separation of buffalo and mouse LDHB, PEPB and TPI was visualized in the form of dark bands migrated anodal from the origin.

4.5.1 Zymogram Pattern of LDHB

Buffalo cells showed four anodal bands. Most anodal (band-4) was approximately five cm away from the origin. Intensity of all the bands was almost same. Mouse cells showed three anodal bands migrated almost equal to bands -2, -3 and -4 of buffalo isozymes. Band-1 was the most intense and band-3 the least. There was no difference in electrophoretic mobility of mouse and buffalo isozymes. However, buffalo isozymes had an extra anodal band. Hybrid clones produced either three or five anodal bands. Clones A9, G3, B10, E2, E9 and E11 exhibited three bands with identical electrophoretic migration to that of mouse isozymes and there was an absence of band corresponding to buffalo isozyme bands -1. Clones D12, A5, C3, C5, F6 and H4 exhibited five bands corresponding to all the bands expressed by buffalo cells and an extra band even more anodal to buffalo isozyme band-4. Also, bands-2, -3, and -4 were more intense than that of either parents. Zymogram pattern of LDHB in parental cells and representative hybrid clones is shown in Plate 4.9 with its diagramatic presentation.

4.5.2 Zymogram Pattern of PEPB

Buffalo cells showed one band migrated approximately five cm anodally. Mouse cells also showed one band but migrated less anodally, approximately four cm from origin. Hybrid clones A9, G3, E2, E9, E11 and B10 showed only one band corresponding to the band exhibited by mouse parent. Clones D12, A5, C3, C5, F6 and H4 expressed two bands each one corresponding to the bands shown by both the parents. Zymogram pattern of



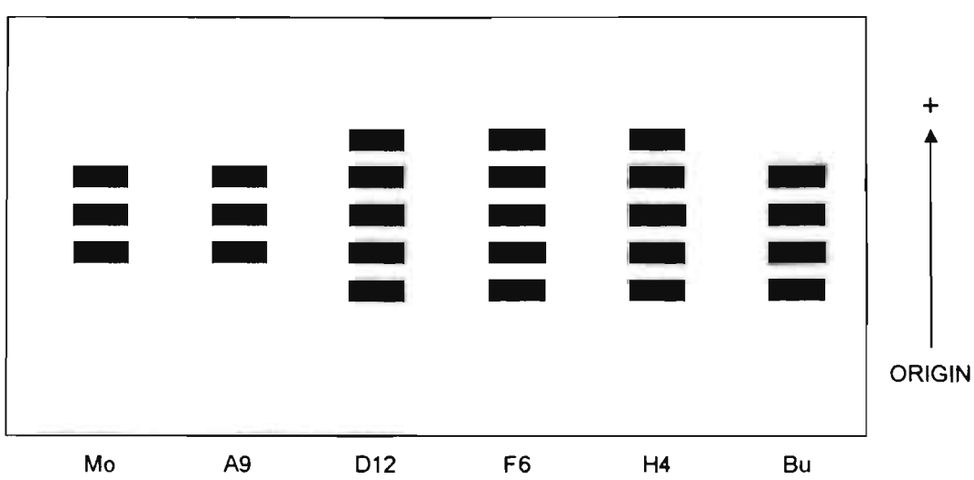


Plate 4.9 Zymogram pattern of LDHB in parental cells and representative hybrid clones

PEPB in parental cells and representative hybrid clones is shown in Plate 4.10 with its diagramatic presentation.

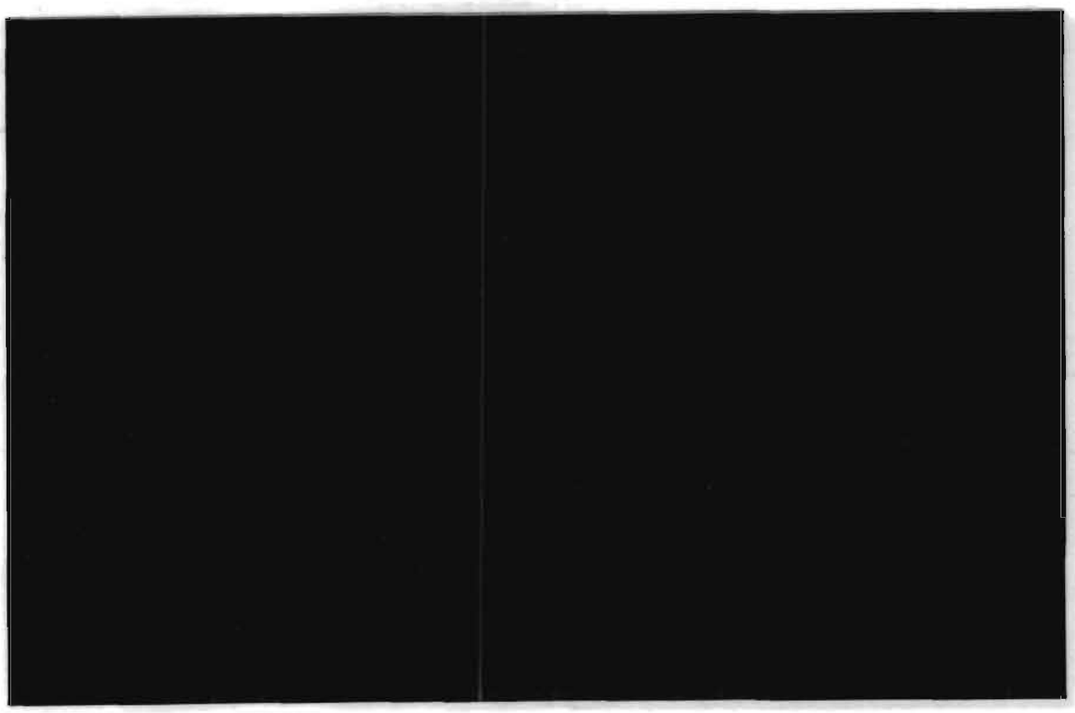
4.5.3 Zymogram Pattern of TPI

Buffalo cells showed only one anodal band, migrated approximately four cm from origin. Mouse cells also showed one anodal band but migrated faster than and more anodal to buffalo isozyme. Hybrid clones A9, G3, B10, E2, E9 and E11 showed only one band corresponding to that expressed by their mouse parent. Clones D12, A5, C3, C5, F6 and H4 showed two bands, one each corresponding the bands shown by both the parents. Zymogram pattern of TPI in parental cells and representative hybrid clones is shown in Plate 4.11 with its diagramatic presentation.

4.6 SYNTENY IDENTIFICATION AND CHROMOSOME ASSIGNMENT

Syntenic relationship between LDHB, PEPB and TPI was established by concordance/discordance analysis. Occurrence of buffalo isozymes and buffalo chromosome 4 in the hybrid clones is summarized in table 4.9.

The enzymes were found to be concordantly present or absent in all the clones studied. Discordance was not observed in any of the clones. This allows to propose a synteny among these genes in buffalo. Again these genes were expressed together in the clones which retained buffalo chromosome 4 and not expressed in the clones which segregated it. This further allows to assign this synteny to buffalo chromosome 4.



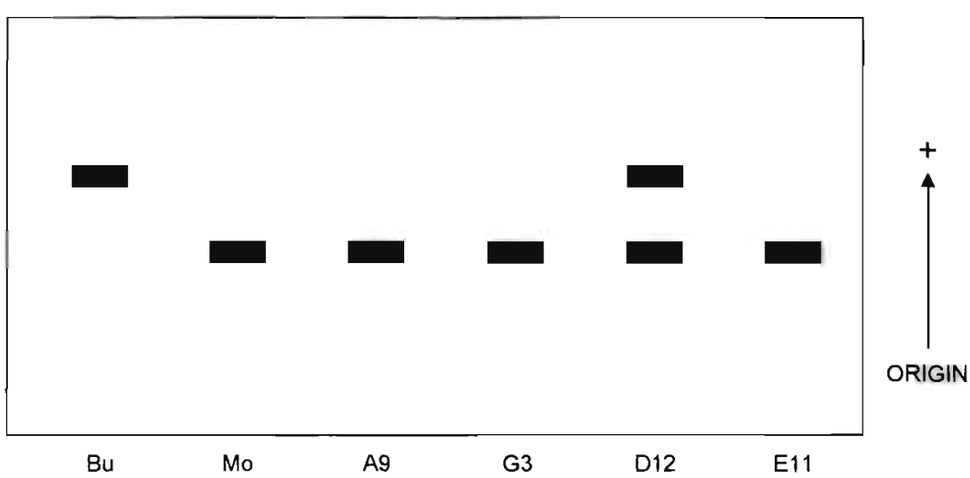
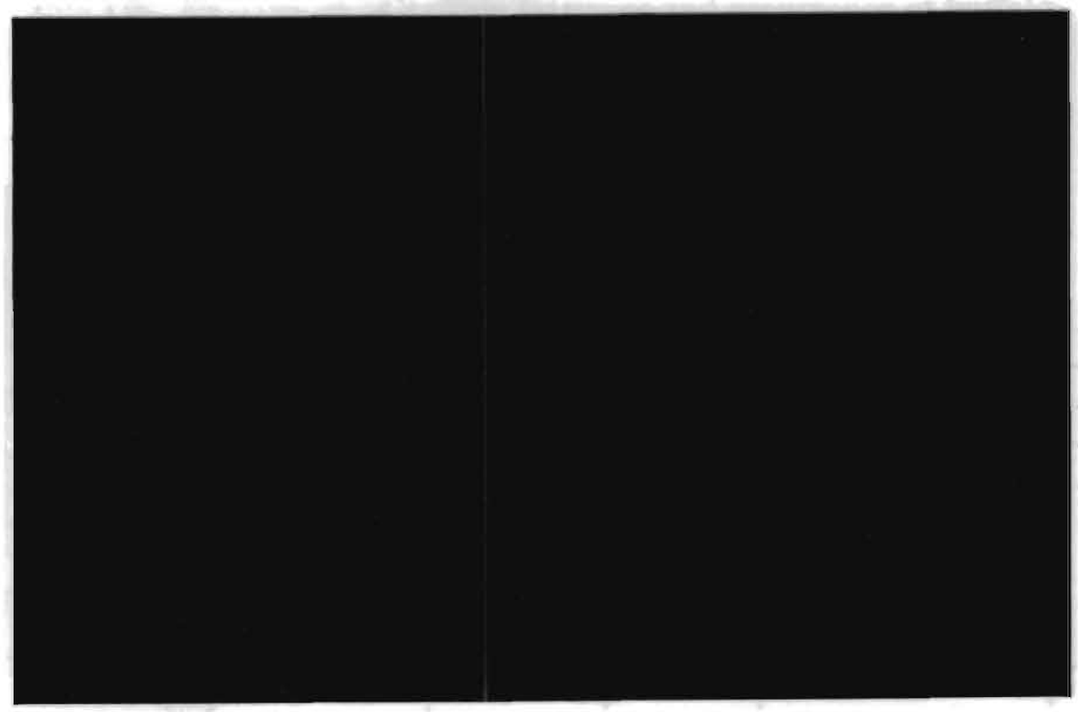


Plate 4.10 Zymogram pattern of PEPB in parental cells and representative hybrid clones



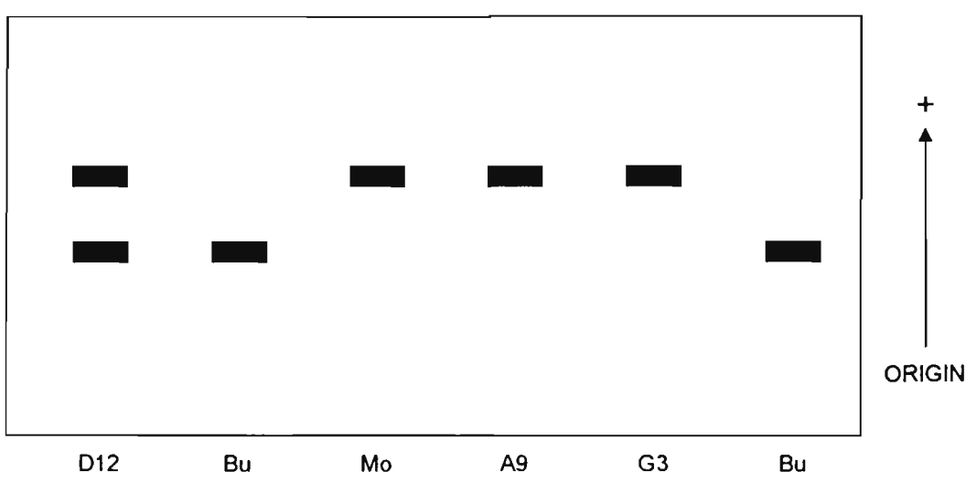


Plate 4.11 Zymogram pattern of TPI in parental cells and representative hybrid clones

Table 4.9: Expression of buffalo isozymes and occurrence of buffalo chromosome 4 in the hybrid clones

Hybrid clones	Buffalo isozymes			Buffalo chromosome 4
	LDHB	PEPB	TPI	
A9	-	-	-	-
D12	+	+	+	+
G3	-	-	-	-
A5	+	+	+	+
B10	-	-	-	-
C3	+	+	+	+
C5	+	+	+	NA
E2	-	-	-	NA
E9	-	-	-	NA
E11	-	-	-	NA
F6	+	+	+	NA
H4	+	+	+	NA

+ Presence, - Absence, NA not analyzed

Discussion

DISCUSSION

5.1 SOMATIC CELL HYBRIDIZATION

5.1.1 Selection of Parental Cell Line

Several mouse and hamster cell lines deficient in HPRT or TK have been used to develop somatic cell hybrids. Hamster cell lines, Wg3h and E-36 are the commonest among them, the reason being very low chromosome numbers in these cell lines, 21 (El-Nahas *et al.*, 1993) and 22 (Saidi-Mehtar *et al.*, 1981) respectively. The resultant hybrids are easily analyzable owing to less number of chromosomes. Also the hamster cell hybrids segregate donor chromosomes more rapidly than mouse cell hybrids (Kao and Puck, 1967, 1968). Thus, hybrids carrying only a few donor chromosomes can easily be obtained in limited passages. Unfortunately, these cell lines are not available with the repository in India (National Facility for Animal Tissue and Cell Culture, Pune). Efforts to procure either of the cell lines from abroad were futile due to longer transit period in shipment. Hence, it was opted to use mouse cell line, LM(TK⁻). This cell line has fairly large chromosome content (modal number 44). The hybrids developed in our laboratory using this cell line were found to be slow growing (Rank *et al.*, 1996) thus, unsuitable for genetic analysis. Hence, the mouse cell line, RAG was finally employed.

5.1.2 Hybridization Techniques

5.1.2.1 Pancake Fusion

This is one of the most widely followed fusion approaches, as evident from the literature. In the present study also, hybridization attempts were

initiated by this technique. However, all the attempts to isolate the hybrids were unsuccessful since the artificial monolayer of parent cells on glass coverslip peeled off from the surface on PEG dips and could not be recovered. It is surmised that the smooth surface of glass coverslip was not a suitable substrate for attachment of cells. Plastic coverslip and not the glass coverslip has been used in the original method described by O'Malley and Davidson (1977). However, due to non-availability of any suitable substrate, the trial could not be carried further and option for an alternative fusion technique resorted to.

5.1.2.2 Suspension Fusion

Hybridization between buffalo leucocytes and RAG cells was successfully achieved by suspension fusion technique. Here, attempts were made to maximize the yield of hybrid cell colonies by optimizing various parameters involved in the precipitation of hybridization.

5.1.3 Factors Affecting Hybridization Frequency

5.1.3.1 Effect of Duration of PEG Exposure

Although there are sporadic reports on successful hybridization at a prolonged exposure to PEG for five to fifteen minutes (Pontecorvo, 1975), majority of gene mapping studies report the duration of PEG exposure for two or less than two minutes. In the present study too, parent cells were exposed to PEG for 30 to 120 seconds to assess an optimum time of PEG exposure for fusion of leucocytes by suspension fusion technique. When the cells were exposed for 30 seconds, it did precipitate the hybridization but, the frequency

of hybridization was unsatisfactory. When the duration of PEG exposure was prolonged, hybridization frequency also increased and the maximum hybridization frequency was achieved at 90 seconds. Any further exposure diminished the hybridization frequency. Work on hybridoma (Goding, 1983) has revealed that the rate of fusion and also the toxicity increases with the duration of PEG exposure. In the present study, it appears that increased output of hybrid cell colonies was due to the improvement in rate of fusion but the drop in hybrid cell output at 120 seconds was most logically due to reduced cell viability on account of PEG approaching toxic level. Gefter *et al.* (1977) also observed that parent cells could tolerate prolonged exposure (of upto seven minutes) to PEG at lower concentration (35-40 per cent) however, toxicity was evident in most circumstances, beyond two minutes of exposure to PEG at 50 per cent concentration. Davidson and Gerald (1976) have shown that PEG exposure for 60 seconds in suspension fusion of leucocytes and mouse cell line gave hybridization frequency better than that obtained at PEG exposure for 30 or 120 seconds. However, they did not evaluate the effect of PEG exposure for 90 seconds. In this context it is pertinent to mention that there are abundant reports on gene mapping describing somatic cell hybridization effected by PEG exposure for 60 or 120 seconds. However, in majority of experiments the hybrids were derived either by monolayer or by pancake technique and only a few reports described suspension fusion.

In the present study, it was found that PEG exposure for 90 seconds was optimal for suspension fusion of leucocytes. The finding seems justifiable with the earlier observation that the cells which fused well at a particular

exposure in one technique required a different exposure time in another technique (O'Malley and Davidson, 1977).

5.1.3.2 Effect of Concentration and Grade of PEG

Fifty per cent is widely recognized as an optimum concentration of PEG for somatic cell hybridization. Any concentration higher than this results into marked cell damage and lower than this gives suboptimum hybridization frequency (Davidson and Gerald, 1976; Gefter *et al.*, 1977; Goding, 1983). However, certain cell types are sensitive to this concentration and do not yield acceptable level of hybridization (Davidson and Gerald, 1976). Under such circumstances dilution of PEG concentration is warranted. Hence, in the present study, two concentrations, 50 and 45 per cent were employed. DMSO was incorporated in latter at 10 per cent level, as it reduces toxicity and widens the range over which PEG is effective (Norwood *et al.*, 1976). Change in concentration was combined with the change in grade of PEG (PEG-3350 and 1450).

Reduction in PEG concentration with an inclusion of DMSO resulted in an improvement in hybridization frequency. However, reduction in PEG concentration did not have equal quantitative effect at different grades and it was evident that there was a marginal decline in hybrid cell colony output when PEG-1450 was used as fusogen. High molecular grade PEG (PEG-3350) at 50 per cent concentration might be toxic to the cells, hence dilution of PEG concentration along with protective coverage conferred by DMSO might have resulted in improvement of hybridization frequency. But lower molecular grade PEG (PEG-1450) at same concentration might not be toxic and hence, dilution of

concentration could not have improved the hybridization frequency. Beneficial effect of dilution of PEG concentration to 45 per cent and incorporation of DMSO was also reported by Rank *et al.* (1996) in somatic cell hybridization involving buffalo fibroblasts and RAG.

Changes in molecular grade of PEG from 3350 to 1450 also improved the hybridization frequency at either concentration. PEG-1450 was less sensitive to dilution, also it was more suitable for practical purposes because of ease in dilution and removal due to its low viscosity. Use of PEG in wide range of grades for successful hybridization of variety of cells has been described without critical effect of grades of PEG when used at 50 per cent concentration (Ryttman *et al.,* 1986; Hallerman *et al.,* 1988 ; Joshi *et al.,* 1995).

Among the four concentration - grade combinations, PEG-1450 at 50 per cent concentration gave the best results. However, PEG-1450 at 45 per cent concentration with 10 per cent DMSO also yielded almost comparable results. Thus, PEG-1450 at either concentrations could be effective as well. Davidson and Gerald (1976) observed peak hybridization at 50 per cent concentration of PEG-1000 among the range of concentrations (40 to 70 per cent) and grades (PEG-200 to PEG-6000) tested.

5.1.2.3 Effects of Cell Density

Density dependent growth of hybridomas is well documented in literature. Hence, to explore possible effect of cell density on growth of buffalo-mouse hybrids PEG-treated cells were seeded at varied cell densities in 96-well

microtitre plates. Effect was evaluated by screening the wells carrying the expanding colonies on day - 10.

In the present experiment growth of hybrid cell colonies was also found to be highly density-dependent. Maximum hybrid cell colonies could be obtained at 1 x 10^5 cells/well seeding rate. Cell density lower or higher than this, resulted in a diminished output. Wells which received cell population higher than 1 x 10^5 i.e. 1 x 10^6 and 2 x 10^6 cells/well are also expected to receive proportionally more hybrid cells. Despite more hybrid cells in these wells, number of hybrid cell colonies obtained was lesser than those wells which received 1 x 10^5 cells. This refractile growth of hybrid cell colonies at higher cell concentration might be due to competence among cells for nutrients and substrate. On the other hand very less hybrid cell colonies at 2 x 10^4 cells/well seeding rate, could be attributed to less number of hybrid cells available. Density depending nature of hybridomas was also observed by Olsson and Kaplan (1983). They found maximum yield of viable hybrids at 2 x 10^5 cells/well, drastically low at 5 x 10^5 cells/well, and absolutely no colonies beyond this density.

The density dependent nature of hybrid cells is important while cloning by limiting dilution method. Feeder cells are generally used to support the growth of newly formed hybridomas. In the present trial, cloning was not done, instead the PEG treated parent cells were placed in HAT medium in which they could not grow, but remained alive atleast for few days to serve as feeder cells to newly formed highly fragile hybrids.

It is noteworthy that the studies which demonstrated the effect of cell concentration refer to concentration of parent cells before fusion (Davidson *et al.*, 1976; O'Malley and Davidson, 1977; Joshi *et al.*, 1995). These studies have shown that cell to cell contact during PEG treatment was an important factor in deciding the frequency of hybridization. Unlike those studies, in the present experiment parent cells in equal proportion were subjected to hybridization and no attempt was made to compare the effect of prehybridization cell concentration.

It is speculative to mention that the factors which determine the hybridization frequency were studied during the course of standardization of fusion between buffalo leucocytes and mouse cell line, and possible interactions among them could not be evaluated as these were studied independently. Existence of them, however, can not be ruled out.

5.2 KARYOTYPIC CHARACTERIZATION OF PARENTAL CELLS

5.2.1 Buffalo

Present karyotype was prepared from GTG-banded metaphase chromosomes of buffalo arranged and numbered as per the standard karyotype generated by Iannuzzi (1994). However, the karyotype prepared by Iannuzzi (1994) is based on prometaphase chromosomes harvested from synchronized cultures, while the present karyotype was prepared from metaphase chromosomes obtained from the non-synchronized cultures. GTG-banding of metaphase chromosome has been successfully employed for individual identification of chromosomes of cattle (Dain *et al.,* 1984), pig (Forster and

Hecht, 1986) and recently of buffalo (El-Nahas *et al.,* 1993) from hybrid complements.

5.2.2 RAG

RAG is karyotypically heteroploid and possesses chromosomes in excess of its normal diploid parental species; mouse, 2n=40 (ATCC, 1988). This is also clear from the fact that RAG complement possessed several biarmed chromosomes which most likely, might have originated from the fusion of normal mouse chromosomes as the mouse karyotype does not possess any biarmed chromosomes.

Heteroploid cell populations grown *in vitro* are generally thought to be heterogenous with marked variation in chromosome constitution from cell to cell. However, present study revealed that although chromosomes varied from 61 to 72 per cell, large proportion of cells (above 70 per cent) possessed 64 to 67 chromosomes. Chen (1987) reported the karyotypic characteristics of RAG and chromosome variation in narrow range (62 to 67) with modal number 65/66. He had also described marked similarity between karyotypes of cells from different harvest with variation only in a few marker chromosomes. Karyotype homogeneity is also evident from the two investigations done twenty years apart. Chromosome number reported by Hashmi *et al.* (1974) and Joshi (1995) differed only by a few chromosomes indicating only a little variation in chromosome composition over a long period of cultivation *in vitro*.

5.3 KARYOTYPIC CHARACTERIZATION OF BUFFALO-MOUSE HYBRIDS

5.3.1 Uncloned Hybrids

Chromosome number in these hybrid cells was very high and distributed over a wide range (76 to 146). Wide dispersion of chromosomes is quite expected in the uncloned hybrids. These hybrid cells exhibited two modal values, *94* and *122 indicating that these hybrid cells might have resulted due* to fusion of two or more cells followed by loss of chromosomes from the hybrid complement. Joshi *et al.* (1995) have recently described buffalo-mouse hybrids with 92 to 172 chromosomes.

5.3.2 Hybrid Panel

Twelve clones which constituted the hybrid panel showed marked variation in their chromosome composition. Their modal values ranged from as low as 66 in A9 to as high as 164 in H4. None of the clone possessed exact sum of chromosome numbers of both the parents. Many of the clones possessed modal number which exceeded the sum of those of both the parents. This could have resulted due to fusion of more than two cells followed by partial segregation of complement. As there is no control over the number of cells fusing in the hybridization event, possibility of fusion of more than two cells could not be ruled out.

It is interesting to note that clone A9 had modal number of chromosomes just equal to that of RAG. This raised some doubt whether they are really hybrids. However, this clone could be grown successfully in HAT

medium, which is lethal to RAG, suggesting that A9 was a hybrid clone. On karyotypic evaluation, it was observed that it contained a few buffalo chromosomes or their rearranged product. However, the origin of translocation could not be ascertained. These chromosomes might have complemented the deficiency of HPRT and permitted further loss of mouse chromosomes, which normally does not occur. Kao and Puck (1970) also observed extensive loss of human chromosomes from human-hamster hybrids immediately after fusion and chromosome number fallen to a point close to and sometimes even less than of original hamster cell line.

5.4 CHROMOSOME SEGREGATION

Chromosome segregation from the hybrid cells, particularly interspecific somatic cell hybrids, is an universal phenomena and is known since the first observation in rat-mouse hybrids (Weiss and Ephrussi, 1966). In the present study chromosome segregation was estimated by periodically studying the chromosome content in the hybrid clones and the change over passaging. Chromosome content was reduced in all the clones, reflected in decline in modal values and left shift of histograms representing the frequency distribution of chromosomes in the hybrid cells.

The chromosome loss although variable in different clones, was not much in proportion to total chromosome content. Plausible explanation could be that much of the chromosome loss might have occurred before the clones were examined. This is clear from the observation that all the clones possessed chromosome number much less than the expected sum of those of both the parents. Weiss and Green (1967) have proposed that bulk of chromosome loss

occurs immediately after fusion. Although there is lack of informations on generationwise segregation of chromosomes, considerable loss of chromosomes from rat-mouse (Weiss and Ephrussi, 1966) and human - mouse (Nabholz *et al.,* 1969) was observed in as early as 40 generations of cell cycle.

Progressive loss of chromosomes was observed even upto 20th passage in D12 and G3 clones. Thus, it seems that these clones are still under karyotypic evolution and undergoing the process of karyotypic stabilization. Clone A9 has probably stabilized in its karyotypic evolution, as there was no apparent loss of chromosomes over passaging. This is further substantiated by the observation that A9 had reached very close to RAG in its chromosome composition. Karyotypic evolution of hybrid clones was also evident from the change in symmetry of histograms. The histograms have become narrower indicating that the clones have become more homogeneous in their composition and less dispersed in distribution.

Chromosome segregation is usually considered to be unilateral. Normally, chromosomes of non-mouse or non-hamster species are segregated. However, in the present study, mouse chromosomes were also segregated. If this is examined in the light of an assumption that several clones might have resulted due to the fusion of two mouse and a buffalo cell, it is reasonable to believe that the hybrids can survive the loss of additional mouse chromosomes.

Pattern of occurrence and segregation of biarmed chromosomes was not similar to that observed for whole complement. Distribution of biarmed chromosomes was less variable as reflected by narrow histograms. Also, the loss of biarmed chromosomes was less frequent. Such non-random segregation

of chromosomes was also observed in pig-mouse hybrids (Leong *et al.,* 1983; Echard *et al.,* 1984; Forster and Hecht, 1986; Ryttman *et al.,* 1986; Zijlstra *et al.*, 1994).

Presence of buffalo chromosomes was judged based on their characteristic banding pattern of centromeric region. All the buffalo chromosomes showed G-negative centromeric region. Di Berardino *et al.* (1991) have also reported similar observations. Recently, Iannuzzi (1994) has described this intrinsic feature of buffalo chromosomes, in standard banded karyotypes of buffalo. Very few buffalo chromosomes were retained in the hybrid clones. Further, the variation in number of buffalo chromosomes was comparatively lesser than that in total number of chromosomes. Thus, the variation in the hybrid complement was mainly due to the variability in mouse chromosomes, Recently, El-Nahta *et al.* (1994) explored the parental source of chromosomes in the buffalo-hamster hybrids using chromosome painting. They also reported that the mouse chromosomes were the major source of variation in the hybrid complement.

The hybrid clones were also evaluated for the retention or segregation of buffalo chromosome 4. The chromosome in question was considered retained, if observed in more than 20 per cent cells. In the present investigation, buffalo chromosome 4 was segregated in clones A9, C5 and B10 and retained in A5 and C3. In clone D12, buffalo chromosome 4 was observed in 66.67 per cent cells at 6th passage but in less than 20 per cent cells in subsequent passages. Hence, it was found retained at 6th passage and segregated in subsequent

passages. However, if the ambiguity in identification is taken into consideration, its presence exceeded 20 per cent cells at 16 and 22 passages.

5.5 ZYMOGRAM ANALYSIS

Buffalo and mouse isozymes were resolved from the hybrid cell lysate by enzyme electrophoresis followed by specific histochemical staining. The hybrid clones were evaluated for the expression of buffalo isozymes of LDHB, PEPB and TPI and thus, the presence of encoding genes.

5.5.1 LDHB

LDHB is a tetramer and may contain peptides derived from the same or different loci. It is produced in four different isozyme forms in the same species due to combination of different subunits. (Harris and Hopkinson, 1976). Each band represents one isozyme. However, as the expression of an isozyme is tissue and time (age) specific, number of bands exhibited depends on the type and physiological growth of tissue used as a source of isozymes. Buffalo cells exhibited all four anodal bands while mouse cells only three. Less number of bands in RAG might be due to extremely low intensity of band below the level of detection or an absence of the isozyme. As the RAG is not the normal mouse cells but adenocarcinoma in origin, possibility of altered expression of some of the isozymes can not be ruled out. Variable number of bands, from two to five have been reported in different mouse cell lines (Nabholz *et al.*, 1969; Dain *et al.*, 1984, Forster and Hecht, 1986; Ryttman *et al.*, 1986).

91

The mobility of both the parental isozymes was identical. Hence, it was not possible to resolve the parental isozymes in the hybrid clone. However, presence of buffalo isozymes in the clones D12, A5, C3, C5, F6 and H4 could be predicted from the expression of all the bands corresponding to those exhibited by buffalo cells and an additional band. The additional band indicated the presence of an interspecific heteromeric molecule of LDHB which could have resulted from the polymerization of subunits from different parents. Presence of heterometeric isozymes have been earlier described by de Hondt *et al.* (1991) in the buffalo-hamster somatic cell hybrids. The situation of active heteropolymers of LDHB has been observed in case of other interspecific somatic cell hybrids also (Saidi-Mehtar *et al.,* 1981; Forster and Hecht, 1986). The isozyme bands in the hybrids were more intense. This could be due to the overlapping of identical buffalo and mouse isozymes. Identical mobility of parental isozymes has also been reported in human - mouse and human - Chinese hamster hybrids (Meera Khan, 1971). Contrarily, buffalo and Chinese hamster isozymes were reported to be distinguishable (de Hondt *et al.,* 1991).

5.5.2 PEPB

Both, buffalo and mouse cells exhibited only one anodal band migrating differentially. Thus, it is possible to distinguish buffalo and mouse isozymes in buffalo-mouse hybrids by their differential migration rate. Clones A9, G3, B10, E2, E9 and E11 expressed only one band corresponding to mouse PEPB indicating absence of buffalo PEPB, while the other clones exhibited two bands corresponding to both mouse and buffalo PEPB suggesting presence of buffalo PEPB in these clones. There was no any intermediate bands suggesting

an absence of heteromeric hybrid molecule. It is not clear at present whether the absence of the heteromolecule is an intrinsic to buffalo PEPB or the present assay is not sensitive enough to detect it. Heuertz and Hors-Cayla (1981) and Saidi-Mehtar *et al.* (1981) have also reported one anodal band in cattle, sheep and Chinese hamster with differential migration. Human and Chinese hamster forms of PEPB found to be electrophoretically identical (Chen *et al.,* 1973).

5.5.3 TPI

Buffalo and mouse, both expressed one TPI band but because of differential mobility of buffalo and mouse isozymes, it was possible to distinguish them in hybrid clones. Clone D12, A5, C3, C5, F6 and H4 expressed both buffalo and mouse isozymes while, the other clones displayed zymogram pattern identical to mouse cells indicating absence of buffalo isozymes. There was no any intermediate bands suggesting an absence of heteromeric hybrid molecule.

TPI isozymes are dimeric. One major band (of primary isozymes) and few minor bands (of secondary isozymes) are detected (Harris and Hopkinson, 1976). However, in the present study no minor bands could be visualized probably due to extremely low intensity of the bands.

Heuertz and Hors-Cayla (1981) observed one parental band with non-identical migration rate in cattle-hamster hybrids. Sheep and Chinese hamster also displayed one band each with different electrophoretic mobility. Their

somatic cell hybrids also did not express any heteromeric TPI isozyme molecules (Saidi-Mehtar *et al.,* 1981).

5.6 SYNTENY IDENTIFICATION AND CHROMOSOMAL ASSIGNMENT

When developing the physical gene map of the buffalo, high degree of chromosome homology existing between buffalo and cattle enables to extrapolate the extensive information already available for cattle gene map to the buffalo. Previously it was shown in a panel of bovine - hamster hybrid clones that genes coding for the enzymes LDHB, PEPB and TPI were syntenic in cattle (Womack and Moll, 1986). This group constitute cattle syntenic group U3 and is assigned to chromosome 5 (Fries *et al.,* 1991). Based on the banding homology, cattle chromosome 5 corresponds to the long arm of buffalo chromosome 4 (Iannuzzi, 1994). Therefore,it is expected that in the buffalo, the genes coding for LDHB, PEPB and TPI are located on long arm of chromosome 4. In order to test this hypothesis a buffalo mouse hybrid panel was developed and screened for simultaneous presence or absence of these three enzymes and buffalo chromosome 4.

These enzymes were concordantly present (or absent) in the hybrid clones. Discordance was not observed in any of the clones. Hence, the genes coding for these enzymes are likely to be located on the same chromosome and thus syntenic. Again the concordance between occurrence of buffalo chromosome 4 and expressions of these buffalo enzymes in the hybrid clones could also be established. Hence, these genes are most likely to be located on buffalo chromosome 4. These genes are also syntenic in human (Chen *et al.,*

1973; Kao *et al.,* 1976), cattle (Heuertz and Hors-Cayla, 1981; Womack and Moll, 1986), sheep (Saidi-Mehtar *et al.,* 1981; Jones *et al.,* 1985), and pig (Ryttman *et al.,* 1986) and located on cytogenetically homologous chromosomes. The synteny between LDHB and TPI was investigated in Egyptian buffalo by de Hondt *et al.* (1991). The synteny was also found to be conserved in buffalo and later assigned to chromosome 4 (El-Nahas *et al.,* 1993). The present investigation confirms this syntenic identification and its chromosomal assignment.

Summary

SUMMARY

Gene mapping in buffalo is of recent origin. First report on buffalo gene mapping appeared only during 1991. Present study was undertaken to establish a synteny between the genes LDHB, PEPB and TPI and to assign to the specific buffalo chromosome through somatic cell hybridization.

Hybridization between buffalo leucocytes and mouse cell line, RAG was achieved by suspension fusion technique using polyethylene glycol as fusogen. Effect of duration of PEG exposure, concentration and grade of PEG and parent cell concentration were evaluated to maximize the hybridization frequency. Among the four durations of PEG exposure, 30, 60, 90 and 120 seconds, 90 second exposure to 50% PEG was found to be an optimum one. Reduction in concentration from 50 to 45 per cent along with incorporation of DMSO and reduction in grade of PEG from 3350 to 1450 resulted in improvement of hybridization frequency. PEG-1450 at 50 per cent concentration gave the highest hybridization frequency. However, PEG-1450 at 45 per cent concentration also gave comparable results. Hybrid cell colony output was also influenced by density of PEG-treated cells in the microtitre plates. The density, 1×10^5 cells/well gave maximum hybrid cell colonies.

Buffalo-mouse hybrids were selected in HAT medium and subsequently maintained in growth medium. Fast growing twelve clones (A9, B12, G3, A5, B10, C3, C5, E2, E9, E11, F6 and H4) were propagated for karyotypic and isozymic analyses. Both parental cells as well as uncloned hybrids were also analysed karyotypically. Buffalo karyotype was prepared from GTG-banded

metaphase chromosomes. It consisted of five pairs of metacentric and twenty pairs of acrocentric chromosomes including the sex chromosomes. The X-chromosome was the largest acrocentric while, the Y-chromosome was similar in size to chromosome 22. All the buffalo chromosomes were having G-negative centromeric regions. RAG was heteroploid in nature. Chromosome number in RAG ranged from 61 to 72 with modal number being 66. Large proportion of cells possessed 64 to 67 chromosomes. GTG-banding of RAG chromosomes revealed that it did not possess diploid configuration and few chromosomes were observed in single and few in multiple copies. All RAG chromosomes exhibited G-positive centromeric regions.

Uncloned hybrids exhibited wide distribution of chromosomes and two modal values. Twelve clones which constituted the hybrid panel, showed marked variation in their chromosome composition. The modal chromosome numbers in these clones varied from 66 to 164. Several clones possessed modal number which exceeded the sum of those of both the parents indicating that they might have resulted from the fusion of more than two cells.

The clones maintained beyond first passage were evaluated on every fourth or fifth passage to ascertain their chromosome content. Chromosome content was reduced in all the clones except in A9, indicating slow and progressive segregation of chromosomes from the hybrid complement. Much of the chromosome loss, however, occurred before the clones were analysed, most expectedly, immediately after fusion. Chromosomes of both parental origin were segregated. However, the segregation was non-random and the loss of biarmed chromosomes was less frequent. Clone A9 was very similar to RAG in

its chromosome composition and there was no apparent loss of chromosome in this clone. These hybrid clones were screened for the presence of buffalo chromosomes. in their composition. Presence of buffalo chromosomes was judged based on characteristic G-negative banding of their centromeric region. Very few buffalo chromosomes were retained in these clones. These clones were also evaluated for the retention or segregation of buffalo chromosome 4. It was scored retained if observed in more than 20 per cent cells. It was segregated in clones A9, C5 and B10 and retained in D12, A5 and C3.

The hybrid clones were evaluated for the expression of buffalo isozymes of LDHB, PEPB and TPI and thus, the presence of encoding genes. Buffalo and mouse isozymes were resolved from the hybrid cell lysate by enzyme electrophoresis followed by specific histochemical staining. Clones D12, A5, C3, C5, F6 and H4 expressed both buffalo and mouse isozymes, while the remaining six clones expressed only mouse isozymes, and no buffalo isozymes.

The enzymes, LDHB, PEPB and TPI were concordantly present or absent in all the clones. Discordance was not observed in any of the clones. Hence, the genes coding for these enzymes are likely to be located on the same chromosome and thus, syntenic. Also, the concordance was observed between occurrence of buffalo chromosome 4 and expression of these enzymes in those clones. Hence, these genes are likely to be located on buffalo chromosome 4.

The physical gene map of buffalo can be enriched further on availability of information regarding syntenic relationship of genes in cattle and the chromosomal homologies between these species.

References

REFERENCES

Adkison, L.R., D.W. Leung, and J.E. Womack, 1988a. Somatic cell mapping and restriction fragment analysis of bovine alpha and beta interferon gene families. Cytogenetics and Cell Genetics. **47** : 62-65.

Adkison, L.R., L.C. Skow, T.L. Thomas, M. Petrash, and J.E. Womack, 1988b. Somatic cell mapping and restriction fragment analysis of bovine genes for fibronection and gamma crystallin. Cytogenetics and Cell Genetics. **47** : 155-159.

Arruga, M.V., L.V. Monteagudo, and M.T. Tejedor, 1992. Assignment of two markers carried by human chromosome 1 to different cattle synteny groups : FH to U1 and PEPC to U17 (chomosome 8). Cytogenetics and Cell Genetics. **59** : 45-47.

ATCC, 1988. American Type Culture Collection, Catalogue of Cell Lines and Hybridomas. Robert, H., M. Macy, T.R. Chen, P. McClinton, and Y. Reid (eds). 6th edition, Maryland, USA.

Baker, R.M., D.M. Brunette, R. Mankovitz, L.H. Thompson, G.F. Whitemore, L. Siminovitch, and J.E. Till, 1974. Ouabian - resistant mutants of mouse and hamster cells in culture. Cell. **1** : 9-21.

Barski G., S. Sorieul, and F. Cornefert, 1960. Production dans des cultures in vitro de deux sauches cellulaires en association, de cellules de caractere 'hybride'. C.R. Acad. Sci. (Paris). **251** : 1825-1827. Cited from Weiss and Ephrussi, 1966.

Basrur, P.K., T.A. Bongso, M. Harisah, M.Hilmi, T.I., Azmi, and M.K.Vidyadaran, 1988. Recent advances in cytogenetics of water buffaloes. Proceedings of Second World Buffalo Congress. New Delhi. pp. 295-318.

Beckmann, J.S. and M. Soller, 1987. Molecular markers in the genetic improvement of farm animals. Bio/Technology. **5** : 573-576.

Beckmann, J.S. and M. Soller, 1988. Detection of linkage between marker loci and loci affecting quantitative traits in crosses betwen segregating populations. Theoretical and Applied Genetics. **76** : 228-236. Cited from Fries *et al.*, 1989.

Bodmor *et al.*, 1969. Cited from Kao and Puck, 1970.

Bongso, T.A. and M.Hilmi, 1982. Chromosome banding homologies of a tandem fusion in river, swamp and crossbred buffaloes (*Babulus bubalis*) Canadian Journal of Genetics and cytology. **24** : 667-673.

Broad, T.E., C. Jones, and D. Patterson, 1984. Gene mapping in the sheep : Synteny of SOD 1, PRGS and PAIS. Eighth International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **37** : 427 (Abst).

Bruns, G.A.P., A.C. Leary, R.E. Eisenman, C.W. Bazinet, V.M.Regina, and P.S.Gerald, 1978a. Expression of ACONs and GALT in man-rodent somatic cell hybrids. Cytogenetics and Cell Genetics. **22** : 172-176.

Bruns, G.A.P., A.C. Leary, V.M. Regina, and P.S.Gerald, 1978b. Lysosomal ß-D-Galactosidase in man-hamster somatic cell hybrids. Cytogenetics and Cell Genetics. **22** : 177-181.

Chen, T.R. 1987. Cytogenetics of somatic cell hybrids. II. Modal karyotypes and mosaicism in mouse mutant cell lines : LM(tk$^-$), A9, Rag. Cancer Genetics and Cytogenetics. 24 : 95-107.

Chen, T.R., F.A. McMorris, R.Creagan, F.Ricciuti, J. Tischfield, and F.Ruddle (1973). Assignment of the genes for malate oxidoreductase decarboxylating to chromosome 6 and peptidase B and lactate dehydrogenase B to chromosome 12 in man. Americal Journal of Human Genetics. **25** : 200-207.

Chowdhary, B.P., I. Harbitz, W.Davies, and I.Gustavsson, 1991. Tentative chromosomal assignment of the glucose phosphate isomerase gene in cattle, sheep and goat by *in situ* hybridization. Genetics Selection Evolution. **23** (Suppl 1) : 91s-95s.

Croce , C.M., 1976. Loss of mouse chromosomes in somate cell hybrids between HT-1080 human fibrosarcoma cells and mouse peritoneal macrophages. Proceedings of National Academy of Sciences, USA. **73** : 3248-3252.

Dain, A.R., E.M. Tucker, R.A. Donker, and S.W. Clarke, 1984. Chromosome mapping in cattle using mouse myeloma/calf lymphnode cell hybridomas. Biochemical Genetics. **22** : 429-439.

Davidson, R.L. and B. Ephrussi, 1965. A selective system for the isolation of hybrids between L cells and normal cells. Nature. **205** : 1170-1171.

Davidson, R.L. and P.S. Gerald, 1976. Improved techniques for the induction of mammalian cell hybridization by polyethylene glycol. Somatic Cell Genetics. **2** : 165-176.

Davidson, R.L., K.A. O'Malley, and T.B. Wheeler, 1976. Polyethylene glycol induced mammalian cell hybridization: Effect of polyethylene glycol molecular weight and concentration. Somatic Cell Genetics. **2** : 271-280.

de Hondt, H.A., A.A. Bosma, M. den Bieman , N.A. de Haan, and L.F.M. van Zutphen, 1991. Gene mapping in river buffalo *(Bubalus bubalis L.)*. Genetics Selection Evolution. **23** (Suppl 1): 104s-108s.

Di Berardino, D. and L. Iannuzzi, 1981. Chromosome banding homologies in Swamp and Murrah buffalo. The Journal of Heredity. **72** : 183-188.

Di Berardino, D. and L. Iannuzzi, 1982. Detailed description of R-banded bovine chromosomes. The Journal of Heredity. **73** : 434-438.

Di Berardino, D. and L. Iannuzzi, 1984. Detailed description of R-banded water buffalo chromosomes. Genetics Selection Evolution. **16** : 249-260.

Di Berardino, D., L. Iannuzzi, T.M. Bettini, and D. Matassino, 1981. Ag-NOR variation and banding homologies in two species of Bovidae : *Bubalus bubalis L.* and *Bos taurus L.* Canadian Journal of Genetics and Cytology. **23** : 89-99.

Di Berardino, D., M.B. Lioi, and D.Matassino, 1991. Sequential GTG-RBA banding pattern in prometaphase chromosomes of cattle (Bos taurus L.) Genetics Selection Evolution **23** : 273-279.

Dietz, A.B., M. Georges, D.W. Threadgill, J.E. Womack, and L.A. Schuler, 1992a. Somatic cell mapping, polymorphism and linkage analysis of bovine prolactin-related proteins and placental lactogen. Genomics. **14** : 137-143.

Dietz, A.B., H.L. Neibergs, and J.E. Womack, 1992b. Assignment of eight loci to bovine syntenic groups by use of PCR : Extension of a comparatine gene map. Mammalian Genome. **3** : 106-111.

Echard, G., J. Gellin, F. Benne, and M.Gillois, 1984. Progress in gene mapping in cattle and pigs using somatic cell hybridization. Seventh International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **37** : 458-459.

Echard, G., J.Gellin, F.Benne, and M.Gillois, 1982. Progress in gene mapping of rabbits, cattle and pigs using somatic cell hybridization. Proceedings of Fifth European Colloquium on Cytogenetics of Domestic Animals. Milano, Italy. pp.237-252.

El-Nahas, S.M., H.A. de Hondt, O.S.Othman, A.A.Bosma, and N.A. de Haan, 1993. Assignment of genes to chromosome 4 of the river buffalo with a panel of buffalo-hamster hybrid cells. Journal of Animal Breeding and Genetics. **110** : 182-185.

El-Nahta, S., C. Zijlstra, H.A. de Hondt, N.A. de Haan, A.A. Bosma, and S.M. El-Nahas, 1994. Evaluation of buffalo-hamster somatic cell hybrids using chromosome painting. Proceedings of 11th European Colloquium on Cytogenetics of Domestic Animals. Cophenhagen, Denmark.

Ephrussi, B. and M.C. Weiss, 1965. Interspecific hybridization of somatic cells. Proceedings of National Academy of Sciences, USA. **53** : 1040-1042.

FAO, 1993. FAO Quarterly Bulletin of Statistics. **6** : 33 - 39.

Ford, C.E., D.L. Pollock, and I. Gustavsson, 1980. (eds). Proceedings of First International Conference for the Standardization of Banded Karyotypes of Domestic Animals. Reading, England. Hereditas. **92** : 145-162.

Forster, M., and W. Hecht, 1984. Some provisional gene assignments in pig. Proceedings of 6th European Colloquium on Cytogenetics of Domestic Animals. pp. 351-355.

Forster, M., and W. Hecht, 1986. Assignment of genes for lactate dehydrogenase A and B in the pig to chormosome No.4 and No.5 by somatic cell hybrids. Journal of Animal Breeding and Genetics. **103**: 46-50.

Fries, R., D.W. Threadgill, R.Hediger, A.Gunawardana, M.Blessing, J.L.Jorcano, G.Stranzinger, and J.E.Womack, 1991. Mapping of bovine cytokeratin sequences to four different sites on three chromosomes. Cytogenetics and Cell Genetics. **57** : 135-141.

Fries, R., A. Eggen, and J.E. Womack, 1993. The bovine genome map. Mammalian Genome. **4** : 405-428.

Galfre, G., S.C. Howe, C.Milstein, G.W. Butcher, and J.C. Howard, 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature. **266** : 550-552.

Gall, J.G. and M.L. Pardue, 1969. Formation and detection of RNA - DNA hybrid molecules in cytological preparations. Proceedings of National Academy of Sciences, USA. **63** : 378-383.

Gallagher, D.S., L.S. Gibbs, J.B. Shaffer, and J.E. Womack, 1992a. Somatic cell mapping of bovine EC-SOD and SOD1L loci. Genomics. **12**: 610-612.

Gallagher, D.S., A.M. Ryan, L.S. Liou, K.N.Sastry, and J.E. Womack, 1993. Somatic cell mapping of conglutinin (CGN1) to cattle syntenic group U29 and fluorescence *in situ* localization to chromosome 28. Mammalian Genome. **4** : 716-719.

Gallagher, D.S., D.W. Threadgill, A.P. Jackson, P. Parham, and J.E. Womack, 1991. Somatic cell mapping of bovine clathrin light chain genes : identification of new bovine syntenic group. Cytogenetics and Cell Genetics. **56** : 154-156.

Gallagher, D.S., J.E. Womack, W. Baehr, and S.J. Pittler, 1992b. Syntenic assignments of visual transduction genes in cattle. Genomics. **14**: 699-706.

Gefter, M.L., D.H. Margulies, and M.D. Scharff, 1977. A simple method of polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genetics **3** : 231-236.

Goding, J.W. 1983. Monoclonal Antibodies: Principles and Practices. Academic Press, New York. pp.56-97.

Graves, J.A.M., 1984. Chromosome segregation from cell hybrids. I. The effect of parent cell ploidy on segregation from mouse - chinese hamster hybrids. Canadian Journal of Genetics and Cytology. **26** : 557-563.

Graves, J.A.M. and I. Barbieri, 1992. Chromosome segregation from cell hybrids. VII. Reverse segregation from karyoplast hybrids suggests control by cytoplasmic factors. Genomics. **35** : 537-540.

Gustavsson, I. and G.Rockborn, 1964. Chromosome abnormality in three cases of lymphatic leukemia in cattle. Nature. **203** : 990.

Hallerman, E.M., J.L.Theilmann, J.S. Beckmann, M.Soller, and J.E.Womack, 1988. Mapping of bovine prolactin and rhodopsin genes in hybrid somatic cells. Animal Genetics. **19** : 123-131.

Harris, H. and D.A. Hopkinson, 1976. Handbook of Enzyme Electrophoresis in Human Genetics. American Elsevier Publishing Company, Inc., New York.

Harris, H. and J.F. Watkins, 1965. Hybrid cells derived from mouse and man : artificial heterokaryons of mammalian cells from different species. Nature. **205** : 640-646.

Harper, M.E., and G.F. Saunders, 1981. Localization of single copy DNA sequences on G-banded human chromosomes by *in situ* hybridization. Chromosoma. **83** : 431-439.

Hashmi, S., P.W. Allderdice, G.Klein, and O.J.Miller, 1974. Chromosomal heterogeneity in the Rag and MSWBS mouse tumor cell lines. Cancer Research. **34** : 79-88.

Hassanane, M.S., F.Gu, B.P.Chowdhary, L.Anderson, and I. Gustavsson, 1993. *In situ* hybridization mapping of the immunoglobulin gamma heavy chain (IGHG) gene to chromosome 20q23-q25 in river buffaloes. Hereditas. **118** : 285-288.

Hayes, H., E. Petit, and B. Duterillauxe 1991. Comparison of RBA-banded karyotypes of cattle, sheep and goats. Cytogenetics and Cell Genetics. **57** : 51-55.

Hayes, S., E.Petit, C.Bouniol, and P. Popescu, 1993; Localisation of the α-S2-casein gene (CASAS2) to the homoeologous cattle, sheep, and goat chromosomes 4 by *in situ* hybridization. Cytogenetics and Cell Genetics. **64** : 281-285.

Hediger, R., H.A. Ansari, W.Backfisch, R.Fries, and G. Stranzinger, 1989. Banding homology and chromosomal localization of genes in cattle and sheep indicate extensive conservation of bovid genomes. Animal Genetics. **20** : (Suppl 1) : 95.

Hediger, R., H.A. Ansari, and G.P. Stranzinger, 1991.Chromosome banding and gene localizations support extensive conservation of chromosome structure between cattle and sheep. Cytogenetics and Cell Genetics. **57**: 127-134.

Hediger, R., J.E. Johnson, W.Barendse, R.D. Drinkwater, S.S. Moore, and D.J.S. Hetzel, 1990. Assignment of the growth hormone gene locus to 19q26→qter in cattle and 11q25→qter in sheep by *in situ* hybridization. Genomics. **8** : 171-174.

Heuertz, S. and M.C.Hors-Cayla, 1978. Carte genetique des bovins por la technique d'hybridation cellulaire. Localisation sur le chromosome X de la G6PD, la PGK, l'd-GAL a et l'HGPRT. Annales de genetique. **24** : 197-202. Cited from Heuertz and Hors-Cayla, 1981.

Heuertz, S. and M.C. Hors-Cayla, 1981. Cattle gene mapping by somatic cell hybrdization study of 17 enzyme markers. Cytogenetics and Cell Genetics. **30**: 137-145.

Hunter, R.L., and C.L. Markert, 1957. Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science. **12** : 1294-1295.

Iannuzzi, L., 1992. The standardization of river buffalo (*Bubalus bubalis L.*) banded karyotype. Proceedings of 10th European Colloquium on Cytogenetics of Domestic Animals, Utrech, The Netherlands. pp. 15-17.

Iannuzzi, L., 1994. Standard karyotype of the river buffalo *(Bubalus bubalis L.*2n=50). Report of the committee for the standardization of banded karyotypes of the river buffalo. Cytogenetics and Cell Genetics. **67** : 102-113.

Iannuzzi, L., and D. Di Berardino, 1985. Diagramatic representation of RBA-banded chromosomes of swamp buffalo *(Bubalus bubalis L.)* and sex chromosomes banding homologies with cattle *(Bos taurus L.)*. Caryologia. **34**: 281-295. Cited from Iannuzzi, 1994.

Iannuzzi, L., G.P.Di Meo, D.S. Gallagher, A.M. Ryan, I. Ferrara, and J.E. Womack, 1994a. Gene mapping in river buffalo by FISH: Present status and localization of UMPS and CGN1 genes to chromosomes 1q31 and 4p16. Proceedings of 11th European Colloquium on Cytogenetics of Domestic Animals. Copenhagen, Denmark.

Iannuzzi, L., G.P. Di Meo, A. Perucatti, and L. Ferrara, 1990a. A comparison of G- and R-banding in cattle and river buffalo prometaphase chromosomes. Caryologia. **43** : 283-290. Cited from Iannuzzi *et al.*, 1994b.

Iannuzzi, L., G.P. Di Meo, A. Perucatti, and L. Ferrara, 1990b. The high resolution G- and R-banding pattern in chromosomes of river buffalo *(Bubalus bubalis L.)*. Hereditas. **112** : 209-215.

Iannuzzi, L., D.S.Gallagher, G.P.Di Meo, A.M. Ryan, A.Perucatti, L.Ferrara, D.M.Irwin, and J.E. Womack, 1993a. Chromosomal localization of the lysozyme gene cluster in river buffalo (*Bubalus bubalis L.*). Chromosome Research. **1** : 253-255. Cited from Iannuzzi, 1994.

Iannuzzi, L., D.S. Gallagher, L.S. Liou, G.P.Di Meo, A.M.Ryan, K.N. Sastry, and J.E. Womack, 1994b. Chromosomal localization of conglutinin (CGN1) gene to river buffalo by sequential RBH-banding and FISH. Hereditas. **120**: 283-286.

Iannuzzi, L., D.S. Gallagher, A.M. Ryan, G.P.Di Meo, and J.E. Womack, 1993b. Chromosomal localization of omega and trophoblast interferon genes in cattle and river buffalo by sequential R-banding and fluorescent *in situ* hybridization. Cytogenetics and Cell Genetics. **62** : 224-227.

Iannuzzi, L., D.S.Gallagher, J.E.Womack, G.P. Di Meo, L.C. Skow, and L.Ferrara, 1993c. Chromosomal localization of the major histocompatibility complex in cattle and river buffalo by fluorescent *in situ* hybridization. Hereditas. **118** : 187-190.

Iannuzzi, L., I.Gustavsson, G.P. Di Meo, and L. Ferrara, 1989. High resolution studies on late replicating segments (G + C bands) in mammalian chromosomes. Hereditas. **110**: 43-50.

Imam-Ghali, M., N.Saidi-Mehtar, and G.Guerin, 1992. Sheep gene mapping : synteny between COL2A1 and LDHB-PEPB-TPI-GAPD-LALBA-IGF1 group. Animal Genetics. **23** : 529-532.

Imam-Ghali, M., N.Saidi-Mehtar, and M.C.Hors-Cayla, 1987. Sheep gene mapping by somatic cell hybridization : synteny of PEPC, ENO1 and PGD. Nineth International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **46** : 632 (Abst.)

ISCNDA, 1989. International System for Cytogenetic Nomenclature of Dometic Animals. Di Berarding, D., H. Hayes, R. Fries, and S. Long (eds). Cytogenetics and Cell Genetics. **53** : 65-79.

John, H.A., M.L. Birnstiel, and K.W. Jones, 1969. RNA-DNA hybrids at the cytological level. Nature. **223** : 582-587.

Johnson, S.E., W. Barendse, and D.J.S.Hetzel, 1993. The γ fibrinogen gene (FGG) maps to chromosome 17 in both cattle and sheep. Cytogenetics and Cell Genetics. **62** : 176-180.

Jones, C., H.G. Morse, D.Geyer, I.S. Scott, and T.E. Broad, 1985. Gene mapping in the sheep: assignment of LDHB, SHMT and PEPB to chromosome M3. Eighth International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **40** : 662 (Abst.)

Jongsma, A.P.M., A. Hagemeijer, and P. Meera Khan, 1974. Regional Mapping of TPI, LDHB and PEPB on chromosome 12 of man. In : Rotterdam Conference. Second International Workshop on Human Gene Mapping. Birth defects. Original Article Series. **11** (3) : 189. The National Foundation, New York. Cited from Dain *et al.*, 1984.

Jongsma, A.P.M., W.R.T. Los, and A. Hagemeijer, 1973. Evidence for synteny between the human loci for triose phosphate isomerase, lactate dehydrogenase B, and peptidase B and the regional mapping of these loci on chromosome 12. In : New Haven Conference. First International Workshop on Human Gene Mapping. Birth defects. Original Article Series. **10**(3) : 106. The National Foundation, New York. Cited from Dain *et al.*, 1984.

Joshi, C.G. 1995. Identification and assignment of syntenic group to chromosome using interspecific cell hybrids in buffalo. Ph.D. Thesis Submitted to Tamil Nadu Veterinary and Animal Sciences University, Madras.

Joshi, C.G., D.N. Rank, P.Kanakaraj, K.Kumanan, and A.R.Krishnan, 1996.

Joshi, C.G., D.N. Rank, P.Kanakaraj, V.Thiagarajan, and A.R.Krishnan, 1995. Interspecific somatic cell hybridization studies between buffalo fibroblast and RAG cells. Indian Veterinary Journal. **72** : 66-70.

Kao, F.T., C. Jones, and T.T. Puck, 1976. Genetics of somatic mammalian cells : Genetics, immunologic and bio-chemical analysis with Chinese hamster cell hybrids containing selected human chromosomes. Proceedings of National Academy of Sciences, USA. **76** : 193-197.

Kao, K.N. and M.R. Michayluk, 1974. Planta (Berlin). **115** : 355-367. Cited from Pontecorvo, 1975.

Kao, F.T. and T.T. Puck, 1967. Genetics. **55** : 513. Cited from Kao and Puck, 1970.

Kao, F.T. and T.T. Puck, 1968. Proceedings of National Academy of Sciences, USA. 60 : 1275. Cited from Kao and Puck, 1970.

Kao, F.T. and T.T. Puck, 1970. Genetics of somatic mammalian cells : linkage studies with human - Chinese hamster cell hybrids. Nature **228** : 329-332.

Kashi, Y., E.M. Hallerman, and M.Soller, 1990. Marker assisted selection of candidate bulls for progeny testing programmes. Animal Production. **51**: 63-74.

Kohler, G. and C. Milstein, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. **256** : 495-497.

Lalley, P.A., and V.A. McKusick, 1985. Report of the committee on comparative mapping. Eighth International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **40** : 536-566.

Leong, M.M., C.C.Lin, and R.F.Ruth, 1983. The localization of genes for HPRT, G6PD and α-GAL onto the X-chromosome of domestic pig *(Sus scrofa domesticus)*. Canadian Journal of Genetics and Cytology. **25** : 239-245.

Littlefield, J.W., 1964. Selection of hybrids from matings of fibroblasts *in vitro* and their presumed recombinants. Nature. **256** : 495-497.

Meera Khan, P., 1971. Enzyme electrophoresis on cellulose acetate gel : Zymogram patterns in man-mouse and man-Chinese hamster somatic cell hybrids. Archives of Biochemistry and Biophysics. **145** : 470-483.

Migeon, B.R. and C.S. Miller, 1968. Science. **162** : 1005. Cited from Kao and Puck, 1970.

Minna, J.D., and H.G. Coon, 1974. Human x mouse hybrid cells segregating mouse chromosomes and isozymes. Nature. **252** : 401-404.

Monteagudo, L.V., M.V.Arruga, M.T. Tejedor, D.Savva, K. Mazhar and C.J. Skidmore, 1992. Mapping of six genes in cattle by using a panel of interspecific hamster x cattle hybrid clones. Animal Genetics. **23** (Suppl 1) : 82.

Monteagudo, L.V., M.T. Tejedor, and M.V. Arruga, 1991. Preliminary studies on the gene map of cattle. Genetics Selection Evolution. **23** (Suppl 1): 173s-175s.

Morton, N.E., 1955. Sequential tests for the detection of linkage. American Journal of Human Genetics. **7** : 277-318. Cited from Fries, R., J.S.Beckmann, M.Georges, M.Soller, and J.Womack, 1989. The bovine gene map. Animal Genetics. **20** : 3-29.

Nabholz, M., V.Miggiano, and W.Bodmor, 1969. Genetic analysis with human-mouse somatic cell hybrids. Nature. **223** : 358-363.

Norwood, T.H., C.J.Zeigler, and G.M.Martin, 1976. DMSO enhances PEG mediated somatic cell fusion. Somatic Cell Genetics. **2** : 263.

O'Brien, S.J. 1993. (ed). Genetic Maps: Locus Maps of Complex Genomes. 6th edition, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, USA.

O'Brien, S.J. and J.A. Marshall-Graves, 1992. Report of the Committee on Comparative Mapping. Eleventh International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **58** : 1124-1151.

Okada, Y. 1958. Biken's Journal **1** : 103-110. Cited from Ephrussi and Weiss, 1969.

Olsson, L. and H.S. Kaplan, 1983. Human-human monoclonal antibody-producing hybridomas: Technical aspects. Methods in Enzymology. **92** : 3-16.

O'Malley, K.A. and R.L. Davidson, 1977. A new dimension in suspension fusion techniques with polyethylene glycol. Somatic Cell Genetics. **3** : 441-448.

Othman, O.E., D.Gallagher, H.A. de Hondt, S.M. El-Nahas, H. Oraby, A.A. Bosma, and J.Womack, 1994. Gene mapping in river buffalo : Five syntenic groups. Proceeding of 11th European Colloquium on Cytogenetic of Domestic Animals. Copenhagen, Denmark.

Pontecorvo, G., 1975. Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. Somatic Cell Genetics. **4** : 397-400.

Puck, T.T., and F.T. Kao, 1982. Somatic cell genetics and its applications to medicine. Annual Review of Genetics. **16** : 225.

Rank, D.N., C.G.Joshi, P.Kanakaraj, B.P.Brahmkshtri, V.B. Kharadi, A.M.Nainar, and A.R.Krishnan, 1996. Interspecific somatic cell hybridization between buffalo and mouse cells. Indian Journal of Animal Science. (in press).

Ruddle, F.H., 1973. Linkage analysis in man by somatic cell genetics. Nature. **242** : 165-169.

Ryan, A.M. and J.E. Womack, 1993. Somatic cell mapping of the bovine prion protein gene and restriction fragment length polymorphism studies in cattle and sheep. Animal Genetics. **24** : 23-26.

Ryttman, H., P.Thebo, I.Gustavsson, B.Gahne and R.K.Juneja, 1986. Further data on chromosomal assignements of pig enzyme loci LDHA., LDHB, MPI, PEPB and PGM1 using somatic cell hybrids. Animal Genetics. **17** : 323-333.

Saidi-Mehtar, N., M.C. Hors-Cayla and N. van Cong, 1981. Sheep gene mapping by somatic cell hybridization : Four syntenic groups : ENO1 - PGD, ME1-PGM3, LDHB-PEPB-TPI and G6PD-PGK-GALA. Cytogenetics and Cell Genetics. **30** : 193-204.

Saidi-Mehtar, N., M.Imam-Ghali, S.Heuertz, and M.C. Hors-Cayla, 1991. Sheep gene mapping by somatic cell hybridization. Genetics Selection Evolution. **23** (Suppl 1) : 196s-200s.

Scaletta, L.J. and B.Ephrussi, 1965. Hybridization of normal and neoplastic cells *in vitro*. Nature. **205** : 1169.

Seabright, 1971. A rapid banding technique for human chromosomes. Lancet. **2** : 971-972.

Siciliano, M.J. and C.R. Shaw, 1976. Separation and visualization of enzymes on gels. In : Smith, I. (ed). Zone Electrophoresis, 4th edition, pp.185-209.

Shimizu, N, Y. Shimuzu, I.Kondo, C.Woods, and T.Wegner, 1981. The bovine genes for phosphoglycerate kinase, glucose-6-phosphate dehydrogenase, alpha-galactosidase and hypoxanthine phosphoribosyl transferase are linked to the X chromosome in cattle-mouse hybrids. Cytogenetics and Cell Genetics. **29** : 26-31.

Snedecor, G.W. and W.G.Cochran, 1980. Stastical Methods. 6th Edn. The Iowa State University Press, Iowa, USA.

Soller, M., 1990. Genetic mapping of the bovine genome using DNA level markers to identify loci affecting quantitative traits of economic importance. Journal of Dairy Science. **73** : 2628-2646.

Soller, M., 1991. Mapping quantitative trait loci affecting traits of economic importance in animal populations using molecular markers. In : Schook, L.B., H.A. Lewin, D.G.McLaren (eds). Gene Mapping: Strategies, Techniques and Applications. Marcel Dekker Inc., New York. pp.159-176.

Sorieul, S. and B.Ephrussi, 1961. Karyological demonstration of hybridization of mammalian cells *in vitro*. Nature. **190** : 653-654.

Sparkes, R.S., T. Mohandas, M.C.Sparkes, and J.D.Shulkin, 1978. Aconitase (E.C.4.2.1.3) mitochondrial (ACONM) mapped to human chromosome 22. Cytogenetics and Cell Genetics. **22** : 226-227.

Thiagarajan, V., 1987. Karyological studies on buffaloes. Ph.D. Thesis Submitted to Tamil Nadu Agricultural University, Coimbatore.

Threadgill, D.W., L.R. Adkison, and J.E. Womack, 1990. Syntenic conservation between humans and cattle. II. Human Chromosome 12. Genomics. **8**: 29- .

Threadgill, D.S. and J.E. Womack, 1991a. Mapping HSA10 homologous loci in cattle. Cytogenetics and Cell Genetics. **57** : 123-126.

Threadgill, D.W. and J.E. Womack, 1991b. Synteny mapping of human chromosome 8 loci in cattle. Animal Genetics. **22** : 117-122.

Trask, B.J., 1991. Fluorescence *in situ* hybridization : application in cytogenetics and gene mapping. Trends in Genetics. **7** : 149-154.

Weiss, M.C., and B. Ephrussi, 1965. Genetics **54** : 1095. Cited from Weiss and Green, 1967.

Weiss, M.C., and B. Ephrussi, 1966. Studies of interspecific (rat x mouse) somatic cell hybrids. I. Isolation, growth and evolution of the karyotype. Genetics. **54** : 1095-1109.

Weiss, M.C. and H. Green, 1967. Human-mouse hybrid cell lines containing partial complements of human chromosomes and functioning human genes. Proceeding of National Academy of Sciences, USA. **58** : 1104-1111.

Wijnen, L.M.M., K.H. Grzeschik, P.L. Pearson, and P. Meera Khan, 1977. The human PGM-2 and its chromosomal localization in mouse-man hybrids. Human Genetics. **37** : 277-278.

Womack, J.E. 1990. Gene mapping in the cow. In : R.A. McFeely (ed). Advances in Veterinary Science and Comparative Medicine. **34** : 251-271. Academic Press Inc., San Diego.

Womack, J.E., L.R.Adkison, J.C. McAvin and L.C. Skow, 1987. Comparative gene mapping: assignment of PRGS, PAIS, GH, IFNA, IFNB, IFNG, CRYA1, CRYG, 210H and the MHC to bovine syntenic groups. Nineth International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **46** : 719 (Abst.)

Womack, J.E. and J.M. Cummins, 1984. Comparative gene mapping : interferon sensitivity (IFREC) and cytoplasmic superoxide dismutase (SOD1) are linked in cattle. Cytogenetics and Cell Genetics **37** : 612.

Womack, J.E., A.B. Dietz, D.S.Gallagher, L.Li, N. Zhang, H.L. Neibergs, Y.D. Moll, and A.M. Ryan, 1991. Assignment of 47 additional comparative loci to the bovine synteny map. Eleventh International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **58** : 2131 (Abst.)

Womack, J.E., J.C. McAvin, and L.C. Skow, 1986. Comparative mapping of human chromosome 21 markers : somatic cell studies of IFREC, PRGS PAIS, SOD1, and CRYA1 in cattle. American Journal of Human Genetics. **39** : 4173 (Abst.)

Womack, J.E. and Y.D. Moll, 1986. Gene map of the cow : conservation of linkage with mouse and man. The Journal of Heredity. **77** : 2-7.

Womack, J.E., D.W. Threadgill, Y.D. Moll, L.K. Faber, M.L. Foreman, A.B.Dietz, T.C. Tobi, L.C.Skow, S.M. Zneimer, D.S.Gallagher, and D.S.Roger, 1989. Syntenic mapping of 37 loci in cattle : chromosomal conservation with mouse and man. Tenth International Workshop on Human Gene Mapping. Cytogenetics and Cell Genetics. **51** : 1109 (Abst.)

Yerganian, G. and M.B.Nell, 1966. Hybridization of dwarf hamster cells by UV inactivated Sendai virus. Proceedings of National Academy of Sciences, USA. **55** : 1066-1073.

Zijlstra, C., A.A.Bosma, and N.A. de Haan, 1994. Comparative study of pig-rodent somatic cell hybrids. Animal Genetics. **25** : 319-327.

Zimmermann, 1986. In : Bartal, A.H. and J.Hirschaut (eds). Hybridoma Formation. Humana Press, Clifton New Jersey. Cited from Zimmermann and Urnovitz, 1987.

Zimmermann, U. and H.B.Urnovitz, 1987. Principles of electrofusion and electropermeabilization. Methods in Enzymology. **151** : 195-221.

Zneimer, S.M. and J.E.Womack, 1989. Regional localization of the fibronectin and γ-crystallin genes to bovine chromosome 8. Genomics. **5** : 215-220.