USE OF FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) FOR STUDYING CENTROMERE ORGANIZATION AND CENTRIC FUSIONS IN CATTLE

by

JIANZE ZHENG

B.SC (Hons.) (China), M.Sc in Cell Biology & Cytogenetics (China)

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Department of Animal Science

University of Adelaide

South Australia

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Abbreviations

AMCA	aminomethylcoumarin acetic acid
bp	base pair
BrdU	5-bromodeoxyuridine
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
cm	centimeter
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytocine 5'-triphosphate
DIG	digoxygenin
dGTP	2'-deoxyguanosine 5'-triphosphate
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's media
DNA	deoxyribonucleic acid
DS	dextran sulphate
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
F-12-dUTP	fluorescein-12-2'-deoxyuridine 5'-triphosphate
FBS	foetal bovine serum
FISH	fluorescent *in situ* hybridization
FITC	fluorescein isothiocyanate
g	gram
G	guanine
gent.	gentamycin
glut.	glutamine
HBSS	Hank's balanced sodium salts (Ca^{2+}, Mg^{2+} free)
hep.	heparin
Leishman's stain	eosin-polychrome methylene blue
L	liter
M	molar
mg	milligram
ml	milliliter

mM	millimolar
ng	nanogram
NSS	new-born sheep serum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PPD9	para-phenylenediamine or para-phenylenediamine dihydrochloride (pH9)
PPD11	para-phenylenediamine or para-phenylenediamine dihydrochloride (pH11)
P/S/F	penicillin/streptomycin/fungizone
PWM	poke weed mitogen, lectin
RPMI	Roswell Park Memorial Institute medium 1640
rRNA	ribosomal RNA
SPRINT	super-fast primed *in situ* targeting
SSC	sodium chloride sodium citrate
ssDNA	salmon sperm DNA
Tdr	thymidine
Tris-HCl	Tris[hydroxymethyl] aminomeethane hydrochloride
TRITC	tetramethylrhodamine isothiocyanate isomer R
Tween 20	polyoxyethylenesorbitan monolaurate
µg	microgram
µl	microliter
µM	micromolar

NAME: JIANZE ZHENG COURSE: .....

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ABSTRACT

The most common chromosome abnormalities in live cattle are various Robertsonian translocations (centric fusions). The progeny of a Robertsonian translocation carrier will be at approximately 50% risk of inheriting the affected chromosome. The only phenotype associated with balanced Robertsonian translocations in cattle is generally thought to be sub-fertility. Some of centric fusion chromosomes are monocentric [eg. t(1;29) in cattle] but most are dicentric [eg. t(14;20), t(16;18) in cattle]. Two hypotheses have been used to explain how monocentric Robertsonian translocation chromosomes form. However, it is still unclear why ancient fusion chromosomes are monocentric and recent fusion chromosomes are dicentric.

In the research herein, four main cattle procentric Satellite sequences were used as single- and two-colour fluorescence *in situ* hybridization (FISH) probes for studying the centromere organization of cattle autosomes and the rearrangement in two cattle Robertsonian translocation chromosomes, the t(1;29) which is monocentric and found in numerous breeds all over the world, and the t(14;20) which is dicentric and found in two breeds. To make the FISH more efficient and reduce the risk of artefacts, cytogenetic techniques for cattle, including leukocyte culture, slide preparation, FISH, and chromosome banding techniques were modified. At the same time, the chromosome association in Jersey cattle previously reported by Gordon (1995) was examined.

The conclusions from this research are as follows:

i) The chromosome association in Jersey cattle is likely to be an artefact.

ii) Satellite II may be the centromeric DNA repeat sequence in cattle autosomes.

iii) The pattern of the four Satellite sequences on procentric region of chromosomes seems to be better explained by a mixed Block/Interspersed model rather than a Block model.

The centromere organization seems to be polymorphic and vary between individual chromosomes. Certainly, the amount of particular satellites on specific chromosomes is polymorphic.

iv) From the order of Satellite I~IV on cattle chromosomes 14, 20 and t(14;20), the formation of the dicentric Robertsonian translocation chromosome t(14;20) can be interpreted by a simple breakage and fusion model with both original centromeres retained in the centric fusion.

v) The formation of the monocentric Robertsonian translocation chromosome t(1;29) cannot be interpreted in simple mechanistic terms. The total loss of Satellite I from both original chromosomes 1 and 29, and the partial loss of Satellite III from the original chromosome 1 precludes a breakage and fusion model. It suggests either a further reduction and rearrangement of satellite sequences subsequent to the centric fusion or some other form of instant loss of Satellite I.

vi) Two new hypotheses are considered to explain how monocentric Robertsonian translocation chromosomes are generated: either direct formation, or evolution from dicentric chromosomes.

Chapter 1

LITERATURE REVIEW

1.1 Introduction

Chromosomes were identified by Waldeyer in 1888 and later proved to be the carrier of hereditary information in the early 1900's (Nora and Fraser, 1981). The chromosome number in different cells of an individual and among individuals of a species is normally constant, eg. human male, 46,XY. For animals, the number of chromosomes is generally double in a somatic cell (called diploid, 2n) compared to the number in a sexual gamete (called haploid, n). The size and shape of each chromosome at metaphase of mitosis or meiosis are relatively stable.

The karyotype of a species is the number, morphology and sex determination pattern of chromosomes in the species. The morphology of a chromosome refers to the size of the chromosome and the location of its centromere. For example, a chromosome will be metacentric, submetacentric or acrocentric depending upon the location of the centromere (Fig 1.1). For metacentric and sub-metacentric chromosomes, the short petite (p) and long (q) arms of the chromosome are evident. However, for acrocentric chromosomes, the short (p) arm may not be visible even under the highest magnification of routine light microscopy (White, 1973).

Chromosomal variation in number or in structure (called chromosomal aberrations) can affect the phenotype of an individual. In mammals, particularly humans, many chromosomal anomalies have been correlated with clinical problems such as early embryonic death, abortion, congenital anomalies and reduced reproductive efficiency (Logue, 1978; Nicholas, 1987). In addition to the study of human chromosomes, many chromosome studies have focused on economically important animals (eg. cattle, sheep and pigs). When such studies relate to the evolution of organisms, the field is termed evolutionary cytogenetics (White, 1973; King, 1995).

1.2 The cattle karyotype

The cattle karyotype was first described by Krallinger in 1927 using testicular tissue (Gustavsson, 1977), and was verified using cultured leukocytes in 1960 to be 2n=60,XY for male and 2n=60,XX for female (Fig 1.2) (Eldridge, 1985).

Breeds of cattle belong to two species in the genus *Bos*: *B. taurus* from Europe and *B. indicus* from Asia (Eldridge, 1985). Normally, both *B. taurus* and *B. indicus* have 60 chromosomes. The only obvious morphological difference between the chromosomes of *B. taurus* and *B. indicus* is the Y chromosome. The Y chromosome is the smallest acrocentric in *B. indicus* and is a small submetacentric or metacentric chromosome in *B. taurus*. All autosomes in both species are acrocentric. The X chromosome, although often described as metacentric, is actually a submetacentric chromosome (Zheng and Webb, unpublished).

The application of chromosome banding techniques since the 1970s has provided better methods for chromosome identification, more detailed information on individual chromosomes and helped to further the research on the structure and organization of chromosomes (Gustavsson, 1977 and 1991; Eldridge, 1985). The banded karyotype of cattle was standardized by the Reading Karyotype Conference in 1976. However, all chromosome pairs except for the longest autosomes and the sex pairs are of similar size and morphology, which has caused great identification problems (Eldridge, 1985).

1.3 Chromosome structure and centromere repeats

A eukaryotic genome typically consists of 3 molecular components (Hartl, 1994): 1) unique (single copy) sequences, which are usually the major component and located in euchromatin (also called the gene rich region); 2) highly repetitive sequences, most of which

are localized in blocks of tandem repeats and located in heterochromatin; and 3) middle-repetitive sequences, which comprise many families of related sequences and include several groups of genes, such as major ribosomal RNA (18s and 28s rRNA).

Chromosomes have a number of structurally important features related to these molecular components, including the centromeres, telomeres and nucleolar organizer regions (NORs). At the ends of chromosomes are telomeres, which attach to the nuclear membrane at interphase. Telomeres are important for the stability of the chromosomes and may help homologous chromosome pairing during meiosis (Suzuki *et al.*, 1989). The centromeric region is the dynamic center of the chromosome and is responsible for the chromosome movement during nuclear divisions. In addition to the narrowing attributed to the centromere, some of chromosomes have secondary constrictions which frequently contain nucleolar organizing regions (NORs). These regions are DNA sequences which encode major ribosomal RNA (rRNA) (Rothwell, 1988). During interphase, the NORs attach to the nucleolus at interphase. Consequently, the residuum of NORs at metaphase may appear to be "associated".

Both the centromeres and telomeres are heterochromatic and consist of abundant repeat elements (de la Sena *et al.*, 1995; Jobse *et al.*, 1995). The telomeric repeats are highly conserved in sequence and amount. However, the centromeric repeats, known as satellite DNA, are species specific and differ in number, exact sequence and organization between species. In human centromeres, for instance, there is an abundance of α-satellite, and some Satelllites 1, 2 and 3, β-satellite, γ-satellite, a 48-bp repeat and a number of dispersed repeat sequences (Kalitsis and Choo, 1997).

In cattle, Satellite I (1.715 gm/cm^3, result of density gradient centrifugation), Satellite II (1.723 gm/cm^3), Satellite III (1.706 gm/cm^3) and Satellite IV (1.709 gm/cm^3) are the major centromeric repeats (Modi *et al.*, 1993; Jobse *et al.*, 1995). The repeat sequences of Satellite I

and II are detected in all autosomes in cattle, but not all cattle autosomes have repeats of Satellite III and IV (Kurnit *et al.*, 1973; Finashin *et al.*, 1992; Modi *et al.*, 1993). The organization of these satellite sequences has not been elucidated yet in cattle. Satelllite sequences on the sex chromosomes have not been described; none of the four major Satellite sequences I, II, III or IV are found in the cattle X- and Y- chromosomes.

1.4 Chromosomal aberrations

The first disorder that could be traced to a chromosomal abnormality was described by Lejeune *et al.* in 1959. The disorder was Down Syndrome in humans and is caused by an extra chromosome 21 (2n=47,XX/XY,+21) (Nora and Fraser, 1981; Gustavsson, 1991). Many studies since then have been devoted to understanding the relationship between phenotypic malformations and chromosomal aberrations. The patterns of inheritance of chromosomal aberrations are characteristically non-Mendelian (Schulz-Schaeffer, 1980; Nora and Fraser, 1981). For example, human carriers of extensive pericentric inversions, which have a high chance of crossing over within the mutually inverted region, would be expected to produce approximately 45% abnormal progeny with a partner of either sex. This outcome is not indicative of dominance, recessiveness nor sex linkage.

1.4.1 Types of chromosomal aberrations

Chromosomal abnormalities are most often caused by errors in meiosis, although mitotic mistakes occurring during early cell divisions of the zygote can also lead to chromosomal abnormalities. Chromosomal abnormalities are divided into two classes, abnormalities in number and in structure (Schulz-Schaeffer, 1980; Nora and Fraser, 1981; Eldridge, 1985).

1.4.1.1 Chromosomal aberrations in number

Numerical chromosomal anomalies include euploid and aneuploid aberrations. Euploid aberration is defined as a change in the number of chromosomes in somatic cells which involves the entire chromosome set, while an aneuploid aberration is the loss or gain of one or more chromosomes, but does not include the entire chromosome set.

The euploid variations, such as haploidy (n, where n refers the chromosome number of a basic genome of a species) and polyploidy (*eg.* 3n), are mostly found in plants and insects. For example, various bread wheats are hexaploids (6n=42), the seedless watermelon is a triploid (3n=33), and the male bee is a haploid (n=16). Another hymenopteran, an ant, *Myrmecia pilosula*, has the lowest possible chromosome number (n=1 in the male) (Crosland and Crozier, 1986). These examples are the results either of natural evolution or artificial culture for breeding and economic purposes (Schulz-Schaeffer, 1980). In mammals, euploid variations can be found in some somatic cells of an individual or in aborted embryos. Triploid (3n) and tetraploid (4n) humans can be liveborn, although invariably are short-lived (Gardiner and Sutherland, 1996). No liveborn cattle have been found with euploid aberrations (Boland *et al*, 1984; Eldridge, 1985; Murray *et al.*, 1985-1; Murray *et al.*, 1985-2).

Aneuploid variations include monosomy (2n-1) and trisomy (2n+1). Autosomal monosomy has never been observed in mammals, even in the extensively investigated human field (Gardiner and Sutherland, 1996). It is presumably lethal at the single cell or very early embryonic level. X-chromosome monosomy is possible in liveborn animals and has been documented for a variety of mammals (Nicholas, 1987). It is widely known as a cause of Turner syndrome in humans. Even though only one X chromosome of a normal mammal female is active during the most of her life, a female that only has one X chromosome is sterile or has extremely low fertility due to very early secondary amenorrhoea. Y chromosome monosomy with consequent X chromosome nullisomy has not been observed in liveborn mammals.

In mammals, trisomy always causes disorders, weak growth, low fertility, early death of individuals or embryo abortion. Trisomy for all of the human autosomes has been observed in aborted material although the reported case of trisomy 19 is probably an error and trisomy 1 has been found only in a 16-cell embryo (Webb, pers. comm.). Humans carrying trisomy 13, 18 and 21 are frequently liveborn, although trisomy 13 and 18 individuals are short-lived. In general, liveborn trisomic mammals are phenotypically abnormal and sterile.

The polysomic aberrations of the X chromosomes are a special case because of the inactivation of all but one X-chromosome (Lyon's single active X hypothesis). However, the effect of additional X chromosomes is more severe in males than in females. A woman with 2n=47,XXX is fertile and looks like a normal female, but a man with 2n=47,XXY is sterile and somewhat feminized. More than one additional X chromosome leads to mental retardation which is more severe in males; for example, 49,XXXXX females are only moderately retarded, whereas 49,XXXXY males are profoundly retarded. Additional Y chromosomes in human males do not have major phenotypic effects, but behavioral changes and reduced fecundity have been noted (Han *et al.*, 1994).

1.4.1.2 Chromosomal aberrations in structure

The structural anomalies of chromosomes are caused by rearrangements occurring, usually during interphase, within chromosomes and between chromosomes. They generally include deletions, duplications, inversions, insertions and translocations. The first three structural anomalies usually only involve one chromosome pair, while translocations involve at least two chromosome pairs. Most of the structural anomalies are the results of incorrect reunion after chromosomal breaks (Schulz-Schaeffer, 1980; Nora and Fraser, 1981).

If all normal chromosomal segments are present, the rearrangement is said to be balanced and the individual carrier is phenotypically normal except in very rare cases where a break has disrupted a gene sequence. However, the carriers of balanced rearrangements often suffer

decreased fertility because they are likely to have unbalanced embryos and are prone to abort. If chromosomal segments are duplicated or deleted, then the rearrangement is said to be unbalanced. Carriers of unbalanced rearrangements are usually phenotypically abnormal with deletions having a more severe effect than duplications.

1.4.1.2.1 Rearrangements within chromosomes

1.4.1.2.1.1 Deletions

In deletions (del or -), chromosomal material is lost (Fig 1.3A). A deletion is usually the loss of an interstitial segment of a chromosome. The loss of a terminal segment is unlikely because of the requirement for telomeres (Blackburn and Greider, 1995). The deletion of a chromosomal fragment usually causes an individual to show phenotypic variation in morphology and physiology, and is often lethal because the hereditary material is unbalanced (Schulz-Schaeffer, 1980).

1.4.1.2.1.2 Duplications (repeats)

In duplications (dup or +), DNA sequences are repeated (Fig 1.3B). In general, the phenotypic expression of duplications is less severe than deletions (Schulz-Schaeffer, 1980). Gene duplication can occur in many places in most genomes with little or no phenotypic effect, but may behave like a dominant mutation in the heterozygous condition. Occasionally, fertility will be affected if the duplication interferes with chromosome pairing. On the other hand, the rare duplication of large, visible chromosomal segments usually produces severe phenotypic effects.

1.4.1.2.1.3 Inversions

An inversion results from two chromosomal breaks and reversal of the intervening segment before reunion. Thus, in an inversion (inv), genes can be in the reverse order (Fig 1.3C). Inversions are of two types: i) paracentric with the breaks in the same chromosome

arm, and ii) pericentric with breaks on each side of a centromere. If a chiasma occurs in the paired, mutually inverted regions of a paracentric inversion during meiosis, then the recombinant strands form a dicentric chromosome and an acentric fragment. This usually leads to loss of the resulting gametes or zygotes. If a similar chiasma occurs in a pericentric inversion, the recombinant strands show duplication of the same telomeres and corresponding deletions of distal segments. Again, the recombinants lead to abnormal gametes and zygotes. However, pericentric inversions are more likely to develop to term, if the rearranged terminal segments are small, because no centromeres are joined or lost from the chromosomes and only small amounts of chromosomal material are duplicated and deleted (Daniel, 1988).

1.4.1.2.2 Rearrangements between chromosomes

1.4.1.2.2.1 Translocations

Translocations (t) are defined as the exchange of genetic material from one chromosome to another, usually between nonhomologous chromosomes (Fig 1.3D) (Emanuel, 1993).

In a reciprocal translocation, two chromosomes are broken and the broken segments exchange locations, with no loss of genetic material. However, this does affect the pairing and segregation of chromosome pairs during meiosis. Therefore, the main effect of reciprocal translocations is semi-sterility in the carrier parent (Schulz-Schaeffer, 1980; Nora and Fraser, 1981; Nicholas, 1987).

Reciprocal translocations are probably the most common type of *de novo* translocation. As with pericentric inversions, the position of breakpoints of a reciprocal translocation may allow only small duplications and deletions, resulting in an abnormal liveborn rather than a spontaneous abortion (Daniel, 1988).

Nonreciprocal translocations occur when only one product is derived from a translocation. However, all such cases are now considered as reciprocal translocations at the

very end of the chromosomes (Nicholas, 1987) with the loss of two telomeres. Nonreciprocal translocations may also involve the loss of a centromere, unless the single product is dicentric. Very small chromosomes or acentric fragments tend to be lost during cell division.

1.4.1.2.2.2 Robertsonian translocations

Robertsonian translocations, also called centric fusions, are a special case of nonreciprocal translocations and involve the fusion of the long arms (q) of two acrocentric chromosomes at their centromeres and the loss of the short arms (p) plus usually one centromere to form a new monocentric chromosome (Nora and Fraser, 1981; Eldridge, 1985; Long, 1985; Nicholas, 1987). However, some fused chromosomes are dicentric where the two centromeres generally are very close and work as one. In dicentric fusion chromosomes the activity of one centromere is often suppressed (Neibuhr, 1972; Wolff and Schwartz, 1992), and the identity of the active and suppressed centromeres can be revealed by special staining (Daniel, 1979). In the present work, whether a fusion chromosome is monocentric or dicentric is largely based on the size and extent of centromeric heterochromatin.

A Robertsonian translocation is a chromosomal aberration not only in structure, but also in number (Schulz-Schaeffer, 1980; Eldridge, 1985). It is a form of unbalanced translocation in that material is lost (the p arms), but the loss usually does not affect the phenotype of the balanced carrier. During meiosis a trivalent is formed, and the segregation from this may be abnormal, resulting in the formation of some partial trisomic and monosomic gametes. This means that the main effect of a Robertsonian translocation is to decrease the fertility of the carriers (Schulz-Schaeffer, 1980; Eldridge, 1985; Long, 1985; Nicholas, 1987). However, it was thought that in Prosimians, some of the Robertsonian translocations constituted a particular type of chromosomal rearrangement that are able to diffuse inside a population and may progressively replace the original karyotype (Dutrillaux, 1979). This means that the Robertsonian translocation plays a role in chromosome evolution, or even in speciation.

1.4.1.2.3 Insertions

Insertions are the result of three chromosomal breaks, at least two of which are in the same chromosome. A segment between two breaks is transferred to the third break. Insertion may be direct, maintaining the centromere to telomere orientation of the arm or inverted. Insertions may occur between two chromosomes or within the one chromosome (in which case they are called shifts) (Webb *et al.*, 1988). Unbalanced karyotypes usually arise through mis-segregation in cases of insertion between chromosomes (Keith *et al.*, 1988), although recombination between the normal and inserted segment could also occur. In the case of shifts, recombination within a directly inserted segment can delete or duplicate the region between the shifted segment (Webb *et al.*, 1988). Recombination within inverted shifted segments can produce dicentric and acentric recombinant chromosomes.

1.4.1.2.4 Complex rearrangements

Rearrangements involving more than three simultaneous breaks are usually referred to as complex if all segments are mutually interchanged. The maximum number of simultaneous breaks recorded in humans is 8, and a complex rearrangement involving 7 breaks in only two chromosomes has been proposed for one case (Voullaire and Webb, 1988). Because of the number of breaks involved, complex rearrangements have been useful in establishing that almost all *de novo* rearrangements arise in the male genome (Gardiner and Sutherland, 1996).

1.4.2 Chromosomal abnormalities in cattle

1.4.2.1 General chromosomal abnormalities in cattle

Some morphological, physiological and fertility anomalies in cattle have been found to be associated with chromosomal aberrations (Gustavsson, 1977; Eldridge, 1985). Chromosome abnormalities in live cattle are mainly translocations, trisomies, inversions and somatic cell mosaics. Most reports are of various Robertsonian translocations. Only chromosome 19 in

cattle has not been reported to be involved in Robertsonian translocations (Table 1.1). The progeny of a Robertsonian translocation carrier will be at approximately 50% risk of inheriting the affected chromosome (Eldridge 1985).

The first chromosomal aberration in cattle was observed by Gustavsson and Rockborn in 1964, and later identified as the 1;29 Robertsonian translocation [2n=59,XY,t(1q;29q),-1,-29]. It is widely distributed all over the world (Eldridge, 1985; Long, 1985). It is interesting that the 1;29 translocation has a higher occurrence than expected among females (Eldridge, 1985). Other Robertsonian translocations are less frequent than the t(1;29) and usually are confined to one or two breeds.

1.4.2.2 Phenotypic effects of Robertsonian translocations in cattle

The only phenotype associated with balanced Robertsonian translocations in cattle is generally thought to be sub-fertility (Long, 1985). The decreased fertility may be caused by unbalanced gametes, where a gamete containing a deficient or duplicated chromosome arm takes part in fertilization (Eldridge, 1985; Long, 1985) and results in early embryo loss.

According to Gustavsson (1977), Robertsonian translocations have the "great risks for extensive distribution" and it is necessary "to keep the cytogenetic situation under control with continuous investigations of breeding animals". Because of the effect of the decrease in fertility, some countries do not permit the importation of animals or semen that has the 1;29 translocation (Eldridge, 1985). In the United States and Canada, Robertsonian translocations tend to be ignored by breeders.

1.4.2.3 Mechanisms of Robertsonian translocation formation

Robertsonian translocations are the main chromosomal abnormalities reported in live cattle (Gustavsson, 1977; Eldridge, 1985; Long, 1985). C-banding indicates that some Robertsonian translocations are monocentric and contain only one centromere [eg. the

chromosome t(1;29), (Fig 1.4A)] (Logue, 1978), while others are dicentric and contain two centromeres [eg. the chromosome t(14;20), (Fig 1.4B)] (Table 1.1).

For both monocentric and dicentric translocations, the two arms of the Robertsonian translocation chromosome are contributed by different autosomes (Eldridge, 1985; Long, 1985). For instance, the two arms of the cattle 1;29 Robertsonian translocation chromosome [t(1q;29q)] are from the longest and the shortest pairs of autosomes (numbered 1 and 29).

Two mechanisms are used to explain the formation of the monocentric centromere in a centric fusion translocation. One is that each of the two original chromosomes contributed only a part of their centromeres (Fig 1.5A). The other mechanism is that one of the two original chromosomes contributes its entire centromere to the fusion chromosome and the other centromere is lost (Fig 1.5B). The explanation for the dicentric chromosome formation is that both of the two original chromosomes contribute their centromeres (Fig 1.5C) (Eldridge, 1985; Miyake *et al.*, 1994). Tyler-Smith *et al.* (1993) found that in humans, one of the two centromeres in a dicentric chromosome was functionally suppressed (Wolff and Schwartz, 1992) when the two centromeres were very close) and the suppression was not usually accompanied by gross changes to the DNA. Chromosomal nondisjunction can occur when when the two centromeres are at a distance suppressed (Neibuhr, 1972).

1.4.3 Molecular constitution of a mammalian centromere

Since molecular biology techniques are widely used now, many researchers are investigating the molecular constitution of a centromere in mammalians, especially in human. Mammalian centromeres are typically composed of large arrays of tendemly repeated DNA families. These repeats are called satellite DNA and classified in many families. Dutrillaux (1979) hybridized human satellite DNA I, II, III and IV on human chromosome slides using *in situ* hybridization techniques and found that the contributions of satellite DNA on centromeres are different. α-satellite is the only repeat sequence that has been found at the primary

constriction of all normal human chromosomes (Tylor-Smith and Willand, 1993). According to the study of Sunckl and Coelho (1995), there is no interruption between two α-satellite families, but there is a complex set of new repeats on the centromere of Y chromosome not found in other centromere.

The studies on cattle satellite DNA sequences are far less than human ones. Main bovine satellite DNA sequences have been localized on cattle chromosomes (Beridze, 1986; Modi *et al.*, 1993). However, no known satellite DNA sequence has been found on the centromeres of cattle sex chromosomes. Furthermore, it is still unknown that the relationship of these satellite DNA sequeces on cattle centromere and how these sequences appear on Robertsonian translocation chromosomes.

1.4.4 Chromosome associations

Some chromosomes occasionally appear to form "associations" in metaphase cells. That is, they are consistently physically close in metaphase spreads. In the chromosomes of the higher primates, there is a strong association between the procentric nucleolar organizer regions (NORs) of chromosomes 13-15 and 21-22 of humans (Barch, 1991) (section 1.3).

Chromosome associations have not been described in many other species. However, Gordon (1995) found that 3 of 170 Jersey cows had a "chromosome association" in cultured blood and fibroblast cells. A Robertsonian translocation chromosome was observed in about 10% of metaphase or late prophase solid stained chromosomes. That is, 2 chromosomes were presumably associated at their centromeres.

1.5 Methods for studying chromosomal abnormalities

Many methods to study chromosomes cytologically in mammals were first used for human chromosomes, and then adapted for other mammalian species by modifying the techniques (Eldridge, 1985). Cell culture is generally the first step.

1.5.1 Cell culture

In 1953, Hsu and Pomerat found that hypotonic solutions make cultured cells swell so the chromosomes are easily separated on glass microscope slides. In 1960, Nowell discovered that leukocytes (t-lymphocytes) can be stimulated by phytohaemagglutinin (PHA) to enter a cycle of cell division from G_0 interphase. Thus, a sufficient number of metaphase spreads of the mitotic chromosomes can be collected after a short period of culturing leukocytes. Moorhead *et al.* (1960) combined the two techniques into a system for studying chromosomes. This technique is now in use in laboratories worldwide. The technique has been used to culture cattle leukocytes, as well, largely replacing meiotic studies with testicular tissue.

Many factors can affect the quality of chromosome slides. Good quality slides have high percentage of metaphase and late prophase cells, well spread chromosomes, and a clean background. Thus, the composition of the medium (Freshney, 1994), culture time and pre- and post-harvest treatments are all important to slide quality.

1.5.2 Chromosome banding

A chromosome number variation can be recognized cytologically without difficulty. Cultured cells are collected at metaphase and used to make slides. Slides are stained without special treatment, and the chromosomes belonging to a cell are counted. However, it is not easy to recognize most chromosomal structural variations, especially in cattle. The application of metaphase chromosome banding techniques can resolve this problem when aberrations of chromosome structure cannot be recognized by a change of shape or length.

Chromosome banding techniques were first used for studies of human chromosomes in the late 1960s and early 1970s. They have since been applied to studies of other mammalian chromosomes (Eldridge, 1985), including sheep, cattle, and goats (Evans *et al.*, 1973).

Metaphase chromosomes show different banding patterns with various treatments. Fluorescent banding using quinacrine compounds (Q-bands) was developed in 1968 (Eldridge, 1985) and was the first banding technique used to identify chromosomes in cells accurately. The procedure for Q-banding is simple, quick and reliable without the need of slide pretreatment before staining. G-bands (produced by using Giemsa stain) are similar to Q-bands, but are more permanent. G-bands can be obtained by several methods, such as treatment of slides with a protease (trypsin) or incubation of the slides in 56°C 2xSSC (Wang, 1987) before putting the slides into Giemsa stain. Dark G-bands in chromosomes contain A+T-rich DNA and appear to contain relatively few active genes (Barch, 1991). R-banding which reverses the dark and light bands seen in Q-bands and G-bands is produced by incubation in 86°C saline solution (Wang, 1987) before fluorescence staining or Giemsa staining.

Constitutive heterochromatin bands (C-bands) usually show the centromeres of a chromosome since this technique stains only the heterochromatin of a chromosome and heterochromatin is procentric in mammals. C-bands are produced by slide pretreatment with acid, alkali and hot saline citrate before Giemsa staining (Benn and Perle, 1986). In plants (Stack and Roelofs, 1996) and some insects (Shaw *et al.*, 1976), C-bands may be interstitial.

All chromosomes in mammals may show Q-, G-, R- and C-bands. However, silver staining which recognizes the nucleolar organizer regions (called NOR-banding) only stains a few chromosomes because the nucleolar organizer regions are only on one or a few pairs of chromosomes (Eldridge, 1985; Sumner, 1989). Only C-banding and NOR staining produce bands in plants.

In cattle, Q- (Hansen, 1972), R- (Gustavsson and Hageltorn, 1976), C- (Hansen, 1973), G- (Lin *et al.*, 1977) and NOR-bands (diBerardino *et al.*, 1979) have been studied since the early 1970s. The standard G-, R- and Q-bands of cattle were given by the Second International Conference on Standardization of Domestic Animal Karyotypes (Fig 1.6, ISCNDA 1989). There are a maximum of 10 NOR regions per metaphase found in cattle and all are located distally at the telomeric ends of chromosomes 2, 3, 4, 11, and 28 (Mayr and Gruber, 1987) or 29 (Eldridge, 1985).

1.5.3 *In situ* hybridization (ISH)

Small structural abnormalities of chromosomes can still be hard to recognize using the banding techniques. However, the application of *in situ* hybridization (ISH) can be very useful in detecting small changes in chromosome structure and studying genomic organization.

In situ hybridization is the "use of a nucleic acid probe to detect the presence of a DNA sequence in chromosome spreads or in interphase nuclei or an RNA sequence in cells" (Emanuel, 1993). This technique relies on the complementarity between the nucleotides in the nucleic acid probe and the target sequence, usually on a microscope slide. ISH is classified as isotopic or nonisotopic depending on the molecules used for the labelling of the probe.

ISH (Buckle and Craig, 1986; Emanuel, 1993) basically has 3 steps (Fig 1.7). Firstly, the appropriate probe DNA is labelled, and chromosome samples or target DNA (metaphase chromosomes or interphase nuclei on slides) are prepared. Secondly, the probe and the target DNA are denatured to form single strands, and the labelled single-stranded probe is hybridized to the target DNA. Thirdly, after the unbound or homologous probe DNA is washed away, the location of the probe/target DNA hybrids is detected by microscopy after chromosome banding.

Use of ISH can localize genes or DNA sequences directly on chromosomes in cytological preparations (called physical gene mapping) (Harper and Saunders, 1981; Lawrence, 1990; Jiang and Gill, 1994; de la Sena *et al.*, 1995; Iannuzzi *et al.*, 1996; Robinson *et al.*, 1996). It can also be very helpful for detecting small structural aberrations, particularly if "chromosome paints" (mixed probes delineating particular chromosomes or segments) are used.

1.5.3.1 ISH with Radioactive Isotopes (RISH)

RISH refers to hybridization with probes labelled with a radioactive isotope. The isotope of choice for good resolution on chromosomes is tritium (H^3) which is used to label one or more of the four nucleotide triphosphates (dATP, dCTP, dGTP, or dTTP). The ^3H-nucleotides are incorporated into DNA by nick translation or primer oligonucleotide extension. Then, the labelled probe DNA is denatured and hybridized on fresh denatured mitotic chromosome spreads on a slide. After the unbound probe DNA is washed off, autoradiography is used to detect the probe hybridized to the chromosome. The silver grains formed by β-particles from the probe/target hybrids is scored in many metaphase cells to determine the location of the DNA hybrids (Buckle and Craig, 1986; Lawrence, 1990). Amplification of the signal is achieved by increasing the time of exposure of the autoradiographs.

RISH remains the only method of detecting short unique probes (down to 200bp) or poor labelling probes, because it is approximately 50 times more sensitive than nonisotopic ISH (Webb, pers. comm.). Slides made by radioactive ISH are permanent, whereas slides made by nonisotopic ISH are wet-mounted and rather delicate. However, a weakness of RISH is the spread of radioactive particles through the emulsion, which spoils the resolution. Thus, the spatial resolution of nonisotopic ISH is better than that of RISH because there is very little scatter of the signal. The spread of radioactive particles in RISH can only be overcome by scoring large numbers of grains and identifying the peak (Board and Webb, 1987). In

addition, nonisotopically labelled probes are stable for some years because they are not subject to radioactive decay, although with a half-life of 12.3 years, tritiated probes are stable over many months.

1.5.3.2 Nonisotopic ISH

In nonisotopic ISH, the molecules used for the probe labelling are not radioactive isotopes. The probes are labelled using one or more chemically modified nucleotide triphosphates. If the signal is not strong, it can be amplified before detection.

The labelled probes can be detected after hybridization using affinity reagents, such as avidin to a biotin label, or antibodies to the label, such as anti-digoxygenin. The secondary reagents are themselves labelled with enzymatic tags or fluorochromes. By using enzymatic reporter molecules which convert a substrate to a visible product, the signal of the location of probe:target hybrids can be detected with the advantage that the signal is stable and visible under simple brightfield microscopy.

Fluorescent tags provide the highest resolution possible with visible light or ultraviolet. They are easily observed and identified. For some applications, fluorescent compounds can be labelled into the probe and detected under the fluorescent microscope directly after hybrids are formed. Alternatively, they can be combined with an affinity reagent or antibody after hybridization to detect the location of the probe:DNA hybrids. Amplification can be achieved by repeated use of affinity reagents and/or antibodies. Moreover, if a number of probes are labelled separately with different fluorochromes (multicolored labelling) and used at the same time for fluorescence *in situ* hybridisation (FISH), then different genes, repetitive sequences or chromosomes can be studied in one cell (Lawrence, 1990; Trask, 1991; Emanuel, 1993; Ford *et al.*, 1994; Han *et al.*, 1994; Jiang and Gill, 1994). Two colour FISH is particularly useful for establishing the order of the labelled probes.

With nonisotopic ISH, the signals from the target sequences can be amplified without waiting many weeks for exposure of the autoradiographs involved in isotopic ISH. Nonisotopic ISH has been increasingly used for detecting single-copy DNA sequences, down to 1000 bp, as the sensitivity of this technique and semiautomated techniques for analysing and recording fluorescent signals has improved (Lawrence, 1990; Jiang and Gill, 1994). A very recently developed technique involving tyramide allows nonisotopic ISH to localize single-copy probes as short as 500 bp (Webb, pers. comm.).

1.5.3.3 Applications of fluorescence *in situ* hybridization (FISH)

FISH can be used for recognising not only single-copy sequences or chromosomal subregions, but also entire chromosomes or genomes of a particular species by chromosome painting either metaphase chromosomes or interphase nuclei (Trask, 1991; Lawrence, 1990; Jiang and Gill, 1994). Thus, the application of FISH provides useful information in many areas, such as physical gene mapping (Korenberg *et al.*, 1992; de la Sena *et al.*, 1995; Iannuzzi *et al.*, 1996), cytogenetics (Boyle *et al.*, 1990; Lawrence, 1990; Trask, 1991; Jiang and Gill, 1994), clinical diagnosis of hereditary disease (Emanuel, 1993), genetic transformations and embryo sexing for animal breeding (Gustavsson, 1991; Ford *et al.*, 1994).

FISH can also be used to study the contribution of the two autosomes in a Robertsonian translocation chromosome. By applying FISH, the chromosomes which contribute to a Robertsonian translocation can be recognized by use of chromosome-specific probes. That is, probes derived from a relevant cattle autosome can be used to check the presence of autosomal sequences in the fused chromosome. This is particularly useful when the simple identification of the chromosomes involved in the fusion from the banding pattern is not easy (eg. small cattle autosomes).

Moreover, specific FISH probes complementary to the repeat sequences at the centromeres or adjacent regions can be used to detect the contribution to the centromeres of

Robertsonian translocation chromosomes. That is, Robertsonian translocations can be studied using procentric satellite probes labelled with two different colored fluorochromes to detect the contribution of both original centromeres and the centromere of the centric fusion chromosome. These will provide more information about the centromere rearrangement than R- or C-banding.

1.6 The aims of this research

Aberrations in the structure and in the number of chromosomes may lead to abnormalities in the phenotype of an individual which carries the aberration (Schulz-Schaeffer, 1980). In cattle, Robertsonian translocations are of particular important because the only major phenotypic defect is reduced fertility (Rangel-Figueiredo and Iannuzzi, 1993; Hanada, 1994; Hanada *et al.*, 1995; Kawarsky *et al.*, 1996; Schmutz *et al.*,1997). Hence, carriers of Robertsonian translocations can go undetected and the defect widely spread. Unfortunately, the mechanism underlying the formation of the translocation chromosomes and the rearrangement of the centromeres are not understood. To understand the formation of Robertsonian translocation chromosomes, it is first necessary to elucidate the organization of normal centromeres and potential factors which may lead to a centric fusion.

FISH is an effective method of studying chromosome structure and organization. By using probes for the major bovine satellite DNA sequences in FISH experiments, it should be possible to delineate the centromere organization of normal and translocated cattle chromosomes.

Therefore, the specific aims of this research were: 1) to modify the cytogenetic techniques for cattle, including leukocyte cell culture, FISH, and chromosome banding to improve the quality of the slides; 2) to examine the chromosome association in the Jersey breed and determine its role, if any, in the formation of Robertsonian translocations; 3) to study of the

procentric organization of cattle satellite DNA sequences in normal cattle chromosome centromeres using single and two-color FISH; and 4) to determine the centromere structure with respect to a cattle monocentric Robertsonian translocation [t(1q;29q)] and a cattle dicentric Robertsonian translocation [t(14q;20q)].

Table 1.1 Robertsonian translocations in cattle

Cs No. involved	Result	Special reports	Breed	Reference
1/4			Czechoslovakian cattle	Lojda *et al.* (1976)
1/21			Holstein-Friesian	Miyake *et al.* (1991)
1/23			Czechoslovakian cattle	Lojda *et al.* (1976)
1/25			Pie Rouge	Stranzinger & Forester (1976)
1/26				Miyake & Kaneda (1988)
1/28			Czechoslovakian cattle	Lojda *et al.* (1976)
1/29	Sub-fertile	Monocentric	Ala-Tau	Kanapin *et al.* (1989)
			Barrosa	Rangelfigueiredo & Iannuzzi (1991)
			Blonde d'Aquitaine	Agerholm *et al.* (1993)
			Branman	Nel *et al.* (1988)
			British Friesian	Wilson (1990)
			Brown Swiss	Christensen *et al.* (1992)
			Charolais	Schmutz *et al.* (1990)
			Criollo	Madriz & Munoz (1991)
			Guernsey	Bongso & Basrur (1976)
			Italian Friesian	Gali & Poloschi (1988)
			Japanese Black	Hanada *et al.* (1991)
			Limousine	Agerholm *et al.* (1993)
			Maronesa	Rangelfigueiredo & Iannuzzi (1990)
			Mirandesa	Rangelfigueiredo & Iannuzzi (1993)
			Nguni	Nel *et al.* (1985)
			Piedmontese	Gali & Poloschi (1988)
			Red Danish Dairy	Agerholm *et al.* (1993)
			Retinta	Moreno-Millan *et al.* (1991)
			Simmental	Schmutz *et al.* (1990)
			Swedish Red & White	Dyrendahl & Gustavsson (1979)
			Sykia Chalkidiki	Vainas *et al.* (1992)
			Venezuelan Creole	Munos *et al.* (1994)?
2/4	Sub-fertile		British Friesian	Pollock & Bowman (1974)
2/8			British Friesian	Pollock (1974)
3/4			Limousin	Popescu (1977)
3/27			Friesian	Samarineau *et al.* (1977)
4/8				Bouvet *et al.*(1989)
4/10		dicentric	Blonde d'Aquitaine	Bohridarwich *et al.* (1993)
4/21			Simmental	Ibrahim *et al.* (1983)
5/18		dicentric	Simmental	Papp & Kovacs (1980)
5/21			Japanese Black	Sakai *et al.* (1991)
5/22		dicentric	(Poland)	Slota & Switonski (1992)
5/23			Romanian Brown	Samarineanu *et al.* (1976)
6/16		dicentric	Dexter	Eldridge (1974)
6/28			Czechoslovakian cattle	Lojda *et al.* (1976)
7/21	Sub-fertile		Japanese Black	Hanada *et al.* (1991)
8/9			Swiss	Tschudi *et al.* (1977)
8/23		dicentric	Grey Ukrainian breed	Biltueva *et al.* (1994)
9/23			Blonde d'Aquitaine	Cribiu *et al.* (1989)
10/15			Petangueiras	Pinheiro and Ferrari (1980)
11/16			Hungarian Simmental	Kovacs and Papp (1977)
11/21			Romanian Brown	Samarineanu et al. (1976)
11/22			Czechoslovakian cattle	Lojda *et al.* (1976)
12/15	Sub-fertile		Holstein-Friesian	Roldan *et al.* (1984)
13/21			Holstein-Friesian	Kovacs and Papp (1977)
			Swiss Simmental	Harvey and Lodge (1975)
13/24		dicentric	(Czech)	Holeckova *et al.* (1995)
14/20	Sub-fertile	dicentric	Simmental	Weber *et al.* (1992)
			Dexter	Logue & Harvey (1978b)
14/24			Podolian	diBerandino *et al.* (1979)
14/28		dicentric	Holstein	Ellsworth *et al.*(1979)
16/18		dicentric	Barrosa	Iannuzzi *et al.* (1993)
16/20		dicentric, *de novo*	Red pied cattle	Rubes *et al.* (1996)
21/27			Blonde d'Aquitaine	Berland *et al.* (1988)
25/27			Alpine Grey	de Giovanni *et al.* (1979)
27/29			Guernsey	Bongso & Basrur (1976)

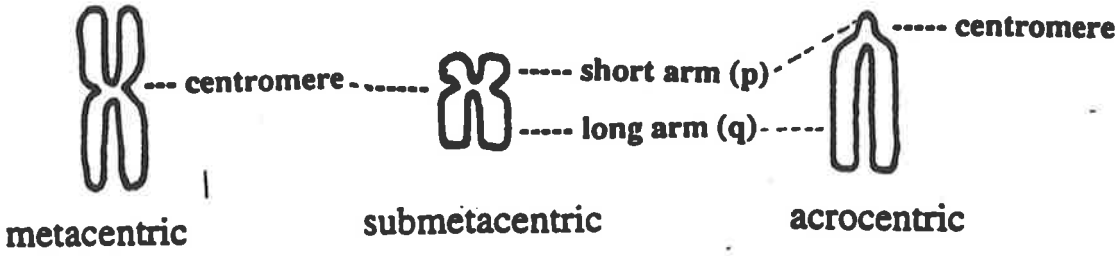


Fig 1.1. Different morphologies of chromosomes.

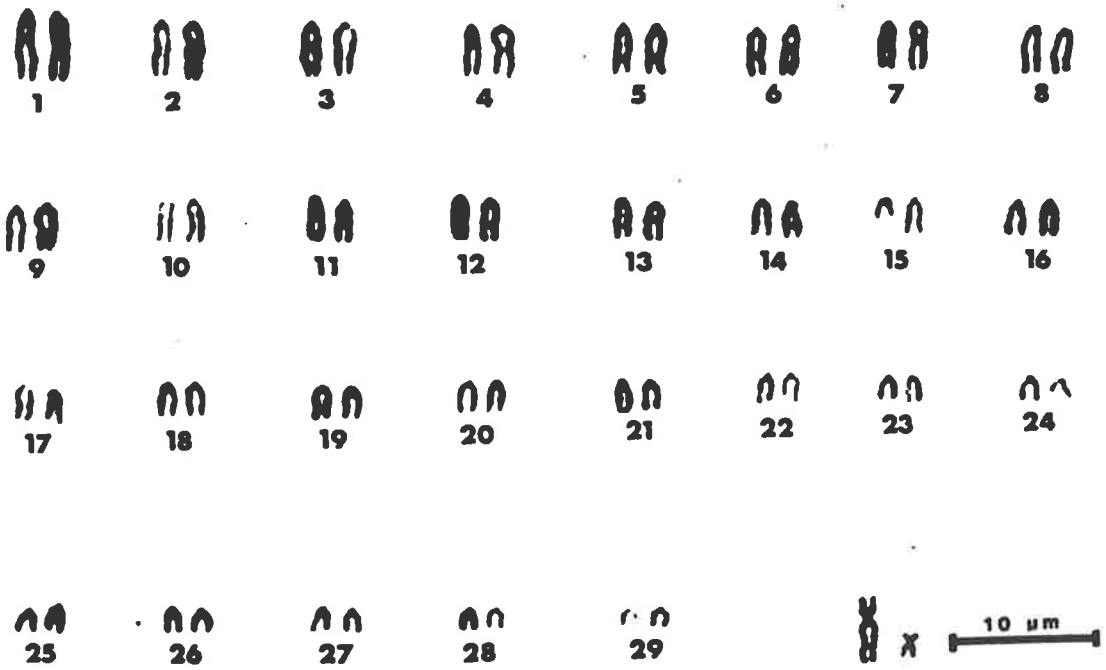


Fig 1.2. Giemsa-stained karyotype of a normal B. taurus male (Brown Swiss bull, from Eldridge, 1985).

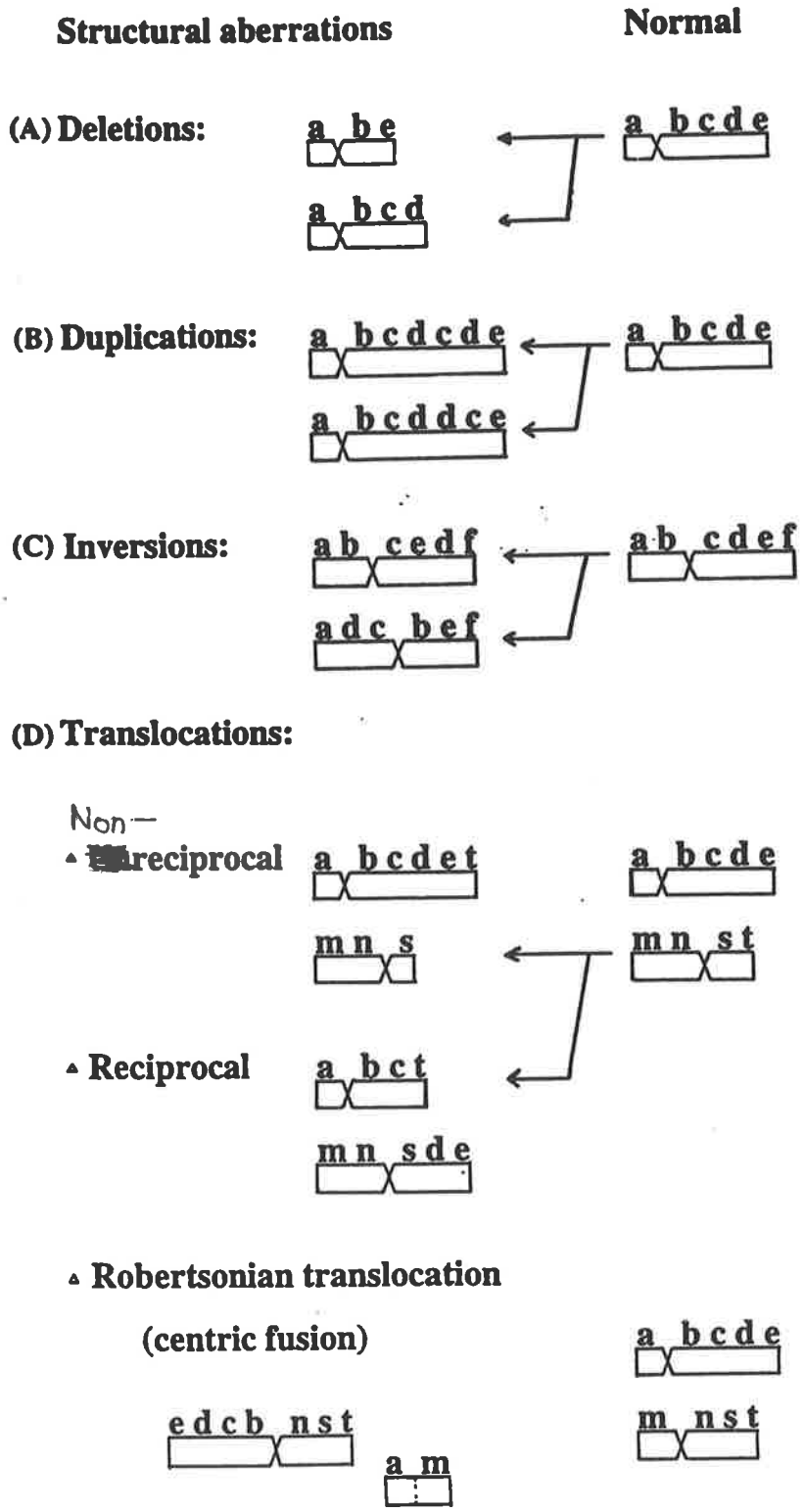


Fig 1.3. Main chromosomal structural aberrations.

(A) Monocentric: t(1;29)

(B) Dicentric: t(14;20)

Fig 1.4. Two types of Robertsonian translocation chromosomes in cattle from C-bands (From Lougue 1978, Logue and Harvey 1978).

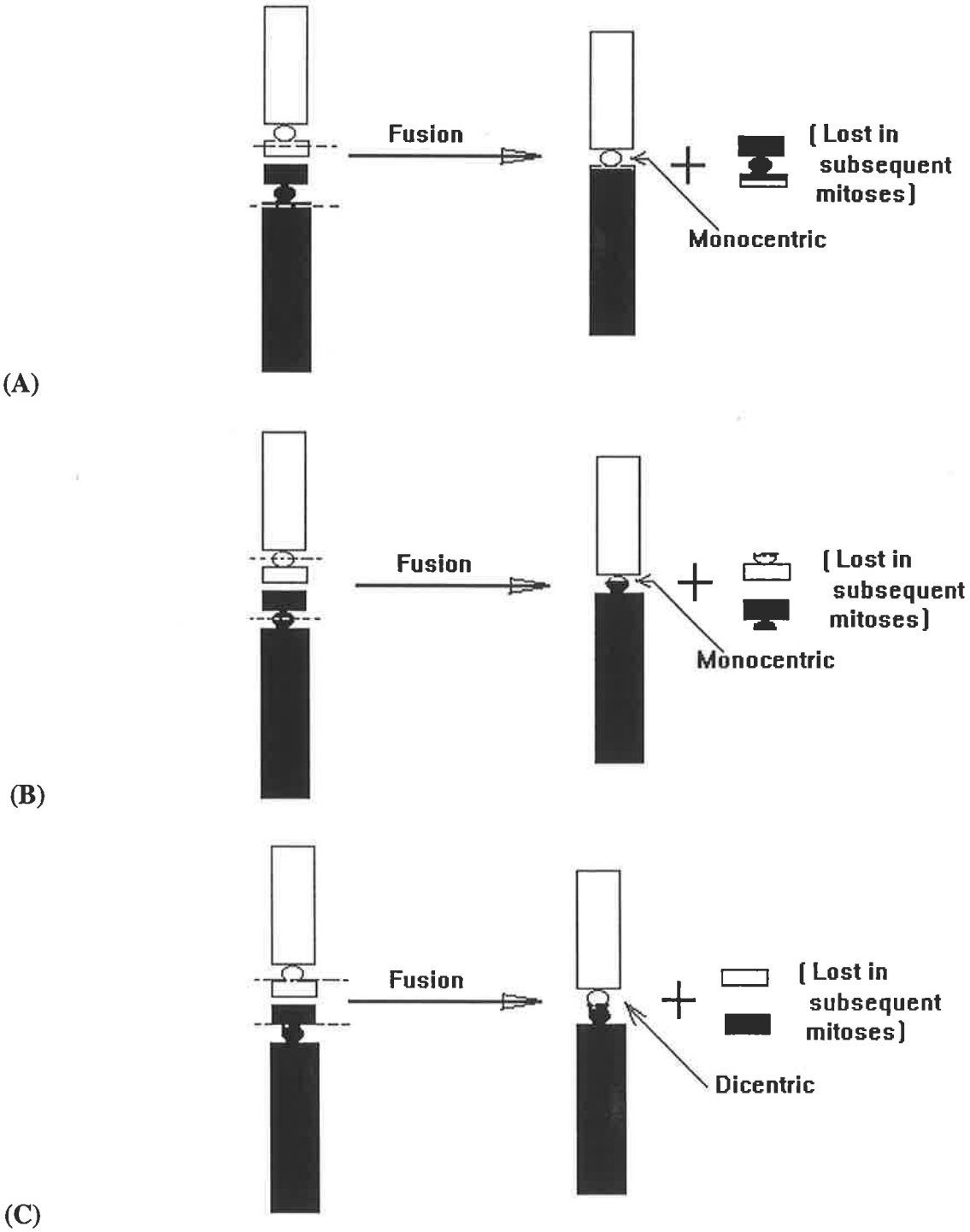


Fig 1.5. Mechanisms of centric fusion formation (Dashed lines indicate the locations of breaks) (From Eldridge1995, modified).

(A) One of the two original chromosomes contributes its entire centromere to the fusion chromosome while the other centromere is lost;

(B) Each of two original chromosomes contributes only a part of the centromere of the new chromosome;

(C) Both of two original chromosomes contribute their centromeres to the new chromosome.

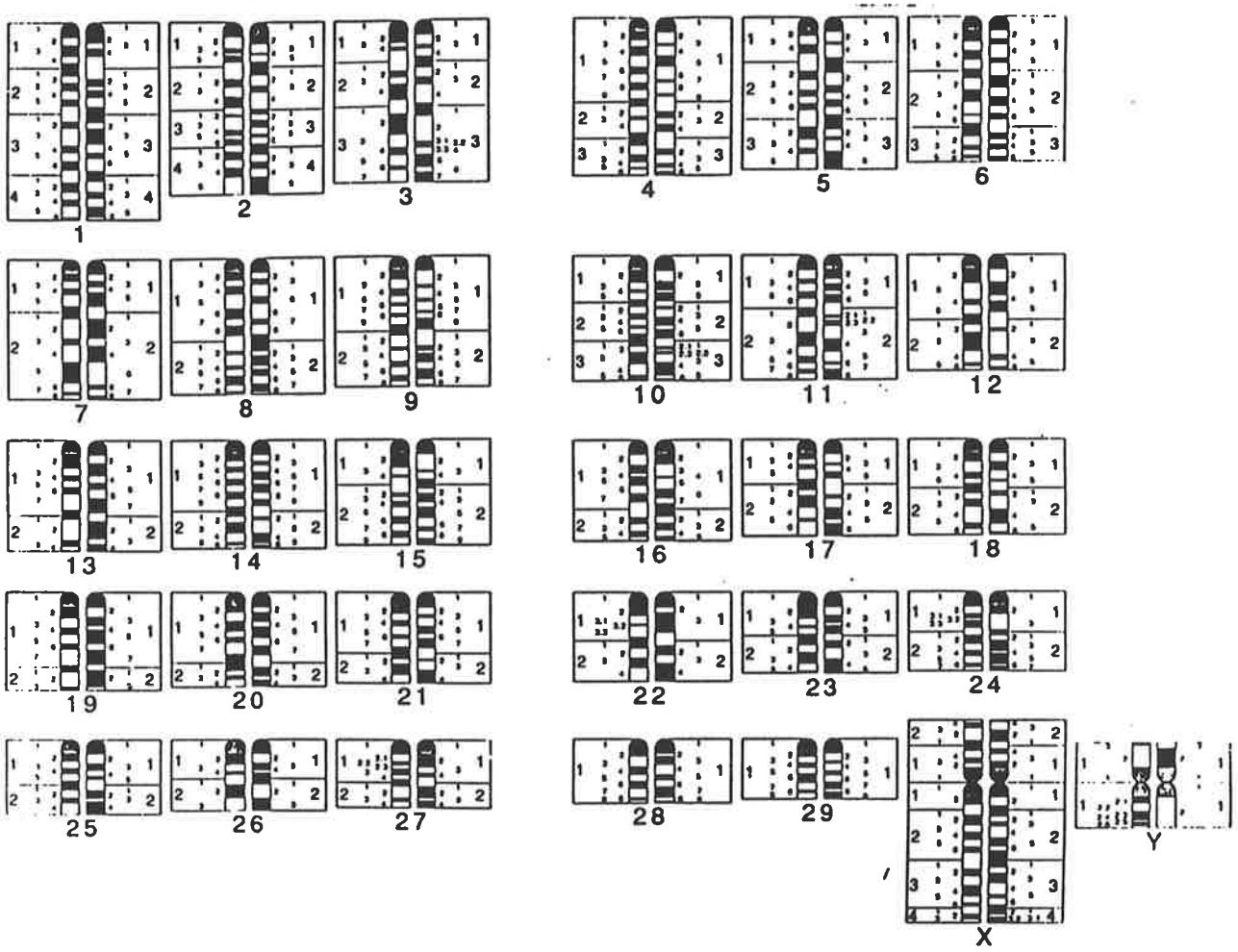


Fig 1.6. The standard G-, R- and Q-bands of cattle chromosomes (ISCNDA, 1989)

(A) Diagrammatic representation of G-bands (left) and R-bands (right)

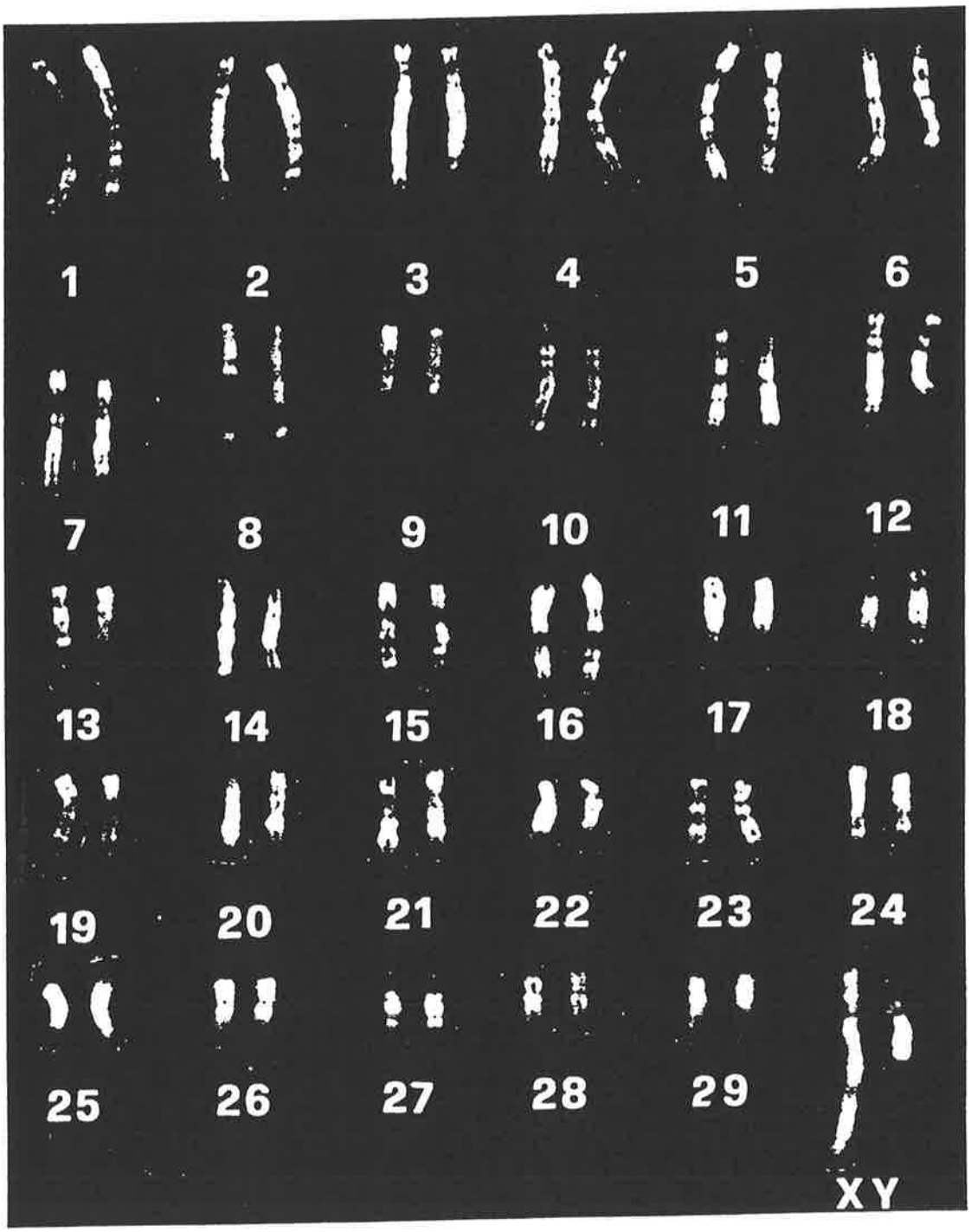


Fig 1.6B The standard Q-bands of cattle chromosomes (ISCNDA, 1989)

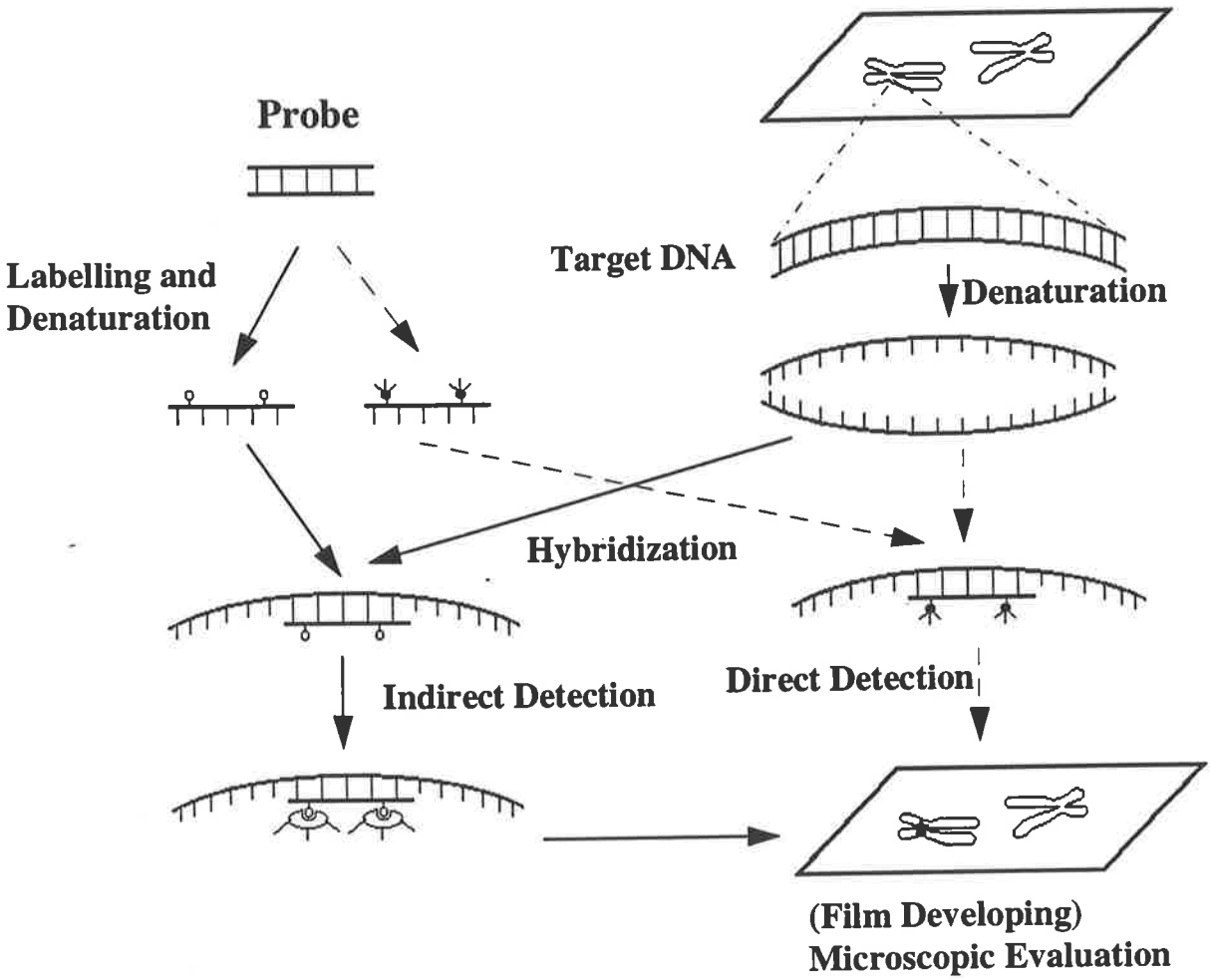


Fig 1.7. ISH protocol (From Pedersen 1995).

Chapter 2

MATERIALS AND METHODS

Recipes for media and buffers appear in Appendix 2.

2.1 Sampling

2.1.1 Animals

a) A Simmental cow was diagnosed by Dr Graham C Webb from the Waite Cytogenetics Service in February of 1995 as a carrier of Robertsonian translocation t(14q;20q). She was produced by artificial fertilization using imported semen from a Canadian bull. Her daughter was born in December of 1996 and was also a carrier of Robertsonian translocation t(14q;20q) [t(14;20)], as diagnosed by Jianze Zheng in January of 1997.

b) The carrier of Robertsonian translocation t(1q;29q) [t(1;29)], a Charolais heifer, was also diagnosed by Dr GC Webb and R-banded by J Zheng from the Waite Cytogenetics Service in August of 1996. The heifer was the single survivor of six embryos imported from the United States and she died not long after the first blood sample was tested.

c) J103 and J111 were two of the three Jersey cows which were determined to have a chromosome association from the analysis of their karyotypes by Mr Benjamin C Gordon (Honours thesis, University of Adelaide, 1995).

d) A bull (60,XY) from New Zealand was determined to have a normal karyotype by the Waite Cytogenetics Service (tested by Zheng and Webb in 1997).

2.1.2 Sample collection

For the culture of peripheral blood lymphocytes, blood from the jugular vein was drawn into 6 ml vacutainers containing lithium heparin. The tubes were immediately inverted several times to mix the anticoagulant with the blood to prevent clotting, and stored at 4°C. Blood tubes were divided into two sets. One set was used to culture the lymphocytes, while the other set was kept at 4°C and cultured only if necessary. Just before use, the blood tubes

were gently mixed to make the blood homogeneous. Preferably the venous blood samples were collected from animals on the day the cells were to be cultured.

For the culture of fibroblast cells, the ear was cleaned with 70% ethanol and a sample of skin was taken in the form of a 5 mm ear punch. The skin sample was transported and stored at 4°C in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM) plus 2% penicillin/streptomycin/fungizone (P/S/F) until culturing.

2.2 Cell culture

All cell culturing was done using aseptic techniques.

2.2.1 Culture of peripheral blood lymphocytes

Blood lymphocyte cultures were established using 0.5 ml of whole blood per 10 ml of media. The culture media used were Roswell Park Memorial Institute medium 1640 (RPMI) with sodium bicarbonate, mixed with 10-20% v/v foetal bovine serum (FBS), 1% v/v 0.2M glutamine (glut.), 0.2% v/v 10 mg/ml gentamycin solution (gent.), 2% v/v 1M HEPES buffer, 0.1% v/v 25000 unit/ml heparin solution (hep.), and 0.5% v/v P/S/F, or the mixture of AmnioMax Basal and Supplement at the ratio of 90:15, with 0.5% v/v glut. and 0.1% v/v hep. Mitogens were also added to stimulate lymphocytic division [2% v/v phytohaemagglutinin (PHA), M form, 10 ml from lyophilized powder, and 0.5% v/v 1.0 mg/ml poke weed mitogen (PWM)].

The lymphocytes were cultured in 15 ml culture tubes containing 10 ml of the media in a Forma Scientific water-jacketed incubator with 5% CO_2 at 37°C for about 3 days. Cultures were shaken every day to mix the blood cells and medium.

Six to seven hours before harvest, 5-bromodeoxyuridine (BrdU) at a final concentration of 20 μg/ml was added to the culture. Alternatively, cells were suspended in fresh medium

first, and then BrdU was added. The aim was to incorporate BrdU into the DNA as an analogue of the T base during the latter half of S-phase. Therefore, at the staining stage after chromosome preparation, the stain (e.g. Hoest33258) will be quenched to produce R-bands, the reverse of G-banding (Freshney 1994). BrdU was omitted, if banding was not required. In some cases, 300 µg/ml of thymidine (Tdr) was added to arrest the cell cycle in mid S-phase and obtain more cells in mitosis. The Tdr was rinsed out before BrdU was added to produce R-bands. Half to one hour before harvest, 200 µl of 100 µg/ml colchicine was added per 10 ml medium and mixed well to arrest cells at the metaphase stage of cell division.

2.2.2 Culture of skin fibroblast cells

The skin sample was washed 3 times in Petri dishes with 70% ethanol for a few seconds to sterilise the sample. It was then transferred into a drop of DMEM with 10% v/v FBS, plus 1% v/v glut. and 2% v/v P/S/F (DMEM). Using a scalpel and tweezers, the outer layer of the skin sample (epidermis) was removed and discarded. A portion of the dermis and cartilage were returned to the collection tube and kept at 4°C in case of contamination of the original cultures.

The soft tissue layer was washed by transferring the sample from 500 µl of DMEM to another 500 µl of DMEM for a total of 6 washes. The tissue was then cut into very fine pieces (<1 mm). Twenty small pieces of soft layer were placed separately into the grooves of each 50 ml culture flasks. Into each flask, 0.5 ml medium was added and spread over the top of cells. Flasks were placed into an incubator upside down for 6 hours at 37°C, 100% humidity and 5% CO_2 such that the cells did not have contact with the media. After 6 hours, the flasks were gently inverted so that the cells were in the medium.

Every 24 hours, each tissue piece was scanned under a microscope for signs of cell growth and fungal contamination. If contaminated, pieces were removed by aspiration and 0.5

ml of DMEM was changed until the epithelial cells began to grow. Before the elongated fibroblast cells began to grow, 2 ml of DMEM was used in each flask. After the cells began to grow, 5 ml of medium was added and changed daily.

The cells were grown until the cultures were nearly confluent. The medium was aspirated off, and cells were washed thoroughly with 1 ml Hank's balanced sodium salt solution (HBSS, Ca^{2+}, Mg^{2+} free) three times to remove serum. 0.5 ml trypsin/EDTA (1:250) liquid was added and spread evenly across the flask, and then the flask was incubated at 37°C for 2 minutes. The side of the flask was tapped firmly against the edge of a bench several times to completely release the fibroblasts. Then 4.5 ml of DMEM with 10% FBS plus 1% P/S/F medium was added to the flask and the cells were mixed well. The adequate cell suspension was transferred to a new flask containing 5 ml of new medium and incubated at 37°C and 5% CO_2. At the same time, 5 ml of medium was added to the original flask and incubated at 37°C and 5% CO_2 as a spare. The cell growth was checked daily. When the flasks reached confluency, the cells were passaged again.

2.3 Slide preparation

2.3.1 Slide cleaning

New glass slides were placed in a rack, and allowed to stand in 5% Decon 90 from 2 hours to overnight. The slides were then rinsed in running water for 30 minutes, and dH_2O for 2 minutes. Following the dH_2O rinse, the slides were put through a series of 3 rinses in 100% ethanol for 2 minutes each, and then placed with the frosted end downwards on a rack to dry.

2.3.2 Lymphocyte harvesting

Following the colchicine treatment (section 2.2.1), the cultured cells were isolated by centrifugation at 350 x g for 10 minutes. The cells were resuspended in 10 ml of preheated (37°C) 0.075M KCl (hypotonic) solution by inversion and the suspension was incubated in a 37°C water bath for 20 minutes.

After hypotonic treatment, 3 ml fresh fixative (methanol:glacial acetic acid=3:1, v/v) were added and mixed with cells. The tubes were then centrifuged at 350 x g for 10 minutes. The fixative must be fresh to avoid the formation of the ester, methyl acetate.

After the cells were pelleted, the supernatant was removed and 10 ml of fixative was added. The pellet was resuspended using a Pasteur pipette and centrifuged for 10 minutes at 350 x g. After 2 or 3 of such rinses in full fixative, cells were isolated as a white pellet. The pellet was then resuspended in a small volume of fixative (1 ml) using a Pasteur pipette and one or two drops of the cell suspension were placed onto a cleaned slide. The remaining cells were resuspended in 10 ml of fixative and stored at -20°C. The slides were air-dried, used for solid staining, chromosome banding, FISH or put into a sealed slide-box and stored in a slide box with dessicant at -20°C for later use. Before removing a slide from the box, the box was pre-heated to 37°C to prevent moisture collecting on the surface, and the desiccant was always replaced.

2.3.3 Fibroblast harvesting

After passaging, half of the fibroblast cells (1 ml) from a 50 ml-flask were transferred to a new 150 ml-flask containing 8.9 ml DMEM/10% FBS/1% P/S/F/1% glut. BrdU was added to a final concentration of 45 μg/ml. The cells were incubated with loose lids at 37°C and 5% CO_2 for 24 hours, and the medium was then aspirated off. After two rinses with 5 ml HBSS, 5 ml DMEM/10% FBS/1% P/S/F/1% glut. with 10 μM thymidine was added and the cells

were incubated at 37°C and 5% CO_2 for 6 hours. A further 15 minute incubation was done at 37°C and 5% CO_2 after 25 µl of 100 µg/ml colchicine was added to break the spindle fibres.

When the medium was removed, 2 ml of pre-heated 0.075M KCl was added to each flask for 25 minutes. All flasks were vigorously vortexed for 1 minute to dislodge the metaphase cells. Cells from 4 flasks were transferred into a 15 ml Falcon tube. Another 2 ml 0.075M KCl was added to the flasks and the vortexing step repeated. The cells were transferred into the same Falcon tube. The cells were centrifuged at 250 x g for 5 minutes and the supernatant was aspirated off. Approximately 200 µl were left. The cells were mixed and 12 ml 0.075M KCl was added. The cells were incubated in a 37°C for the remainder of the 25 minutes.

After the hypotonic treatment, 3 ml fresh fixative (methanol:glacial acetic acid=3:1) was added and mixed with cells. The tube was then centrifuged at 400 x g for 5 minutes and the supernatant was removed. The cells were homogenized before 12 ml fixative was added and mixed with the cells as a full fix. The tube was centrifuged at 400 x g for 5 minutes and the supernatant was discarded. The full fix and centrifugation steps were repeated twice.

The cell pellet was resuspended using a Pasteur pipette in an appropriate volume for making slides. 2-3 drops of cells were placed onto one clean slide. The slides were air-dried and artificially aged in a slide box for 2 days at 37°C to improve G-banding. The slides were stored with desiccant at -20°C for later use.

2.4 Staining and viewing

2.4.1 Solid staining

Air-dried slides were directly solid stained in 20% v/v Leishman's stain (Sigma, 2g/L in methanol) in pH6.8 buffer (Gurr Tablets). Slides were placed on a staining rack over a sink

with the cells facing up. Leishman's stain was then poured over the slides and allowed to stand for 20 minutes.

After 20 minutes, the slides were rinsed carefully from one corner using dH_2O so that the precipitated layer of the stain was washed off. The slides were air-dried. Along the center of the slide, 3 drops of DEPEX were applied and a 24x50 coverslip was mounted.

2.4.2 Karyotyping and division counting

For karyotyping using solid stained slides, 5 cells were scored by counting the number of chromosomes in the cell, and the sex chromosomes for each animal were identified under magnification of 100X using bright-field microscopy (Olympus BH-2) with a video camera and screen for scoring.

For mitotic scoring using solid stained slides with one drop of cells, all mitotic cell divisions on the slides were scored under magnification of 10X with bright-field microscopy (Olympus BH-2).

2.4.3 Chromosome banding

2.4.3.1 R-banding

The slides were placed in a Coplin jar with a solution of 4 - 6 µg propidium iodide per ml in PBS for 5 minutes. Then the slides were rinsed in PBS 3 times for 20 second each. PBS was tapped off and a 24X60 mm coverslip was mounted with 100µl of 0.1% para-phenylenediamine dihydrochloride in 90% glycerol at pH 11 (PPD11). Excess mountant (PPD11) was squeezed out and the coverslip sealed with nail varnish. The slides were viewed at magnification of 10X under green or blue excitation by fluorescence microscopy (Olympus BX60 System microscope). Photographs were taken at magnification of 100X under blue or ultra violet excitation.

2.4.3.2 Silver staining for active nuclear organizing regions (NOR-banding)

A 50% w/v solution of silver nitrate ($AgNO_3$) in dH_2O was made up 3 hours before the staining and left to stand for 2 hours. The $AgNO_3$ solution was then mixed with seven volume of 0.2% v/v formic acid (HCOOH). The mixture was filtered through a 22 µm filter and 4-6 drops placed on each slide. After each slide was covered with a coverslip and sealed with vulcanizing cement, it was placed on a rack in an air tight humidified container and incubated at 65°C. Slides were checked every 5 minutes. After a golden-brown coloration appeared (approx. 10-15 minutes), the slides were cooled quickly, and the coverslips removed.. The slide was rinsed with running dH_2O for 5 minutes and allowed to air-dry (Hubbell and Hsu 1977). Slides were scored and photographed quickly using a bright-field microscope (Olympus BH-2). The photographed metaphase cells were scored for the location of the nuclear organizing regions (NORs).

2.4.4 Microscopy and photography

1) The bright-field microscope used herein was an Olympus BH-2 equipped with a video camera and screen.

2) The fluorescence microscope used herein was an Olympus BX60 with magnification lens of X10, X40 (oil) and X100 (oil), and green, blue and UV excitation.

3) For colour photography, the film was Fujichrome 400 ASA colour-positive rated at 1600ASA for bright-object fluorescence and corrected at 0.8-2.0 (usually 1.6) times depending on the number of fluorescent objects in the field.

2.5 Single colour fluorescence *in situ* hybridization (FISH)

2.5.1 Probe labelling with Biotin

2.5.1.1 Biotin nick translation using a BioNick™ Labeling System (kit 1)

1 µCi of ^{3}H-dATP was dried in a 1.5 ml Eppendorf tube under vacuum in a Spin-Vac. The ^{3}H-dATP was used to measuring the efficiency of labelling, because the ATP in the BioNick™ Labelling System (kit 1, GibcoBRL, Catalog No. 18247-015) was biotin-labelled.

While on ice, 5 µl of 10x dNTP Mix, 1µg of probe DNA in 40 µl of dH_2O (kit 1) and 5 µl of 10x Enzyme Mix containing DNA polymerase I and DNase (kit 1) were added to the tube. The mix was gently vortexed, microcentrifuged and incubated at 16°C for 1-2 hours. A 5 cm BioGel P-60 or Sephadex G-50 column for each probe was prepared in a short Pasteur pipette plugged with sterile, non-absorbent cotton wool and washed twice with $T_{10}E_1$ (10mM Tris-HCl and 1mM EDTA) at pH 7.6. At the end of the nick translation, 5 µl of EDTA Stop Buffer (kit 1) was added and mixed well.

2.5.1.2 Collection and isolation of nicked probe

The nick mix was layered onto the column, and a drop of $T_{10}E_1$ was added. After the drop of $T_{10}E_1$ was soaked in, additional $T_{10}E_1$ was added and fractions collected. The fractions were as follows: 355 µl (tube number 1), 100 µl (9 times, tubes 2 to 10), 500 µl (4 times, tubes 11 to 14). 2 µl samples from tubes 2-14 were counted in 150 µl of dH_2O and 1.5 ml of BCS scintillation fluid. The tubes containing the probe DNA were determined and the percentage of incorporation calculated. Over 10% incorporation was considered satisfactory. The probe was ethanol-precipitated by adding 10% volume of 3M sodium acetate (pH 5.2) and then 275% (2.5 x 1.1) volume of 100% ethanol.

2.5.2 In situ Hybridization of Satellite DNA Probes to Chromosomes

2.5.2.1 Denaturation of target DNA

50 ml of denaturation mix at pH 7.0 containing 70% deionized formamide in 2x SSC was made and pre-heated in a weighted plastic Coplin jar to 70°C. The slides were marked with a number using a diamond pen overlaid with a carbon pencil against which was recorded, the animal name, type of slide preparation, date, and satellite DNA probe name. The slides were preheated in an empty jar in a 70°C water bath and then transferred into the denaturation mix at 70°C for 2 minutes with gentle agitation. The slides were immediately placed into cold 70% ethanol at 0-10°C, and agitated for 2 minutes. The slides were dehydrated through an ethanol series in Coplin jars at room temperature from 70%, 80%, 95% and 100% for 2 minutes each. The slides were drained, with the frosted end down, to dry.

2.5.2.2 Denaturation of probe DNA

A 20% w/v solution of dextran sulphate in deionized formamide was made in a fume hood and dissolved in a 70°C water bath with intermittent agitation before use. The ethanol-precipitated probe was spun for 15 minutes in an Eppendorf centrifuge at maximum speed (16000 x g) and the supernatant ethanol was carefully removed, either with a pulled Pasteur pipette or by tipping. The probe was dried under vacuum.

The dried satellite probe (66 ng per slide), was dissolved in 10 µl of dH_2O and mixed with 10 µl of 10x SSCP (pH 6.0, Appendix 3) and 2.15 µl of 7.7µg/µl sonicated salmon sperm DNA, so that the ratio of salmon sperm DNA to probe DNA was 250x. After the volume of the above mixture was adjusted to 25 µl with dH_2O, 25 µl of 20% dextran sulphate in formamide was carefully added.

The probe mix was briefly heated to 75°C, mixed thoroughly on a vortex mixer, and spun in a microcentrifuge. The probe was denatured for 10 minutes at 75°C and quickly cooled in an ice/water mix for at least two minutes.

2.5.2.3 Hybridization

The slide was viewed under blue excitation to show the orange propidium iodide staining of the R-banded chromosomes and to show the bright yellow signal indicating the hybridized probe. Good results were photographed (Ford *et al.*, 1994; Verma and Baru, 1995).

2.6 Two colour FISH

2.6.1 Probe labelling

2.6.1.1 Probe labelling with biotin

Probe labelling with biotin was as described above (section 2.5.1).

2.6.1.2 Probe labelling with digoxygenin (DIG)

2.6.1.2.1 Random primed DNA labelling with digoxygenin-dUTP using a DIG DNA Labeling Kit (kit 2)

1 µCi of ^{3}H-dTTP was dried in a 1.5 ml Eppendorf tube under vacuum in a Spin-Vac. The ^{3}H-dTTP was used to measuring the efficiency of labelling, because the TTP in DIG DNA Labeling Kit (kit 2, , Boehringer Mannheim, Catalog No. 1175033) was DIG-labelled.

0.5-1.0 µg of probe DNA in 15 µl of dH_2O was added, and denatured by heating in a boiling water bath (100°C) for 10 minutes and chilling quickly in ice/NaCl. While on ice, 2µl of random hexanucleotide mixture, 2 µl of 10x dNTP Mix and 1 µl of 10x Klenow enzyme (kit 2) were added to the tube. The mix was gently vortexed, microcentrifuged and incubated at 37°C for 18-20 hours. A 5 cm BioGel P60 or Sephadex G-50 column for each probe was prepared in a short Pasteur pipette plugged with sterile, non-absorbent cotton wool and washed twice with $T_{10}E_1$ at pH 7.6. At the end of the DIG labelling, 2 µl of 0.2M EDTA solution (pH 8.0) was added and mixed well to stop the reaction.

2.6.1.2.2 Collection and isolation of DIG labelled probe

The collection and isolation of DIG labelled probe were as described above (section 2.5.1.2).

2.6.2 Two colour *In situ* Hybridization

2.6.2.1 Denaturation of target DNA

Denaturation of target DNA on slides was as described above (section 2.5.2.1).

2.6.2.2 Denaturation of probe DNAs

Two dried satellite probes, 100 ng per probe (one biotin labelled and the other DIG labelled), were dissolved in 8µl 10x SSCP (pH 6.0) each. The two probes were mixed well with 10.4 µl of 7.7µg/µl salmon sperm DNA, so that the ratio of salmon sperm DNA to probe DNA was 400X. After the volume of the above mixture was adjusted to 25µl with dH_2O, 25µl of 20% dextran sulphate in formamide was carefully added. The probe mix was briefly heated to 75°C, mixed thoroughly on a vortex mixer, and spun down in a microcentrifuge. The probe was denatured for 10 minutes at 75°C, and then quickly cooled in an ice/water mix for at least two minutes.

2.6.2.3 Hybridization

50 µl of the appropriate probe mix was added to each slide in a big droplet. A clean 22 x 50 mm coverslip was mounted, avoiding air bubbles. The slide was sealed with rubber cement

and hybridized overnight at 37°C in a sealed humidity box.

2.6.3 Stringency rinsing

The stringency rinsing for two colour label FISH is the same as for single label FISH (see section 2.5.3).

2.6.4 Immunochemistry

The following procedures were done at room temperature (RT) if not specified.

1) The excess moisture of the slide was removed by tapping the edge of each slide on paper towelling and 100 µl of 4 x SSC/1.0% w/v bovine serum albumin (BSA) was dropped on the slide. A 22 x 50 mm coverslip was added, and the slide incubated at 37°C in a moist chamber for 10-20 minutes.

2) 1 µl of avidin-FITC was thoroughly mixed with 200 µl of 4 x SSC/1.0% BSA. After the coverslip was removed, the excess moisture of the slide was drained by tapping the edge of each slide on paper towelling. 80 µl of the avidin-FITC mix was placed on one slide and another 22x50 mm coverslip applied. The slides were incubated at 37°C in the moist chamber for a minimum of 30 minutes. Usually, the biotin-avidin-FITC signal was strong and there was no need for further amplification.

3) The coverslip was removed and the slide was rinsed as follows with agitation: once for 5 minutes in 4x SSC/0.05% Tween 20, and twice for 5 minutes in 0.1M Tris-HCl/0.15M NaCl/0.05%Tween20 (pH7.5). At the same time, 2µl of mouse anti-DIG was thoroughly mixed with 100 µl of 0.1M Tris-HCl/0.15M NaCl/0.5% BSA. 80 µl of mouse anti-DIG mix was applied to the slides as 2-3 drops, and a coverslip was mounted. The slide was incubated at 37°C in a moist chamber for 30 minutes.

4) The coverslip was removed and the slide rinsed with agitation three times for 5 minutes in 0.1M Tris-HCl/0.15M NaCl/ 0.05%Tween20 (pH7.5). At the same time, 2.5 µl of rabbit anti-mouse-rhodamine was thoroughly mixed with 92.5 µl of 0.1M Tris-HCl/0.15M NaCl/0.5% BSA and 5µl of rabbit serum. The edge of the slide was tapped on paper towelling to remove excess moisture. 80 µl of rabbit anti-mouse-rhodamine mix was placed

onto the slide as 2-3 drops. A coverslip was added, and the slide was incubated at 37°C in the moist chamber for 30 minutes.

5) The coverslip was removed and the slide rinsed with agitation three times for 5 minutes in 0.1M Tris-HCl/0.15M NaCl/ 0.05%Tween20 (pH7.5). At the same time, 2.5 µl of swine anti-rabbit-rhodamine were thoroughly mixed with 92.5 µl of 0.1M Tris-HCl/0.15M NaCl/0.5% BSA and 5µl of swine serum. The edge of the slide was tapped on paper towelling to remove excess moisture. 80 µl of swine anti-rabbit-rhodamine mix were placed onto the slide as 2-3 drops. A coverslip was added, and the slide was incubated at 37°C in the moist chamber for 30 minutes.

6) The coverslip was removed and the slide rinsed as follows with agitation: twice for 5 minutes in 0.1M Tris-HCl/0.15M NaCl/ 0.05%Tween20 (pH7.5) and twice for 5 minutes in 0.1M Tris-HCl/0.15M NaCl (pH7.5).

7) If required, biotin-avidin-FITC signal was amplified using either of the two ways:

a) If the biotin-avidin-FITC signal was weak, the coverslip was removed and the slide rinsed once for 5 minutes in 0.1M Tris-HCl/0.15M NaCl/0.05%Tween20 (pH7.5). Then the slide was treated as described [section 2.5.4 (3) and (4)]; or

b) Amplification was performed during detection of the DIG hybrid. That is, at step 3 [section 2.6.4 (3)], the mouse anti-DIG mix was 2µl of mouse anti-DIG, 1 µl biotinylated goat anti-avidin, 94 µl of 0.1M Tris-HCl/0.15M NaCl/0.5% BSA and 5 µl goat serum; and at step 4 [section 2.6.4 (4)], the rabbit anti-mouse-rhodamine mix was 2.5 µl of rabbit anti-mouse-rhodamine, 0.5 µl avidin-FITC, 92.5 µl of 0.1M Tris-HCl/0.15M NaCl/0.5% BSA and 5µl of rabbit serum.

2.6.5 Staining and viewing

The slide was stained in a solution of 50 µg/ml of Hoechst-33258 in dH_2O for 1 minute and rinsed twice for 30 seconds in dH_2O. After the moisture was tapped off, the slide was mounted with a 24x60 mm coverslip and 100 µl of PPD9. Excess mountant was squeezed out, and the slide sealed with nail varnish.

The slide was screened quickly under UV excitation and 10x magnification to find the location of cells with well spread chromosomes. The slide was then viewed under green excitation at 100x magnification to show a red signal where the rhodamine-labelled antibodies were detecting the hybridized probe and under blue excitation at 100x magnification to show a yellow-green signal where the FITC-labelled avidin was detecting the hybridized probe. Finally, the slide was viewd again under UV excitation at 100x magnification to show bright blue staining of chromosomes. Good results were photographed using triple exposure (Ford *et al.*, 1994).

Chapter 3

LYMPHOCYTE CULTURE OPTIMIZATION, SLIDE-PREPARATION AND EFFECTS ON CHROMOSOMAL ASSOCIATION

3.1 Introduction

Good cell culture conditions are essential to provide clear, banded chromosomes to map DNA sequences and genes, to identify chromosomal abnormalities, or to study the evolution of chromosomes. In mammals, cultured peripheral blood lymphocytes are one of the most widely used sources of banded chromosomes.

The results of cell culture can vary significantly depending upon the conditions. A number of factors may affect the de-differentiation and the growth of cells, including the components of the culture media, the temperature, the pH of the culture environment, and the culture time. Different pre- and post- harvesting treatments can also affect the quality of the prepared chromosomes. Because FISH requires high quality slides, some attempts were made to improve the short-term culture of bovine lymphocytes herein. The effect of lymphocyte culture on the chromosomal associations previously observed (Gordon 1995) was also examined.

3.2 Optimization of lymphocyte culture and slide praperation

Lymphocyte enrichment can increase the number of lymphocyte cells being transferred to the culture media and generally provide more metaphase cells for microscopy. However, compared with lymphocyte enrichment, whole blood culture requires less blood to be sampled and is easier to establish. Because the blood is placed directly into media, whole blood culture also decreases the chance of contamination and cell damage. If whole blood cells are cultured in a suitable medium, the number of dividing lymphocytes will multiple to be sufficient for microscopy. Therefore, it was decided not to undertake lymphocyte enrichment.

3.2.1 Culture medium

Many media have been developed for cytogenetic analysis, including Medium 199 and, in recent times, Roswell Park Memorial Institute medium 1640 (RPMI), a very rich medium initially developed for the culture of leukemia cells. AmnioMax C-100, designed for amniotic cell culture, was thought to be comparable to RPMI (GC Webb, pers. com.), and these two media were systematically compared herein.

RPMI requires added foetal bovine serum (FBS) and glutamine, plus mitogen(s) [phytohaemagglutinin (PHA) and/or pokeweed mitogen (PWM)], heparin, antibiotics and fungicide. AmnioMax is a complete medium for amniocyte culture, but can be used for blood cell culture. It also requires mitogens, heparin, fungicide, and occasionally additional antibiotics. If the medium has been kept at 4°C for longer than 20 days, then additional glutamine should be added.

3.2.1.1 Methods

In this study, the following media were used in trials of cattle blood cell culture:

a) MRB10: RPMI with 10% v/v FBS, 1% v/v 0.2M glutamine (glut.) plus 2% M form PHA (PHA), 0.5% v/v 1.0 mg/ml PWM (PWM), 0.2% v/v 10 mg/ml gentamycin solution (gent.), 0.5% v/v penicillin (50 unit/ml)/streptomycin (50 unit/ml)/fungizone (0.125 mcg/ml) (P/S/F), and 0.1% v/v 25000 units/ml heparin solution (hep.);

b) MRB15: as for MRB10 but with 15% FBS;

c) MRB20: as for MRB10 but with 20% FBS;

d) MRS15: as for MRB10 but with 15% newborn sheep serum (NSS);

e) MDB10: as for MRB10 but with Dulbecco's Modified Eagle's Medium (DMEM);

f) MA: AmnioMax liquid basal and frozen supplement plus 2% v/v PHA, 0.5% v/v PWM, 0.5% v/v P/S/F, 0.1% v/v hep., and if the AmnioMax had been stored for longer than 20days, 0.5% v/v glut.

0.5 ml of whole cattle blood was added to 10 ml of each medium and cultured at 37°C and 5% CO_2 for 72 hours. 5-bromodeoxyuridine (BrdU) was added to a final concentration of 20 μg/ml 7 hours before harvesting and 0.2 ml of 100 μg/ml of colchicine was added 45 minutes before harvesting. Slides were prepared from the cells using standard conditions (section 2.3.2). Slides were stained with 20% Leishman's stain at pH 6.8 for 15 minutes. All cells in metaphase on each slide were counted under 10x magnification using a bright-field microscope (Olympus BH-2) to generate a mitotic score.

3.2.1.2 Results

The mitotic score of blood lymphocytes in AmnioMax was twice that of RPMI with all levels of FBS or DMEM with FBS (Fig 3.1). All individual differences were highly significant using χ^2 tests, $p<0.005$**. RPMI with FBS gave significantly higher mitotic scores than RPMI with NSS using χ^2 tests, $p<0.005$**. There were no significant differences between RPMI with different concentrations of FBS from 10% to 20%. These results clearly indicate that AmnioMax improves the mitotic score of cattle blood lymphocytes in culture.

3.2.2 Effect of freezing medium

Usually complete media are used immediately or stored for only a short time at 4°C before use. However, it is not convenient to prepare a small amount of medium every time. It was thought that media might be kept longer if it is stored frozen at -20°C.

In this study, one tube of 5 ml complete AmnioMax was frozen before use and the other tube was fresh. 0.25 ml of the same blood was added to both tubes and cultured as above. The mitotic indices of the two cultures were the same (Fig 3.2, χ^2 test, $0.50<p<0.90$).

3.2.3 Culture time

The lymphocytes in mammalian blood are normally at the G_0 stage in the cell cycle; that is, they are in a differentiated state and do not divide. However, they will divide in response to allergens or infections. In culture, lymphocytes can de-differentiate and return to the division cycle by stimulating with an appropriate antigen (mitogen) and maintaining division by growth factors (cytokinesis).

Since different cell types have different times for cell cycles, it was necessary to determine the time required for cattle lymphocytes to return to the cell division cycle and determine when an optimal number of metaphase cells could be harvested.

3.2.3.1 Methods

Cattle whole blood cultures were established at the same time under the same conditions. Two cattle, J103 and J111, were used for the test. The blood samples were cultured either in MA or MRB15. The conditions for harvesting and slide-preparation were identical except for the culture time. There was no BrdU incorporation step before harvesting. Colchicine was added 1 hour before harvesting. Slides were stained in 20% Leishman's stain at pH 6.8 for 15 minutes. Metaphases from the same volume of culture were counted over whole slides under 10x10 magnification using bright-field optics.

3.2.3.2 Results

In the first 16 hours of the culture, cells were rarely seen in mitosis (Fig 3.3). At the 24th hour of the culture, metaphase cells were still rare, but some cells were in prophase. After that

time, more cells entered the cell division cycle and more metaphase cells were obtained. The metaphase cell number rose between 48 and 72 hours. The peak was at about 60 hours when cells were cultured in MA and at about 72 hours when cells were cultured in MRB15.

3.2.4 Colchicine incorporation

Appropriate colchicine treatment can prevent the formation of the spindle and help the spread of metaphase chromosomes during slide-preparation. In this study, 0.2 ml of 100 μg/ml of colchicine was added to 10 ml of culture media before harvesting at the following times: 2 hours, 1 hour, 30 minutes, and 15 minutes before harvest.

The slides were stained with 20% Leishman's stain in Gurr buffer (pH 6.8) and screened under the microscope. There were more cells on slides with chromosome morphologies suitable for FISH if the cells underwent colchicine treatment for longer than 15 minutes. However, with 2 hours of colchicine treatment, many metaphase cells had chromosomes which were too short and swollen (chromatoclasic effect) and the chromatids have separated (mitoclasic effect). It was found that the metaphase cells had better chromosome morphology for FISH and banding when the colchicine treatment time was between 30~60 minutes.

3.2.5 Thymidine arrest

To produce R-bands, BrdU can be added to the medium 6~7 hours before the cells are harvested. To increase the ratio of metaphase cells, thymidine (Tdr) can be added before BrdU to arrest cells in the middle of the S-phase (thymidine arrest).

Two tubes of blood cells were cultured in MRB10 for 72 hours, and BrdU was added to tube A to a final concentration of 20 μg/ml and Tdr was added into tube B to a final concentration of 300 μg/ml. Cells in tube A were cultured for further 7 hours and harvested.

Cells in tube B were cultured overnight. After washing out Tdr with two rinses with PBS, cells in tube B were cultured in MRB10 containing 20 µg/ml BrdU for another 7 hours.

The mitotic scores were not significantly different between the thymidine-arrested cells and those harvested after 72 hours with only BrdU present. It was concluded that arrest by thymidine was not justified considering of the labour and materials involved.

3.2.6 Lymphocyte storage

Once cultured, it is easier to store lymphocytes in fixative at -20°C than to store lymphocytes on slides at -20°C. However, some lymphocytes can be destroyed or left in the supernatant each time a slide is prepared if the lymphocytes are stored in a single large volume of fixative. Therefore, it was decided to examine whether it was better to store lymphocytes in a large volume of fixative or in small aliquots.

One tube of 1 ml of fresh lymphocyte suspension was mixed with 10 ml of fixative, and 10 tubes of 0.1 ml of fresh lymphocyte suspension were mixed with 1 ml of fixative. All tubes were stored at -20°C.

After 1 week of storage, the 10 ml tube was warmed and the cells were mixed well. 1.1 ml of the mixture was transferred to an empty tube and centrifuged. Cells were resuspended in 0.1 ml of fixative. At the same time, a 0.1 ml tube was warmed, and cells were pelleted by centrifugation and resuspended in 0.1 ml of fixative. Slides were prepared from each suspension and stained with 20% Leishman's stain. All metaphase cells on the slide were counted under the microscope. The above protocol was repeated for 9 weeks.

With one exception, metaphase cell numbers were constant using the small aliquots for storage. The metaphase cell numbers from the large 10 ml volume sample decreased and had more varied numbers than the small aliquot samples (Fig 3.4).

3.2.7 Modified conditions

Given the results above, cattle lymphocyte culture conditions were modified as follows for routine laboratory blood cell culture: 0.5 ml of cattle whole blood was cultured using 10 ml of complete AmnioMax medium at 37°C and 5% CO_2 for 60~72 hours. Seven hours before harvesting, BrdU was added to a final concentration of 20 μg/ml to produce R-bands and 0.5~1 hour before harvesting, colchicine was added to a final concentration of 2 μg/ml. Fixed cells were stored at -20°C in small aliquots before slide preparation. This modified cattle lymphocyte cell culture was used to examine the cattle chromosome association previously reported by Gordon (1995).

3.3 Chromosome association studies

Chromosomes in mitotic metaphase cells will be well separated on a slide if the cells are treated appropriately during culture and slide preparation. However, some chromosomes may occasionally appear "associated" in metaphase cells. For example, in humans, the procentric nucleolar organizer regions (NORs) of chromosomes 13~15 and 21~22 are often physically close in metaphase spreads.

In cattle, Gordon (1995) described a chromosome association. He cultured lymphocyte samples from 170 Jersey cows, 108 Limousin cows and 96 Angus bulls and heifers, and screened solid stained slides made from these samples. He reported that three Jersey cows (Jersey 103 - J103, J111 and J198) 'exhibited a centric fusion between two unidentified chromosomes' and 'this centric fusion was mosaic and appeared in 10% of cells viewed' (Gordon, 1995). However, Gordon did not observe the centric fusions in R-banded chromosomes using fluorescence and suggested that chromosomes involved in the 'centric fusion' may not be a true Robertsonian translocation, but an association at the centromeres.

Because the nucleolar organizer regions (NORs) in cattle are normally found at the q-terminus (ie., telomeric region of the long arm) of 5 pairs of autosomes (Mayr and Gruber, 1987), Gordon postulated that the chromosome associations could be caused by unusual NORs. Presumably, in the three Jersey cows, the NORs had moved from the q-terminus to the p-terminus of the chromosome. Since cattle autosomes are acrocentric, two of the unusual NOR chromosomes might associate near the centromeres in metaphase cells and appear as a centric fusions (Gordon, 1995).

To determine whether the chromosome associations in the Jerseys cows involved their NORs, experiments were designed to confirm the association and the location of the NORs. The experiments involved scoring the slides for the frequency of the chromosome associations, silver staining the NORs and using fluorescence *in situ* hybridization (FISH) of 18s ribosome DNA to localize the NORs on metaphase chromosomes.

3.3.1. Solid staining

Because one of the three Jersey cows (J198) previously scored for chromosomal association died before additional blood samples could be taken, only J103 and J111 were used as experimental animals.

To test for the chromosome association, the original solid-stained lymphocyte slides of one Jersey cow (J103) (Gordon, 1995) were scored. Fifty-six well spread cells had no chromosome association. Only one cell was identified that had two small chromosomes crossing at their centromeres [43,XX,t(?;?)]. Notably, many of chromosomes in this cell were missing.

New lymphocyte chromosome spreads from Jersey cows J103 and J111 were made as previously described (Gordon, 1995). Slides were stained with 20% Leishman's stain at pH 6.8 for 20 minutes and screened at a magnification 100x with an Olympus microscope BH-2.

From the new solid stained slides, 97 good metaphases of J103 were scored and 1 cell was observed to have a chromosome association. No chromosome associations were found in 63 good metaphases of J111. These results did not verify the high frequency of chromosome associations in J103 and J111 observed by Gordon (1995).

3.3.2 Localization of nucleolar organizer regions (NORs)

Since the chromosome association was believed to be involve in the formation of cattle nuclear organizer regions (NORs) (Gordon, 1995), two experiments using silver staining and FISH were performed to determine the chromosomal location of NORs in the Jersey cows J103 and J111.

3.3.2.1 Silver staining (Ag-NOR)

Silver nitrate (Ag-NOR) stains the region adjacent to the NORs because of the presence of an acidic, non-histone protein, called nucleolin (Barch, 1991). Thus, by using of this stain, the NORs in the chromosomes of J103 and J111 could be studied.

Air-dried metaphase slides of J103 and J111 were prepared in usual manner and stained with silver nitrate as described (section 2.4.3.2). Metaphase cells for each animal were screened using an Olympus BH-2 microscope and photographed. All NORs of the chromosomes from J103 and J111 were at the q-terminus. None of the NORs appeared at the p-terminus in any chromosomes (Fig 3.5).

3.3.2.2 Fluorescence in situ hybridization (FISH) of 18s ribosome DNA

The NORs in mammalian chromosomes contain a large number of repeated genes that code for the highly conserved 18s and 28s ribosome RNAs (rRNAs) (Benn and Perle 1986, Nicholas 1987). Therefore, an 18s rDNA probe (5.0 kb insert of rat 18s RNA gene in *pBR332*

at the *Hind III* site, from Drs. Andreas Evdokiou and Prue Cowled) was used as a FISH probe to detect the NORs on cattle chromosomes.

The probe was labelled with biotin-dATP by nick translation (section 2.5.1). The metaphase slides of J103 and J111 were prepared in usual manner and treated with RNase for 1 hour just before FISH. After 18 hours of hybridization, slides were rinsed as described (section 2.5). Avidin-FITC was used for detecting the location of the hybrids. Signals were amplified with goat anti-avidin, followed by avidin-FITC. The slides were screened under the fluorescent microscope and metaphase chromosomes of J103 and J111 were photographed.

The results from the FISH of 18s rDNA on chromosomes from J103 and J111 indicated that all NORs were near the telomeres of the long arms of the chromosomes (Fig 3.6). From these experiments, it appears that the Jersey chromosome associations are not likely to involve NORs.

3.3.3 Blind scoring of chromosome association

Since results from above experiments could not characterise the chromosome association, a blind scoring experiment was designed. Blood samples were collected from four cows: J103 and J111 as the test animals, and Jersey 946 (J946) and Limousin 486 (L486) as control animals. Blood samples were centrifuged at 350 x g for 10 minutes for lymphocyte enrichment. 0.3 ml of lymphocytes was transferred to 4.9 ml of RPMI plus 10% FBS media as described by Gordon (1995). Cells were cultured at 37°C, 5% CO_2 for 72 hours, and then BrdU was at the final concentration of 20μg/ml. The cells were cultured for further 8 hours. Two hours before harvest, two drops of colchicine at 100μg/ml were added to each culture.

For comparing the frequency of the chromosome association in different culture conditions, J103 was also cultured by placing 0.3 ml of whole blood into 5 ml of complete AmnioMax medium and incubated at 37°C, 5% CO_2 for 64 hours. After BrdU was added as

above, and cells were cultured for another 7 hours. One hour before harvest, two drops of colchicine at 100μg/ml were added.

The harvest of cells was performed as described (section 2.3.2). Slides were made from the above 5 cultures, stained with 20% Leishman's stain at pH 6.8 for 15 minutes, air dried and covered with a coverslip and mounted with DEPEX (BDH).

The slides were coded by a third party. Well spread metaphases from each slide were observed and scored, and the scores from the same animal combined (Table 3.1). The results showed that the 'chromosome association' is not particularly frequent in Jersey cows J103 and J111. There is only one metaphase cell with a 'chromosome association' in 219 metaphase cells scored in L486, one in J103 cultured in RPMI and harvested as described by Gordon (1995), and none in J103 when cultured in AmnioMax.

Table 3.1 Blind score results

Culture media	Animal number	Scored metaphase cells	Metaphase cells with chromosome association
AmnioMax	J103	71	0
RPMI1640+FBS	J103	222	1
	J111	34	0
	J946	107	0
	L486	219	1

3.4 Discussion

3.4.1 Cattle chromosome culture

Adequate cell culture and good chromosome slide preparation are essential for cytogenetic studies. Yet few efforts have been made to improve the conditions for livestock species. Therefore, an attempt to optimize conditions was made herein for cattle chromosome

preparation. AmnioMax was a better culture medium for cattle lymphocytes than RPMI with FBS, RPMI with NSS and DMEM with FBS. This is not surprising as AmnioMax was designed for amniotic cell culture. Amniotic cell culture media must be optimal for cell growth because of the low number of viable cells in a sample of amniotic fluid used for routine human chromosomal analysis.

It should be noted that culture temperatures were not examined herein and routine cell culture was done at 37°C. However, culture temperatures at 37.5~38°C are sometimes better for cattle blood culture (W Chen, pers. com.). It might be appropriate to examine the optimal the culture temperature for the different media as this may also affect cell growth.

In this research, the mitotic scores were not much different between thymidine-arrested cells and non-arrested cells harvested after 72 hours. This may be because the peak of growth was over at 72 hours of culture. It would be useful to thymidine arrest the cells after 48 ~ 60 hours culture to determine whether an early arrest improves the bands. To arrest cells at the same phase, there are other methods which could be tried, such as cooling the culture to 4°C overnight (GC Webb, pers. com.). The length of BrdU incorporation might also be optimized to improve banding.

The time and concentration of colchicine is critical for obtaining quality chromosomes. The time was examined herein, however, the colchicine incorporation can be further investigated by varying the concentration to determine the effects on cell toxicity. High concentrations of colchicine are toxic to cells and may cause cell death. On the other hand, lowered concentrations of colchicine in the culture might still prevent the formation of the mitotic spindle but delay chromosome condensation and the chromatid separation. This may decrease the metaphase score but provide better chromosome spreads (Webb, 1973).

It has been suggested that if a higher mitotic ratio was needed, the length of the colchicine incorporation time could be extended (B Gordon, pers. com.). However, according to the results from this study, 2 hours of colchicine treatment made the chromosomes too short and swollen to band well, and more chromatids were separated. Colcemid (N-desacetyl-N-methylcolchicine) can be substituted for colchicine because colcemid is less toxic and can be used at higher concentrations and longer times. However, colcemid shows only 1/10 of the mitoclasic effect of colchicine (Webb, 1973) and is a poorer spindle disruptor.

The quality of slides may be also affected by the hypotonic treatment. If the hypotonic treatment is not adequate, the chromosomes may not spread. The optimal times and temperatures of the hypotonic treatment may vary for different cell types and species. According the experience of our laboratory, the best length of the hypotonic treatment for cattle lymphocytes is 20~30 minutes at 37°C. It is difficult to optimize hypotonic treatment and control the pH. The use of a membrane poison has no detectable effect (GC Webb, pers. com.).

Another area for improvement is storage of fixed cells. Some of the cells are lost during centrifugation (GC Webb, pers. com. and Fig 3.4), and chromosomes made from cells stored in 3:1 of methanol and acetic acid at -20°C are "fuzzy". However, in this study, there was no obvious difference in the chromosomes from fresh and stored cells, although the metaphase score was reduced a little in the latter. When cells were stored in small aliquots, the metaphase score was not usually reduced over several months.

Using the results herein, the protocols in the laboratory were modified accordingly to provide good, consistent cattle chromosome spreads. One of the more important reasons to optimize the cell culture conditions and the preparation of slides is to minimize the cost of karyotype testing for chromosomal abnormalities in terms of both time and money. Lower costs will give cytogenetic services a wider acceptance and utilization by producers, and the

breeding programs will benefit by avoiding the use of animals with chromosome abnormalities. However, optimal cell culture is most useful for gene mapping studies, where high numbers of cells per slide with good banding are required.

3.4.2 Cattle chromosome association

Gordon observed that three Jersey cows had a chromosome association in 10% of metaphase fibroblasts and lymphocytes. The association involved the overlap of two chromosomes at the centromeres (Gordon, 1995). This chromosome association may have been caused by:

a) a rearrangement of the NORs to the centromeres of two chromosomes and a subsequent association of the NORs,

b) a centric fusion which occurred between two autosomes in some cells,

c) the incomplete separation of chromosomes because the spindle fibre was not completely disrupted during the colchicine and hypotonic treatments, or

d) an occasional overlap of chromosomes at the centromeres by chance (Fig 3.7).

The association was re-examined in lymphocytes to determine the most likely cause.

Results from the Ag-NOR banding and FISH of 18s ribosomal DNA experiments showed that the NORs were in the normal q-terminus location on chromosomes of J103 and J111. Thus, the NORs have not rearranged in J103 and J111 to the p-terminus. Consequently, the Jersey chromosome association at the centromeres is not likely to involve NORs.

If a centric fusion has occurred, the Jersey 'chromosome association' should be observed by R-banding to involve only specific chromosomes. However, no association was observed in the R-banded chromosomes from the Jersey cows. Therefore, it is unlikely that the association is a true centric fusion.

Since the blind scoring of the chromosome association indicated that it occurs in less than 1% of well spread metaphase cells, this suggests that the Jersey chromosome association may be an artefact. If Jersey chromosome association occurred by chance, the frequency of chromosome association would be expected to be lower than 10%. Therefore, the original observation may have been made because the chromosomes were not adequately separated.

There are several factors which might prevent chromosomes from being well separated on a slide:

a) The spindle fibre may not be completely disassembled during the colchicine and the hypotonic treatments. Thus, the centromeres of some chromosomes will not separate during the slide preparation.

b) Cells may have been treated with colchicine for too long so that the two chromatids of each chromosome began to separate. This causes the edges of chromosomes to blur, and the chromosomes do not appear to separate.

c) The techniques of slide preparation, such as the concentration of cell suspension in fixative, temperature differences between fixed cell suspension and the glass slide, the height between the cell drop and the glass slide, and the humidity of the environment may all affect the separation of the chromosomes.

d) Cell structures, such as the nuclear membranes, may vary between individuals. The centromeres are attached to the nuclear membrane in interphase, and if the attachment is stronger in some individuals, this may affect the chromosome behaviour during cell division.

In the work herein, every effort was made to duplicate the study of Gordon. This included using the same colchicine and hypotonic treatments. However, fine details (eg. temperature differences, humidity) may have differed. Therefore, it is likely that the chromosomes in Gordon(1995) were not as well spread as those herein and may explain the

higher frequency of chromosome association observed originally (10% vs. 1%). On the other hand, there is no explanation why these specific animals had a higher percentage of association versus other 374 animals studied by Gordon. Nevertheless, since the results cannot be reliably replicated and the chromosome associations were never been observed with R-banded chromosomes, it can be concluded that the associations were most likely an artefact of slide preparation.

Fig 3.1. Culture medium test. Blood lymphocytes were cultured and slides prepared as described (sections 2.3.2 and 3.2.1.1).

a) MRB10: RPMI with 10% foetal bovine serum (FBS), plus mitogens and anti-biotics;

b) MRB15: as for MRB10 but with 15% FBS;

c) MRB20: as for MRB10 but with 20% FBS;

d) MRS15: as for MRB10 but with 15% newborn sheep serum (NSS);

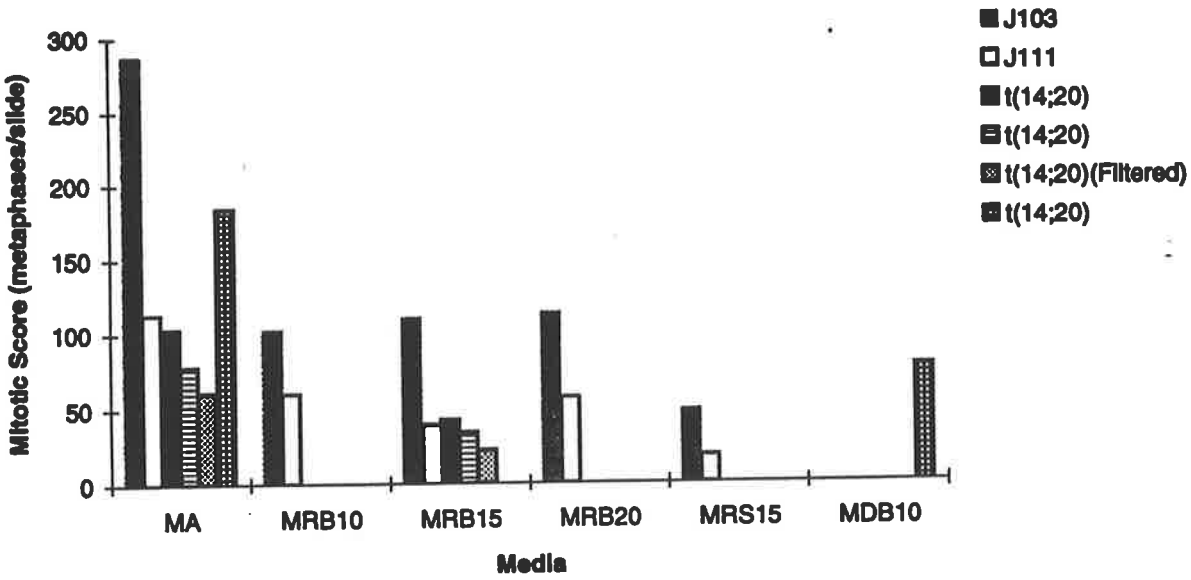
e) MDB10: as for MRB10 but with DMEM;

f) MA: AmnioMax liquid basal and frozen supplement plus mitogens and anti-biotics.

J103 and J111-two Jersey cows;

t(14;20)- Simmental cow carrying the 14;20 Robertsonian translocation used at different times.

Fig 3.2. Culture in fresh and frozen MA media. Blood lymphocytes were cultured and slides prepared as described (sections 2.3.2 and 3.2.1.1). The tested animal was a New Zealand bull. The average of 2 slides is shown for each medium.


Mitotic Score (metaphases/slide)
300
250
200
150
100
50
0
MA
MRB10
MRB15
MRB20
MRS15
MDB10
Media
J103
J111
t(14;20)
t(14;20)
t(14;20)(Filtered)
t(14;20)

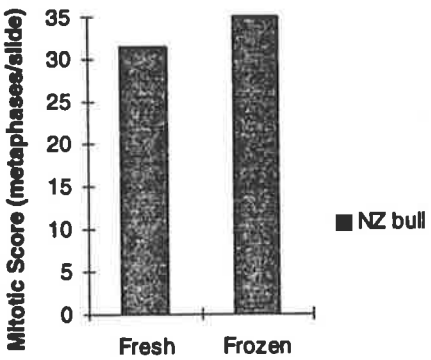

Mitotic Score (metaphases/slide)
35
30
25
20
15
10
5
0
Fresh
Frozen
NZ bull

Fig 3.3. Culture time test. Lymphocyte cultures were prepared and harvested at different times as described (section 2.3.2 and 3.2.3.1).

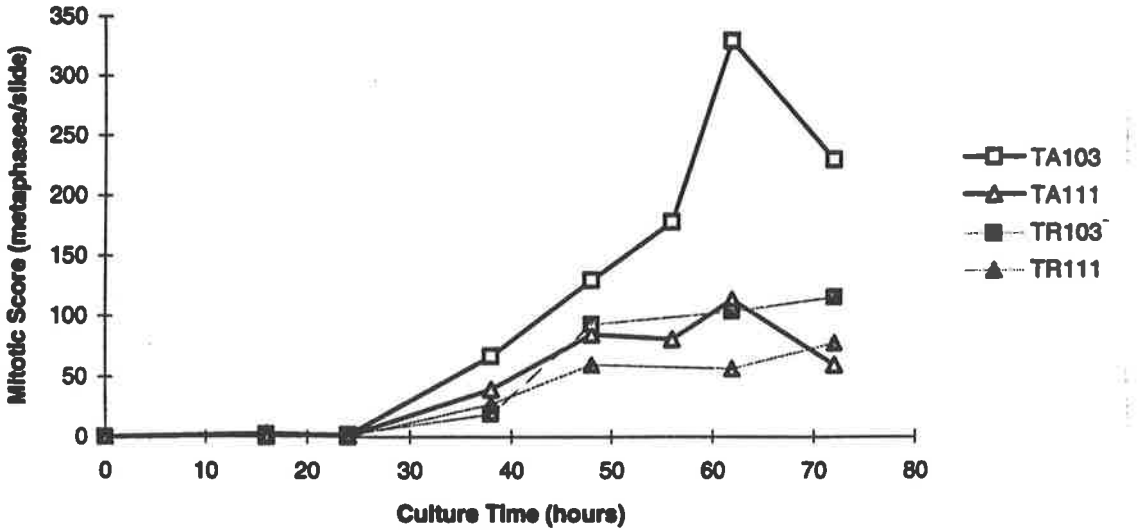
a) TA103: lymphocyte culture of J103 in MA;

b) TR103: lymphocyte culture of J103 in MRB15;

c) TA111: lymphocyte culture of J111 in MA;

d) TR111: lymphocyte culture of J111 in MRB15.

Fig 3.4. Lymphocyte storage test. Lymphocyte cultures were prepared and harvested at different times as described (section 2.3.2 and 3.2.6).


Mitotic Score (metaphases/slide)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
Culture Time (hours)
TA103
TA111
TR103
TR111

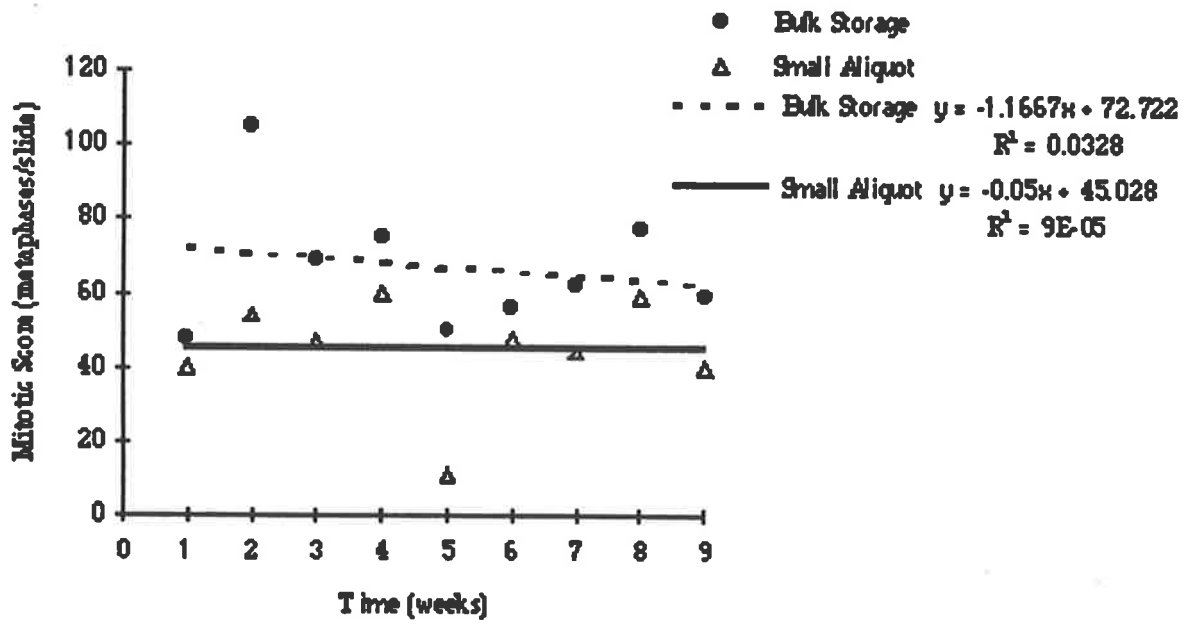
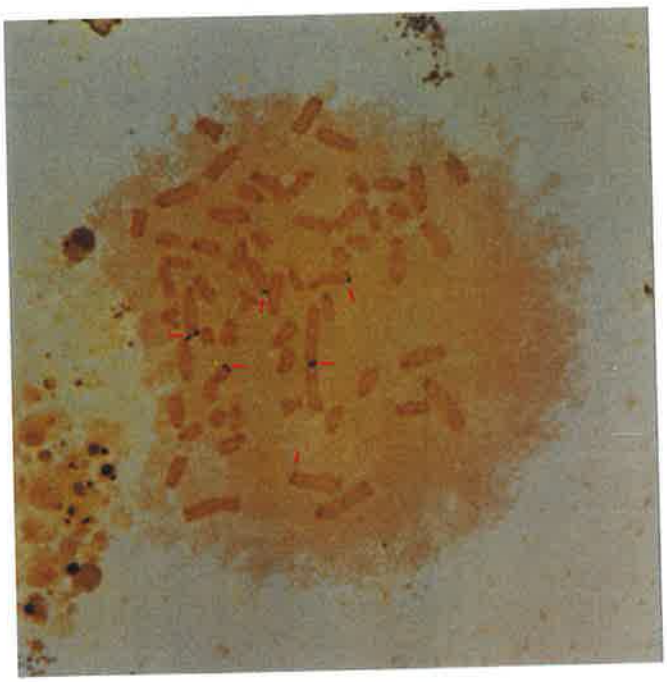
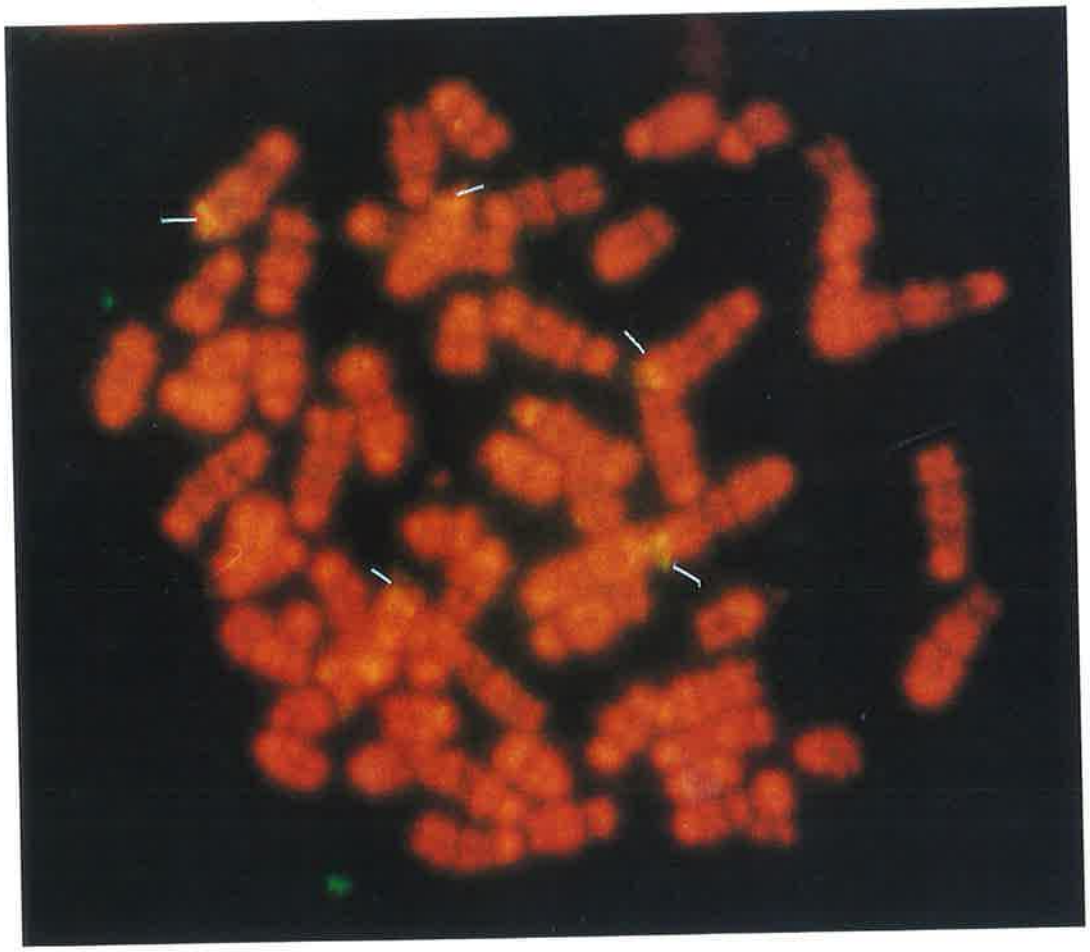

Mitotic Score (metaphases/slide)
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Time (weeks)
Bulk Storage
Small Aliquot
Bulk Storage y = -1.1667x + 72.722
R² = 0.0328
Small Aliquot y = -0.05x + 45.028
R² = 9E-05

Fig 3.5. Silver stain the nucleolar organizer regions (NORs) on the chromosomes of J103 (**short red lines**)

Fig 3.6. FISH on the chromosomes of J103 with rat 18s rDNA as the probe (**short white lines**)





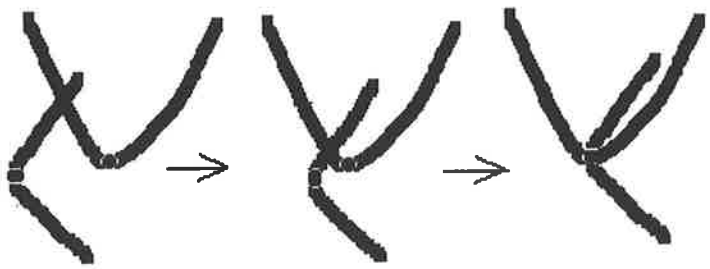


Fig. 3-7 Overlap of chromosomes at centromeres by chance

Chapter 4

LOCALIZATION OF SATELLITES I~IV IN *BOS TAURUS*

4.1 Introduction

The repetitive tandemly arranged DNA sequences at the centromeres of chromosomes are known as satellite DNA because when genomic DNA is separated on a density centrifugation gradient, they appear as satellite bands from the main band of unique DNA. Cattle have at least eight satellite DNA classes based on density. However, some of these are actually subsets of others. There are four main cattle satellite sequences: Satellite I (density 1.715 gm/ml), II (1.723), III (1.706), and IV (1.709), plus sub-classes sub-Satellite III (1.711a-ins, also called Satellite Ia), sub-Satellite III_d (1.711a-Pvu), sub-Satellite III_a/III_c (1.711a-Sau), Satellite Ib (1.711b-ins, sub-Satellite IV) and another sub-Satellite III (1.720b) (Modi *et al.*, 1993; Jobse *et al.*, 1995).

Satellites I, III, and IV are multiple repeats of relatively long sequences: 1.4, 2.3 and 3.8 kb, respectively; whereas Satellite II is somewhat anomalous, having a 686 bp repeat. The satellite sequences I, II, III and IV are found in the procentric heterochromatin of the 29 cattle acrocentric autosomes but not the metacentric sex chromosomes (Beridze, 1986; Modi *et al.*, 1993; Jobse *et al.*, 1995). In this study, FISH (fluorescence *in situ* hybridization) was used to analyse the pattern of localization of the four main cattle satellite sequences and their relationship to each other.

4.2 Materials and methods

4.2.1 DNA probes

Clones of bovine satellite DNA sequences I, II, III and IV were kindly provided by Mrs. H. Cerin Kojevnikoff from Professor C.D.K. Bottema's laboratory (Bottema *et al.*, 1994).

remove the coverslip, and twice to remove the PPD11. Amplification was then performed as above step (1).

4.2.4 Two-colour FISH

4.2.4.1 Probe labelling

Satellite probes I, II, III and IV were labelled with biotin-14-dATP using the biotin nick translation technique. Satellite probes I, II, III and IV were also labelled with digoxygenin (DIG) -11-dUTP using random primed DNA labelling (section 2.6.1).

4.2.4.2 Two-colour in situ hybridization of satellite DNA probes to chromosomes

100 ng biotin-labelled and 100 ng DIG-labelled satellite probes with 200 times ssDNA were denatured in 50 µl of probe mixture and hybridized to denatured cattle metaphase chromosomes on one slide (section 2.6.2). The hybridization was overnight at 37°C in a sealed humidity box. The stringency rinses for two-colour label FISH was the same as for single label FISH (section 4.2.3.2).

Slides used were metaphase chromosome spreads of fibroblasts from a carrier of the Robertsonian translocation t(14;20) and of lymphocytes from a carrier of the Robertsonian translocation t(1;29).

4.2.4.3 Immunochemistry

4.2.4.3.1 Biotin detection with fluoresceine isothiocyanate (FITC)

Detection of biotin by avidin-FITC was as described above (section 4.2.3.3). After incubation with 0.5% avidin-FITC in 4 x SSC/BSA for 30 minutes, the slides were rinsed once for 5 minutes in 4x SSC/Tween 20 with agitation.

4.2.4.3.2 DIG detection

4.2.4.3.2.1 Direct DIG detection with aminomethylcoumarin acetic acid (AMCA)

For the direct DIG detection with AMCA, the slides were rinsed twice for 5 minutes in PBS/0.05% Tween 20 (PBS/Tween 20) with agitation, incubated at 37°C in a moist chamber with PBS/0.5% BSA (PBS/BSA) for 10-20 minutes, and then with 25% sheep anti-DIG-AMCA in PBS/BSA for 30 minutes.

The slides were rinsed as follows: three times for 5 minutes in PBS/Tween 20, and twice for 5 minutes in PBS (pH 7.0) with agitation. All slides were stained with propidium iodide and mounted with a coverglass and PPD11 to obtain R-bands. The slides were sealed with nail varnish.

The slides were viewed under blue excitation to show the orange propidium iodide staining of R-banded chromosomes and a bright yellow signal where the FITC-labelled avidin was bound to the hybridized probe. The slides were also viewed under UV excitation to show the faint orange propidium iodine staining of R-banded chromosomes and a bright blue signal where the AMCA labelled anti-DIG was bound to the hybridized probe. A white or faint pink signal was observed occasionally under UV excitation if the FITC signal was very strong. Good results were photographed separately under blue excitation and under UV excitation.

4.2.4.3.2.2 Indirect DIG detection with tetramethylrhodamine isothiocyanate (TRITC)

For indirect DIG detection with TRITC, the slides were rinsed twice for 5 minutes in 0.1M Tris-HCl/0.15M NaCl/0.05%Tween 20, pH 7.5 (Tris/NaCl/Tween 20) with agitation, and incubated with 2% mouse anti-DIG in 0.1M Tris-HCl/0.15M NaCl/0.5% BSA, pH 7.5 (Tris/NaCl/BSA) at 37°C in a moist chamber for 30 minutes.

The slides were then rinsed three times for 5 minutes in Tris/NaCl/Tween 20 with agitation, and incubated with 2.5% rabbit anti-mouse-TRITC and 5% rabbit serum in Tris/NaCl/BSA at 37°C in a moist chamber for 30 minutes. Subsequently, most slides were

rinsed three times for 5 minutes in Tris/NaCl/Tween 20 with agitation, and incubated with 2.5 swine anti-rabbit-TRITC and 5% swine serum in Tris/NaCl/BSA at 37°C in a moist chamber for 30 minutes. (These steps were omitted in a few slides for comparison.)

The slides were finally rinsed as follows with agitation: twice for 5 minutes in Tris/NaCl/Tween 20 and twice for 5 minutes in 0.1M Tris-HCl/0.15M NaCl (pH 7.5). All slides were stained with Hoescht 33258. Most of the slides were mounted with PPD at pH 9, and some with diluted PPD at pH 9 with 2x SSC in an effort to improve the G-bands.

The slides were screened quickly: 1) under UV excitation and 10x magnification to find cells with well spread chromosomes, 2) under green excitation and 100x magnification to show a red signal where the TRITC-labelled antibodies were bound to the hybridized probe, 3) under blue excitation and 100x magnification to show a yellow-green signal where the FITC-labelled avidin was bound to the hybridized probe, and 4) under UV excitation and 100x magnification to show bright blue staining of chromosomes. Good results were photographed using triple exposure on Fujichrome DX 400 colour slide film (see section 2.6.5).

4.2.4.3.3 Post-amplification for biotin signals

When the biotin-avidin-FITC signal was found to be too weak, the slide was treated as described in section 4.2.3.4 (2), with the final washing, Hoescht staining and PPD9 mounting as described in section 4.2.4.3.2.2.

4.3 Results

4.3.1 Single-colour FISH

The bovine Satellite I, II, III and IV DNA sequences hybridized to the procentric regions of the acrocentric autosomes of *Bos taurus*. None of the four satellite probes hybridized to the

X- or Y-chromosomes. Satellites I and II hybridized to all 29 pairs of autosomes, while Satellites III and IV hybridized to a fraction of the autosomal pairs, as previously documented (Modi *et al.* 1995; Webb, pers. comm.; Table 4.1).

Table 4.1. Localizations and intensities of signal from Satellites III and IV hybridized to the chromosomes of *Bos taurus* (modified from Modi *et al.*, 1993).

Chromosome	Satellite III	Satellite IV
	Average, Range	Average, Range
1	2.7, 2-3	0.4, 0-1
2	2.8, 2-3	0.3, 0-1
3	2.7, 2-3	1.8, 1-3
4	2.0, 1-3	0.8, 0-2
5	2.4, 1-3	0.6, 0-2
6	2.9, 2-3	0.0, 0
7	2.5, 1-3	0.2, 0-1
8	2.6, 1-3	2.1, 1-3
9	2.9, 2-3	0.6, 0-2
10	2.9, 2-3	0.3, 0-1
11	2.8, 2-3	0.5, 0-1
12	2.7, 2-3	1.8, 1-3
13	1.5, 1-3	2.7, 2-3
14	0.6, 0-1	2.1, 0-3
15	2.9, 2-3	1.2, 0-2
16	2.4, 1-3	2.1, 1-3
17	2.8, 2-3	0.7, 0-3
18	1.4, 1-2	2.6, 1-3
19	0.0, 0	2.2, 1-3
20	0.4, 0-1	2.3, 1-3
21	2.7, 2-3	0.1, 0-1
22	2.5, 2-3	0.6, 0-1
23	0.1, 0-1	2.7, 1-3
24	0.6, 0-1	1.9, 1-3
25	0.0, 0	1.4, 0-3
26	1.1, 0-2	3.0, 3
27	0.9, 0-3	1.3, 0-2
28	2.5, 2-3	1.8, 1-3
29	0.0, 0	1.0, 0-3
X	0.0, 0	0.0, 0
Y	0.0, 0	0.0, 0

Average and range of signal intensities from 5-18 assessments where 0=none to very light, 1=light, 2=moderate, 3=heavy

Satellite I tends to produce a large single signal on each autosome, but not always in equal amounts. For example, pair 7 in the t(14;20) carrier female is heterozygous for the intensity of the Satellite I signal (Fig 4.1).

Satellite II produces a signal which is smaller than Satellite I. It often appears as 2 separate signals, one for each sister chromatid, and does not reach the p-terminus (Fig 4.2).

Satellite III has a strong signal on the longer autosomes, but is variable on the shorter chromosomes. Many of the smaller autosomal pairs have little or no Satellite III (Table 4.1). Satellite III often leaves the p-terminus unlabelled ("monk's haircut effect"). Even where present in small amounts, it labels as a line rather than on both chromatids (Fig 4.3).

Satellite IV has a strong signal on the small and medium autosomes, but produces little or no signal on the longer chromosomes (Table 4.1). Satellite IV labels close to the p-terminus. If present in small amounts, Satellite IV tends to label the chromatids separately, but less so than Satellite II (Fig 4.4).

4.3.2 Two-colour FISH

Two-colour FISH experiments were done with six combinations of biotin and DIG labelled probes (I, II, III and IV): biotin-I & DIG-II [direct and indirect DIG detection], biotin-I & DIG-III, biotin-I & DIG-IV, biotin-III & DIG-II, biotin-IV & DIG-II, and biotin-IV & DIG-III (Figs 4.5~4.10). The biotin-I signals were strong and it was not necessary to amplify the signal. Biotin-III and -IV signals were amplified because the signals were weak when the chromosomes were viewed under blue excitation.

Satellites I and II were observed to overlap one another in two patterns. Satellite II was either on the p-terminal side of Satellite I or Satellite II was occasionally in the middle of Satellite I depending on which chromosome was examined (Figs 4.5A and 4.11).

When present, Satellite III was on the long arm, distal to Satellite I (Fig 4.6), and clearly distal to Satellites II (Fig 4.8B) and IV (Fig 4.10B). The two-colour labelling showed that, in general, Satellite III was on the long arm and Satellite II was closer to the p-terminus.

Satellite IV, when present, was also seen to be nearer the p-terminus than Satellite III. With respect to Satellite I, Satellite IV tended to be closer to the p-terminus than Satellite I (Fig 4.7). On the other hand, Satellites II and IV appeared to overlap. However, Satellite IV was usually closer to the p-terminus than Satellite II (Fig 4.9B).

4.3.3 Experimental conditions

4.3.3.1 Comparison of DIG detection with direct AMCA versus indirect TRITC

AMCA signals faded under UV excitation within seconds, even though the slide was mounted with an antifade reagent (PPD11) (Fig 4.11). By comparison, the TRITC signals were much more stable under green excitation. Even when the slides were reviewed under UV for one or two minutes, the TRITC signals could be recognized upon returning to green excitation. On the other hand, AMCA detection had a clear background and TRITC detection resulted in background signals outside the metaphase cells.

The result of omitting the final swine anti-rabbit-TRITC step was that the TRITC detection for DIG-labelled Satellite I gave a reasonable strong signal which was easily separated from the background. However, the DIG-labelled Satellite II signal was weak and not easily to distinguished from the background. Under the same conditions, the DIG-labelled Satellite III and IV signals were of moderate intensity.

4.3.3.2 G-banding improvement

The G-banded t(14;20) fibroblast metaphase chromosomes gave a slightly better banding pattern when the slide was mounted with diluted PPD9 rather than with undiluted PPD9.

However, the Hoescht stained metaphase chromosomes did not give a clear banding pattern and could not be used for chromosome identification.

4.4 Discussion

The presence of Bovine satellite sequences I, III, and IV on specific chromosomes was previously described by Modi *et al.* (1993). However, these authors only performed single labelling experiments and did not determine the relationship between the satellite sequences. The results obtained herein were similar to Modi *et al.* (1993) (Table 4.2), but were extended by also using two-colour FISH in order to establish the organization of the Satellite sequences.

Table 4.2 Localization of Satellites I~IV on cattle chromosomes

Satellite	Localization
Sat I	all autosomes, strong
Sat II	all autosomes, weak to strong depending on chromosome
Sat III	most on large autosomes, variable signal
Sat IV	most on small autosomes, variable signal

4.4.1 Single-colour FISH

The single-colour FISH herein (Figs 4.1~4.4), using the four main bovine Satellite sequences (I~IV), demonstrated that the hybridization signals of a given satellite probe are not always equal even in homologous pairs of chromosomes (eg. Satellite I on pair 7, Fig 4.1A). This indicates that the amount of a particular satellite can be variable for a given pair of chromosomes, and hence, a source of polymorphism.

Satellite III gave little or no signal on the smaller chromosomes, while Satellite IV gave little or no signal on the longer chromosomes. This suggests that these two satellite DNA sequences may have separated at some stage of evolution: long chromosomes accumulated Satellite III with the exclusion of IV, while short chromosomes accumulated Satellite IV, but not III.

No hybridization of Satellites I~IV was observed on X and Y chromosomes. This suggests that X and Y chromosomes are likely to have other specific satellite sequences at their centromeres (Modi *et al.*, 1993).

The Satellite II hybridization signal was observed on the centromere of each autosome in both animals used, but not on any q-arm telomeres as Kurnit *et al.* (1973) described. The difference for this observation is presumably because the Satellite II probe used herein was more specific.

The single-colour FISH provided some information about the location of the bovine Satellite sequences relative to each other. For instance, it could be determined that, when present, Satellite III tends to be more distal than the other three Satellite sequences because of its 'monk's head' appearance. Satellites II and IV appear to be more proximal. However, the order of Satellite I, II and IV was not clear from the results of single-colour FISH, and two-colour FISH was performed in an attempt to clarify their relationships.

4.4.2 Two-colour FISH

Two-colour FISH with all combinations of the four main Satellite sequences (Figs 4.5~4.10) was used to better resolve the organization of the sequences at the centromere. The results did not support a Block model for the order of the four Satellite sequences, where the satellites are present as single consecutive segments (eg. Satellites IV-II-I-III).

Interpretation of the results suggests a Block/Interspersed model might be more appropriate (Fig 4.12), where the different Satellite sequences are intermixed with each other (eg. Satellites IV-II-I-IV-II-I-III). The main evidence for the Block/Interspersed model is the overlap of the Satellites II and IV signals with Satellite I. The overlap may be small, because from the single-colour FISH, the Satellite I signal on each of autosome appears as one large block, while Satellites II and IV are small blocks in the procentric area. From the two-colour

FISH (Figs 4.5 and 4.7), despite the overlap (white-yellow colour), there is still a clear red Satellite II or IV signal that can be distinguished from the green Satellite I signal. The overlap may vary from chromosome to chromosome. The relationship of Satellite III with the other satellites is less dependent on an interspersed model, it appears to flank the other Satellite sequences on the long arm with little or no overlap.

There are other lines of evidence in favour of a Block/Interspersed model which include: 1) the mixing of Satellites I-IV on the long arm of the human Y chromosome (Gosden *et al.*, 1975) and 2) the dispersed repeat families with homologous sequences of Satellite IV in bovine (Skrowronski *et al.*, 1984). In humans, most centromeres have a block of α-satellite DNA and/or its sub-family, but "at least in some situations, α-satellite DNA arrays can be interrupted by or joined directly onto non-α-satellite repeats" (Kalitsis and Choo, 1997). However, the resolution of the present work does not clearly distinguish between the Block and Block/Interspersed models in cattle.

4.4.3 Technical aspects

4.4.3.1 Photography

Most two-colour or multicolour FISH is recorded with electronic digital image analysis systems (Verma and Baru, 1995) which captures the images under different excitations and converts the images from white-and-black into colour. Images are captured quickly, so fading of the signal is not a problem (eg. Fig 4.13). The digital image analysis systems also allow the researcher to improve the signals, reduce the background, show different combinations of images between hybrid signals and the chromosomes, produce artificial colours for signals if the probe is labelled with two or more colours at different ratios, analyze the karyotype on the screen, and modify details of the image. Despite the advantages, digital image analysis is too expensive for use in most laboratories.

The photomicrography herein was done directly using triple exposure with Fujichrome DX 400 colour slide film in an Olympus PM 20 camera (Ford *et al.*, 1994). Neither the images nor background can be changed on the picture. However, the triple exposure picture does give exact information about the hybrid signal on the chromosome when the images from the three different filter blocks are aligned. Alignment of the dichromic mirrors of the three filter cubes in the Olympus BX60 microscope, used for the present work, was found to be almost perfect by using an England Finder slide (Graticules Ltd.) which auto-fluorescences with all combinations of the exciting wavelengths. The alignment of the UV mirror was very slightly different to the two-colours; this error has no effect on the observed relationship of the FITC and TRITC signals (Webb, pers. comm.). The triple exposure recording system is much easier to control and much cheaper to use than digital image analysis systems, so it can be used widely by many research groups.

4.4.3.2 DIG detection with AMCA or TRITC

The DIG signals were detected herein using either AMCA without amplification or TRITC with amplification. The AMCA direct detection system saved time and would suit staining with propidium iodide to show R-bands, but the fading of the AMCA is too quick to perform slide-review and photography without using a digital image analysis system. The TRITC detection system required more work and more chemicals, but the signals are much more stable than AMCA.

One problem of the TRITC detection in this research is that red background signals under green excitation were numerous and strong. This background may be due to the age of the antibody reagents or unclean slides.

4.4.3.3 HoechSt Banding

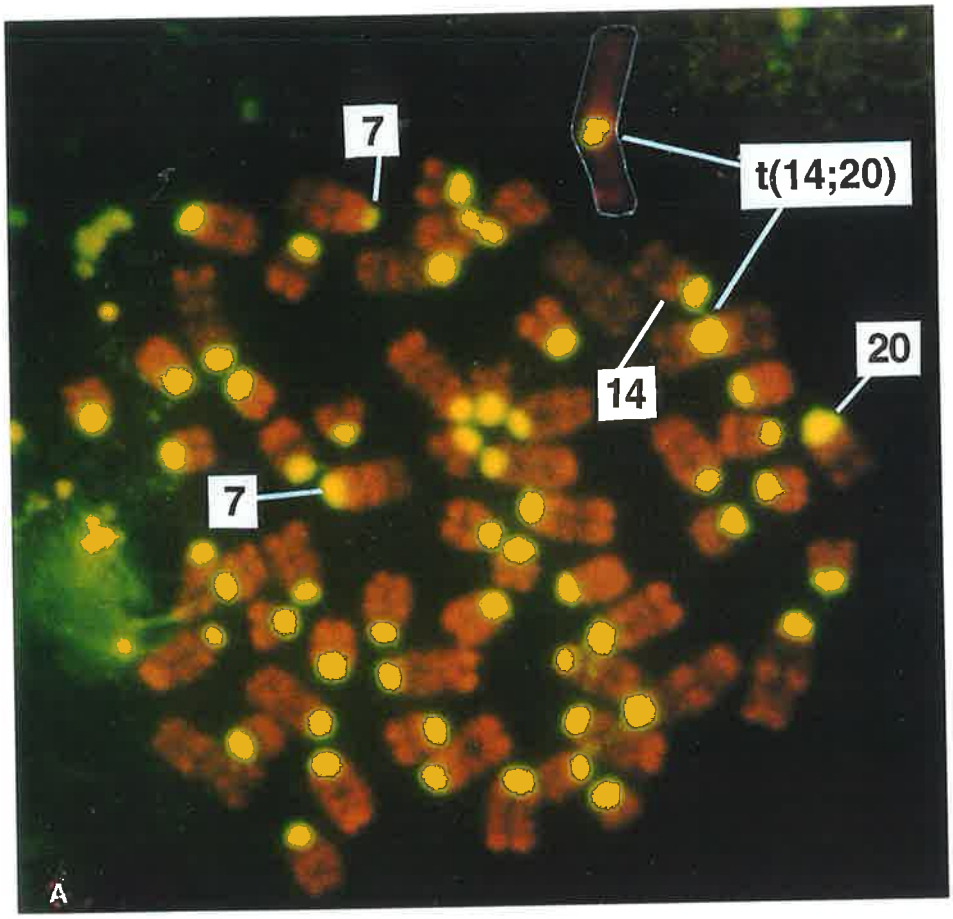
It is possible to band bovine chromosomes with Hoechst 33258 after BrdU has been incorporated during cell culture (Modi *et al.*, 1993). However, bovine fibroblast and

lymphocyte chromosomes with appropriate BrdU incorporation [t(14;20), early S-phase incorporation into fibroblasts for G-banding and t(1;29), late S-phase incorporation into lymphocytes for R-banding] did not band well with Hoechst 33258 herein. One week after the FISH was performed, the lymphocyte chromosomes gave slightly better banding, but the fibroblasts still showed no banding. Hoechst bands can be improved by using diluted antifade mountant (Verma and Baru, 1995), but the chromosomes on the slides mounted with diluted PPD9 still did not band well. It may be worthwhile to use DAPI as a counterstain in future work.

Fig 4.1. Satellite I hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite I does not always hybridize to all of the autosomes in equal amounts [eg. pair 7 indicated by an **arrow** in (A) is heterozygous for the size of the Satellite I signal].

(A) There are two Satellite I signals near the centromere of t(14;20). A second t(14;20) chromosome is shown to better illustrate the gap between the two Satellite I signals.

(B) The t(1;29) chromosome does not have any Satellite I. All other autosomes show a strong Satellite I signal, including chromosome 1 (labelled) and all small chromosomes in the size range of chromosome 29 (not identified, Table 4.1).


7
t(14;20)
14
20
7
A

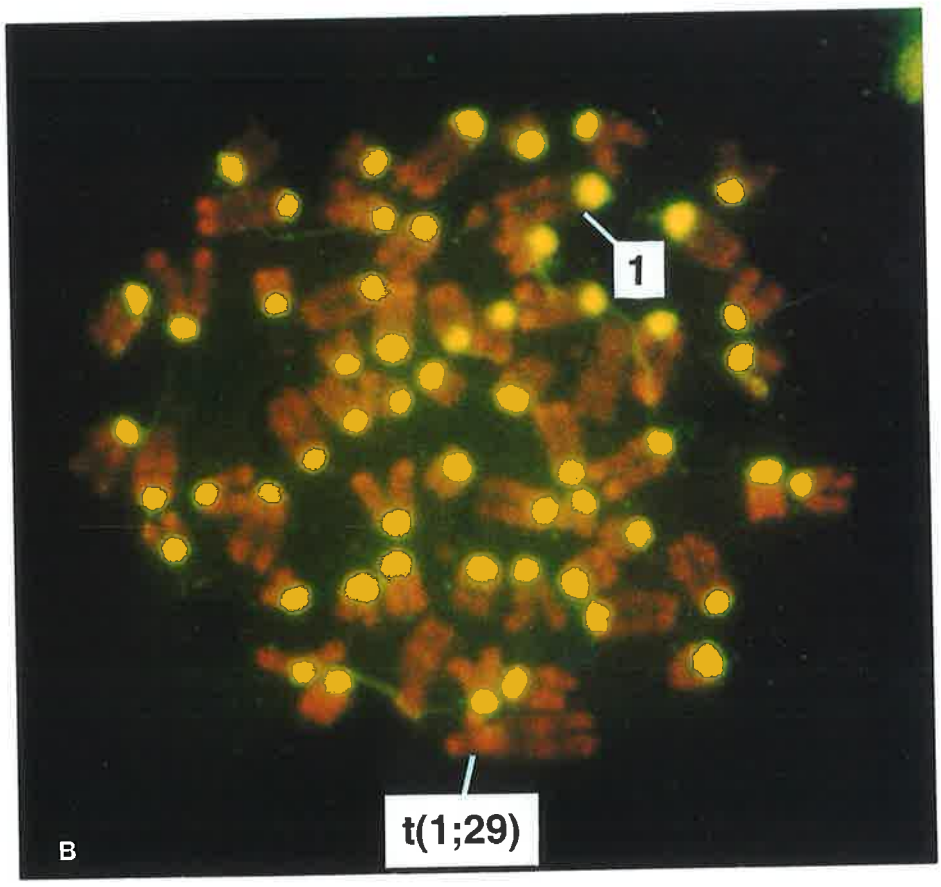
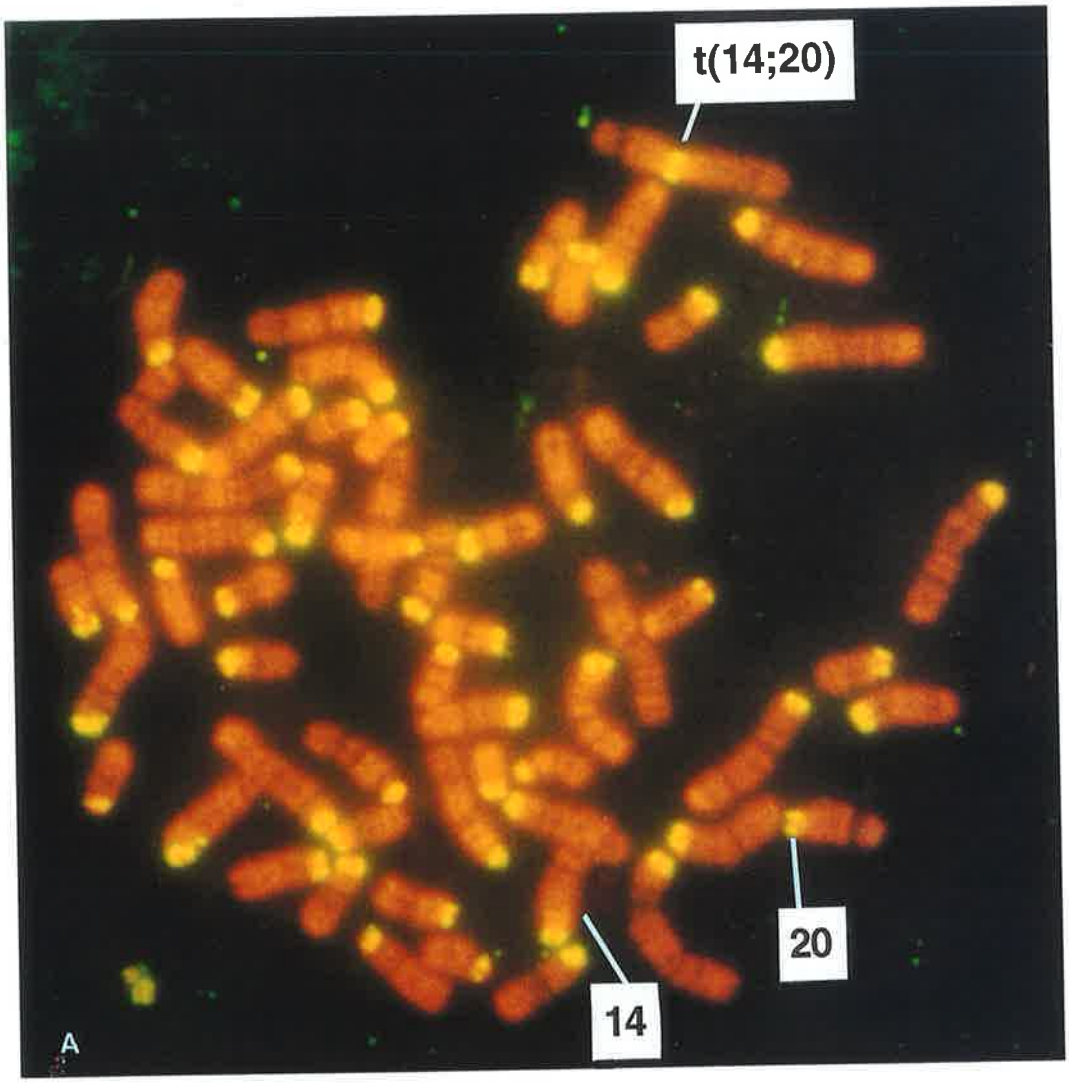

1
t(1;29)
B

Fig 4.2. Satellite II hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite II produces signal which often appears as two separate signals for each chromatid [eg. (A) chromsome 20 and (B) chromosome 1].

(A) Both chromosomes 14 and 20 have Satellite II. However, there is only one Satellite II signal on the t(14;20).

(B) Chromosomes 1 and 29 show a much stronger Satellite II signal than the t(1;29) chromosome.


t(14;20)
20
14
A

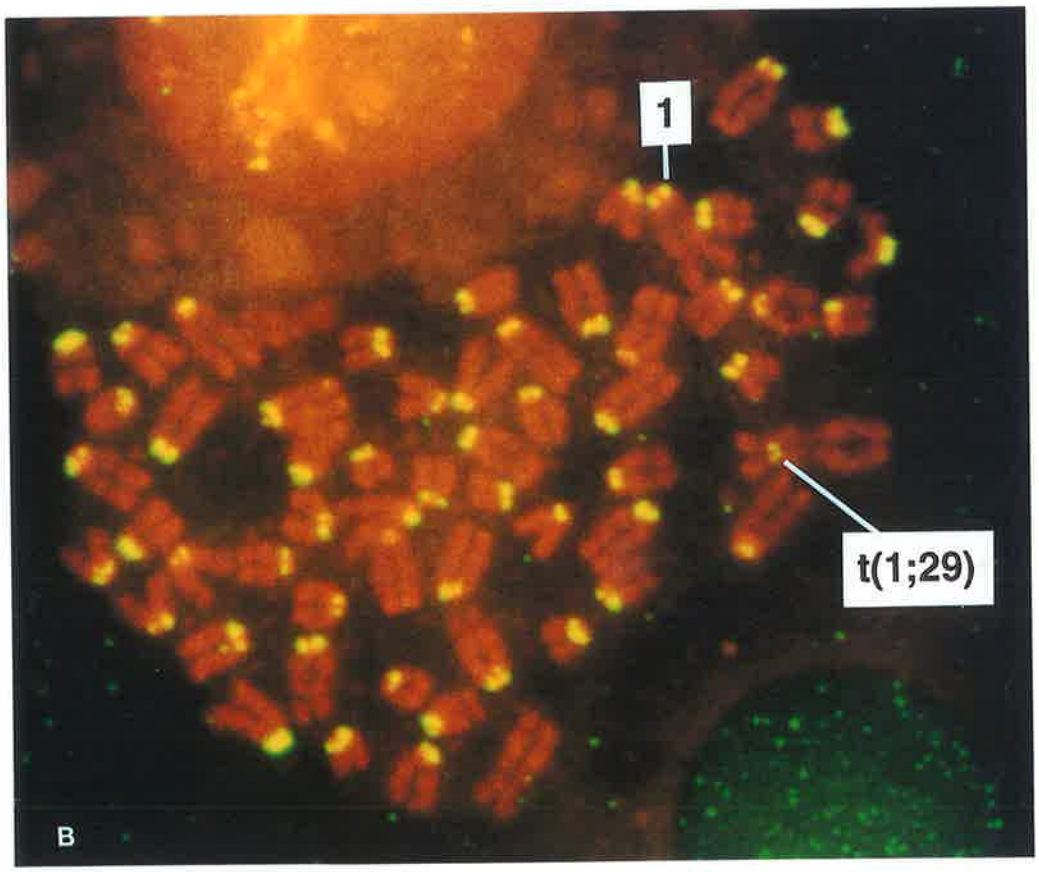
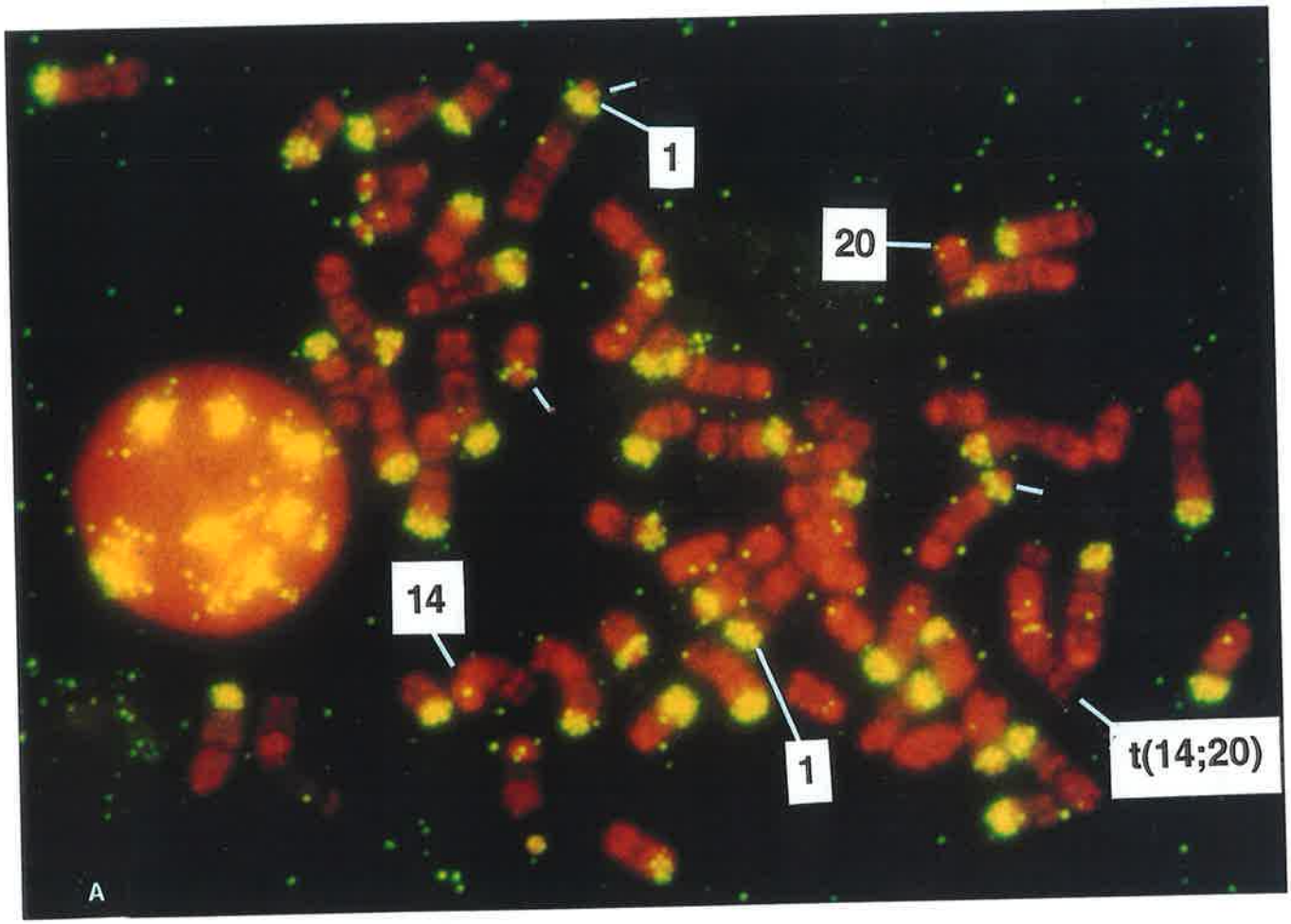

1
t(1;29)
B

Fig 4.3. Satellite III hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite III signals, when present, are generally broad but often leave a considerable length of the p-terminus unlabelled [indicated by **short lines** in (A)].

(A) Both chromosomes 14 and 20 have a little Satellite III. There are two faint Satellite III bands on the t(14;20). Chromosome 1 has a strong Satellite III signal.

(B) Satellite III is absent from chromosome 29 (Table 4-1). Satellite III signal on the t(1;29) appears as two faint bands of different intensities.


1
20
14
1
t(14;20)
A

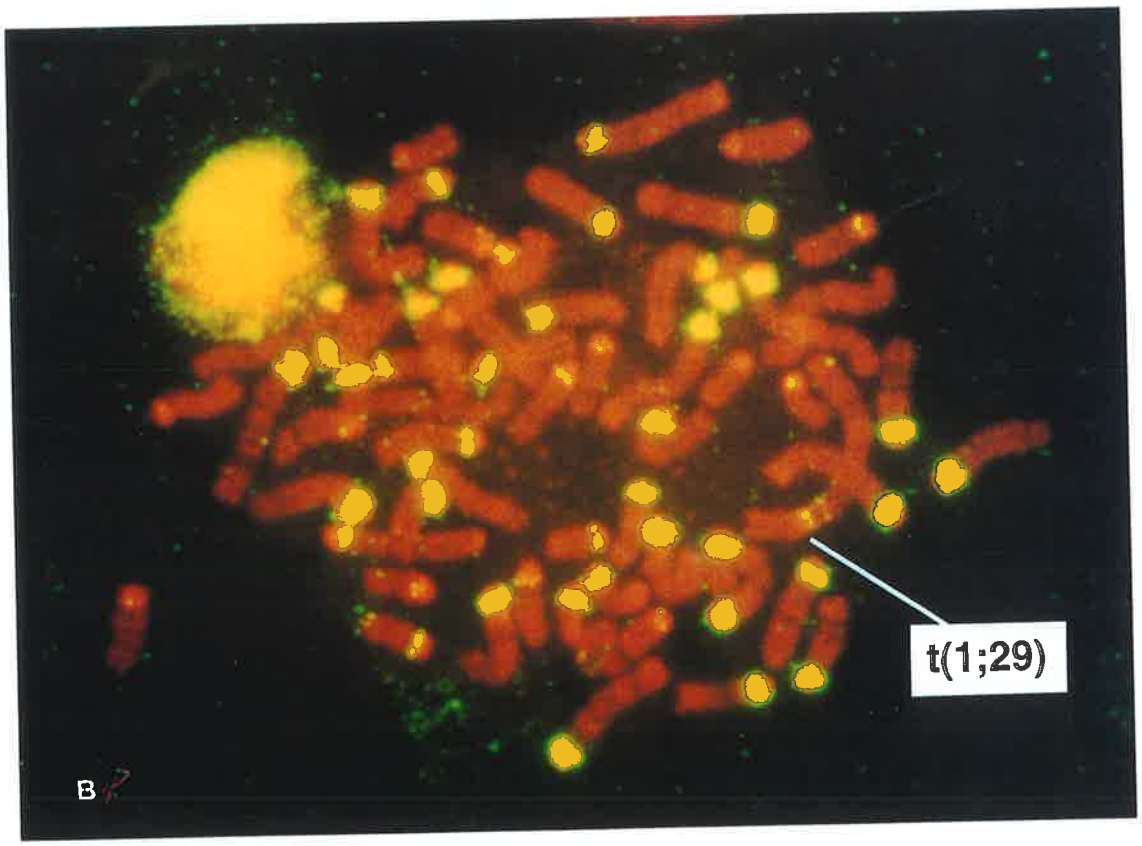
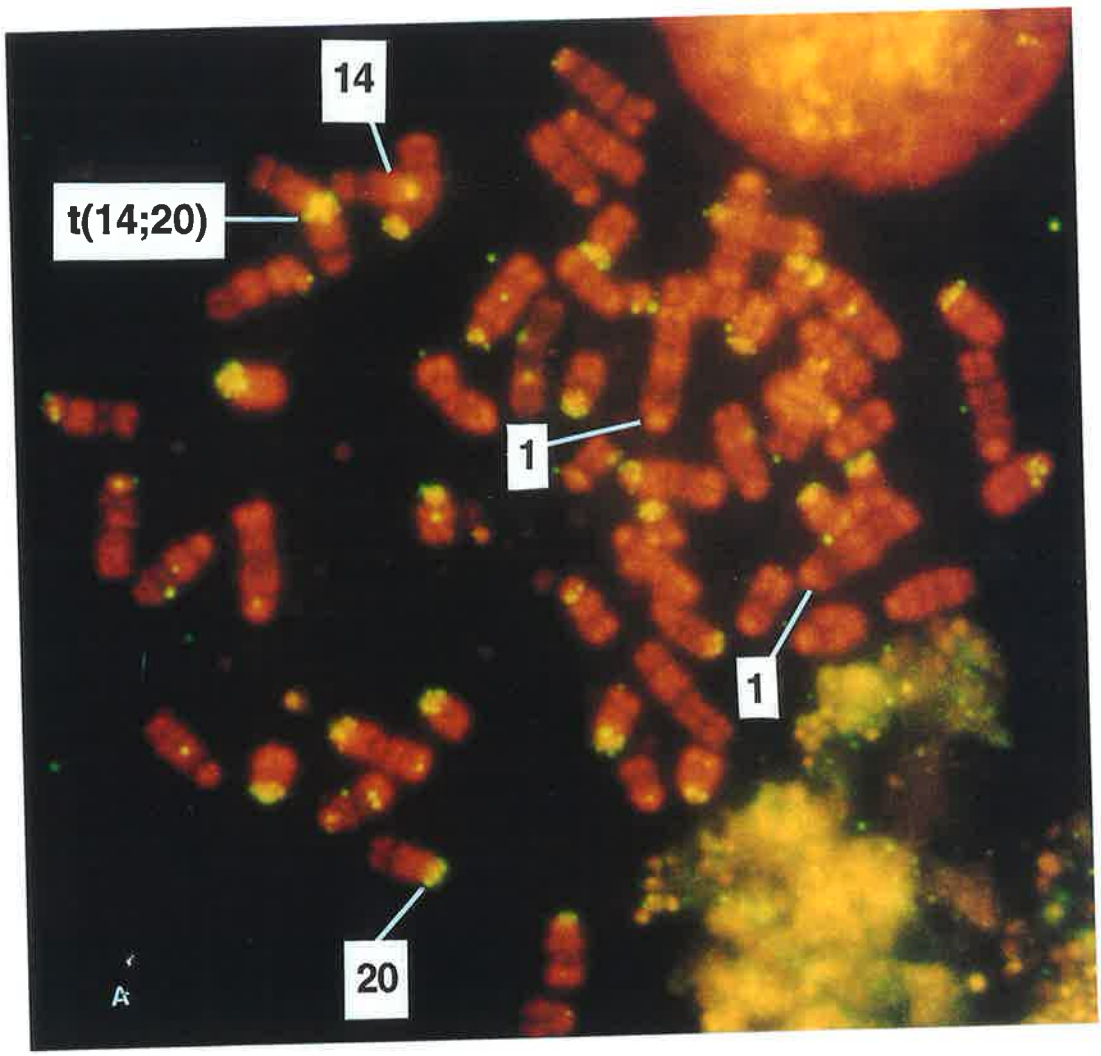

t(1;29)
B

Fig 4.4. Satellite IV hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite IV, when present, usually gives a narrow signal reaching to the p-terminus.

(A) Chromosome 14 has a Satellite IV signal which appears as a small signal towards to the p-terminus. Chromosome 20 has a strong Satellite IV signal which appears as a block. There is a very strong Satellite IV signal on the t(14;20) chromosome.

(B) There is very little Satellite IV on chromosome 1. Chromosome 29 has a strong signal for Satellite IV (Table 4.1). However, chromosome t(1;29) has a weak Satellite IV signal.


14
t(14;20)
1
1
20
A

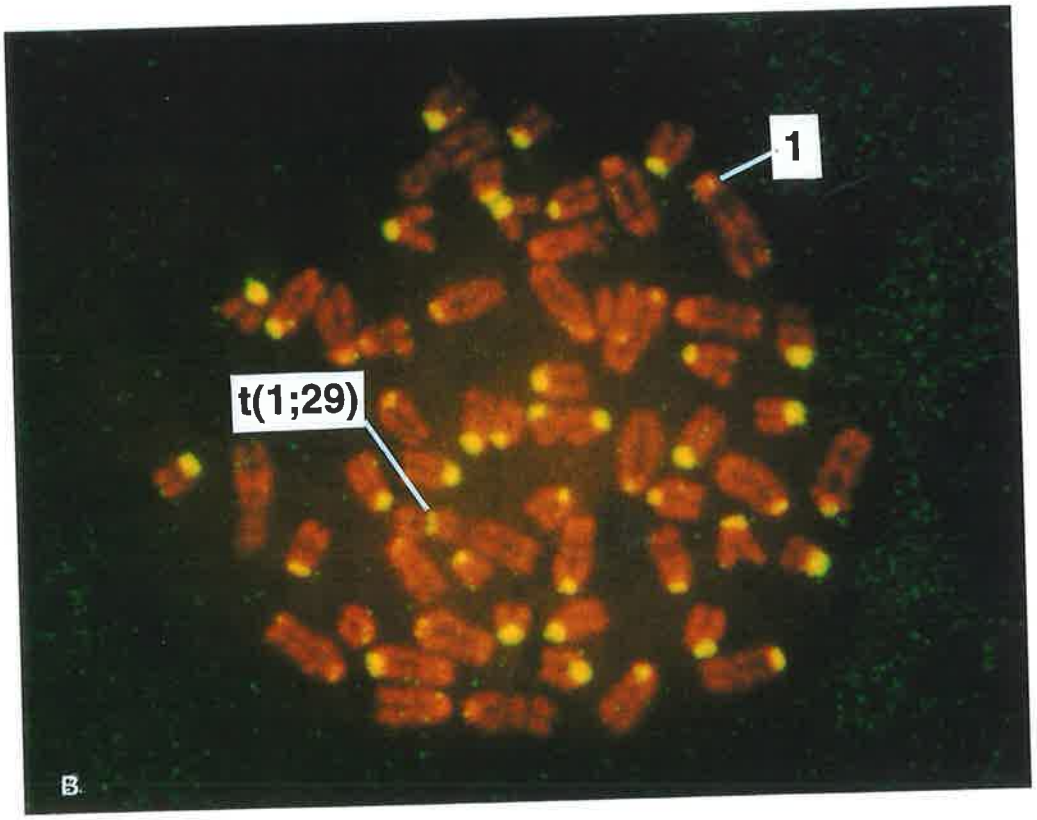
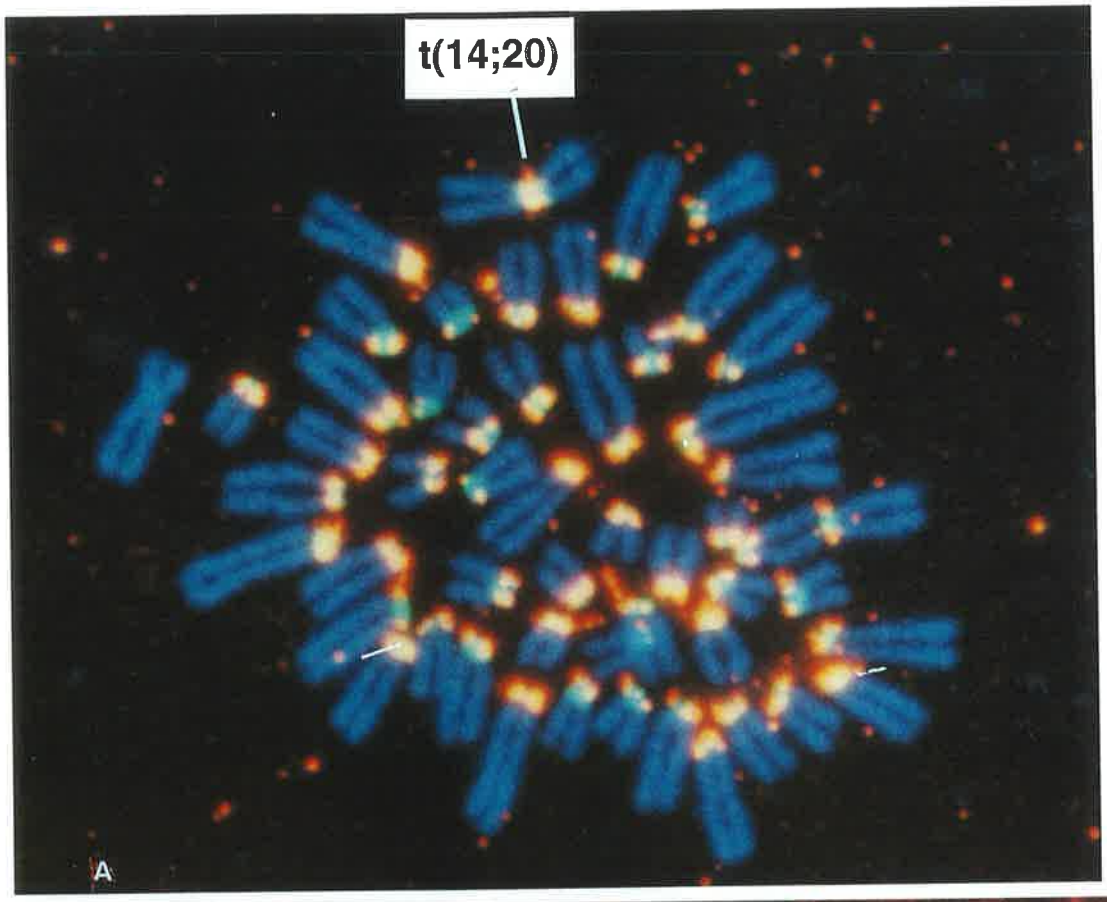

1
t(1;29)
B

Fig 4.5. Satellite I (green signal) and Satellite II (red signal) hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. On normal autosomes, the Satellite II signal is usually closer to the p-terminus than Satellite I. Where the signals overlap, they produce a white-yellow coloration [**short white lines** in (A) and (B)].

(A) The Satellite II signal fills the gap in Satellite I on the t(14;20) chromosome (see also Fig 4.1A).

(B) Satellite I hybrids to all normal autosomes but not to the t(1;29). There is one signal of Satellite II on the t(1;29).


t(14;20)
A

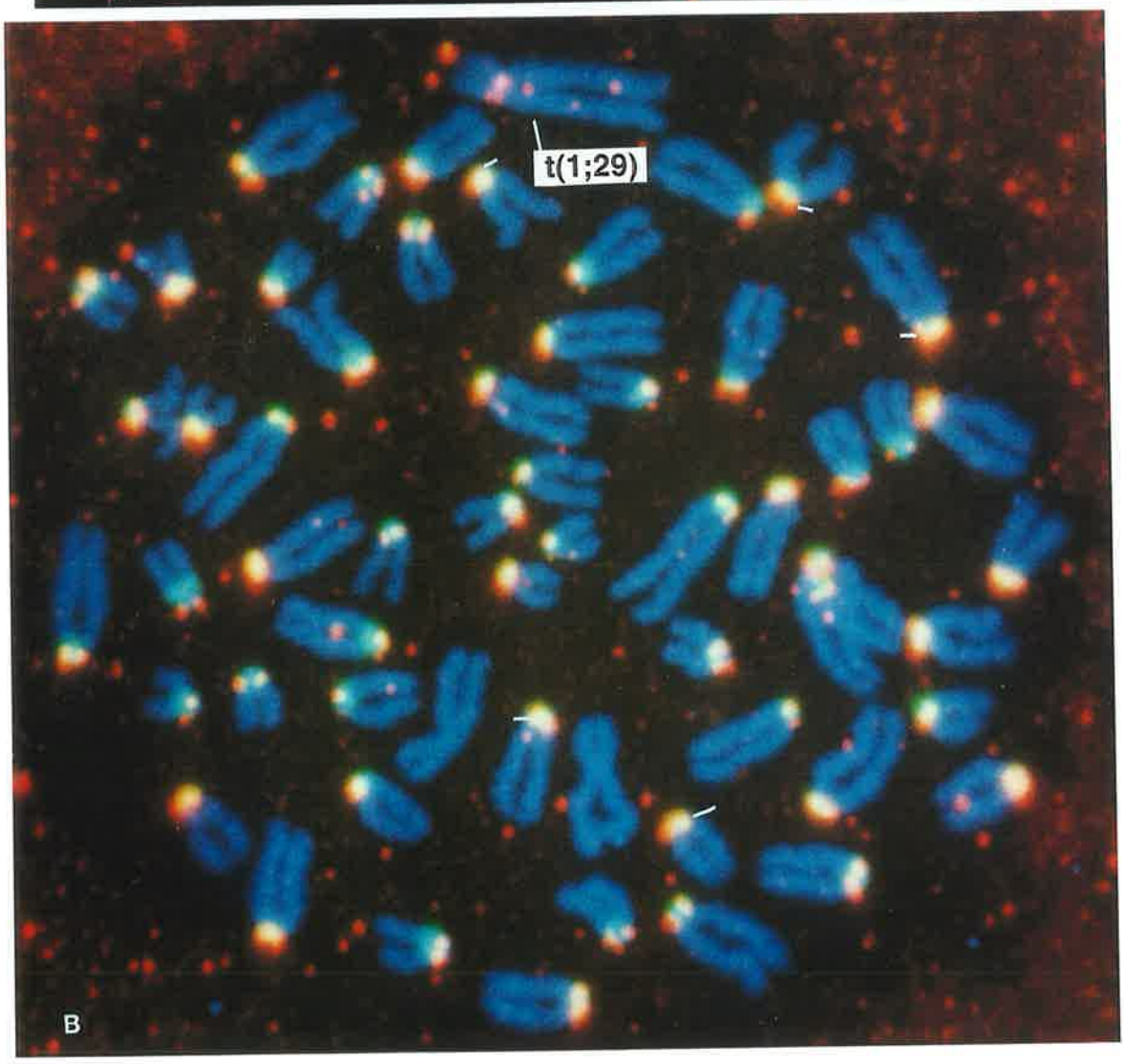
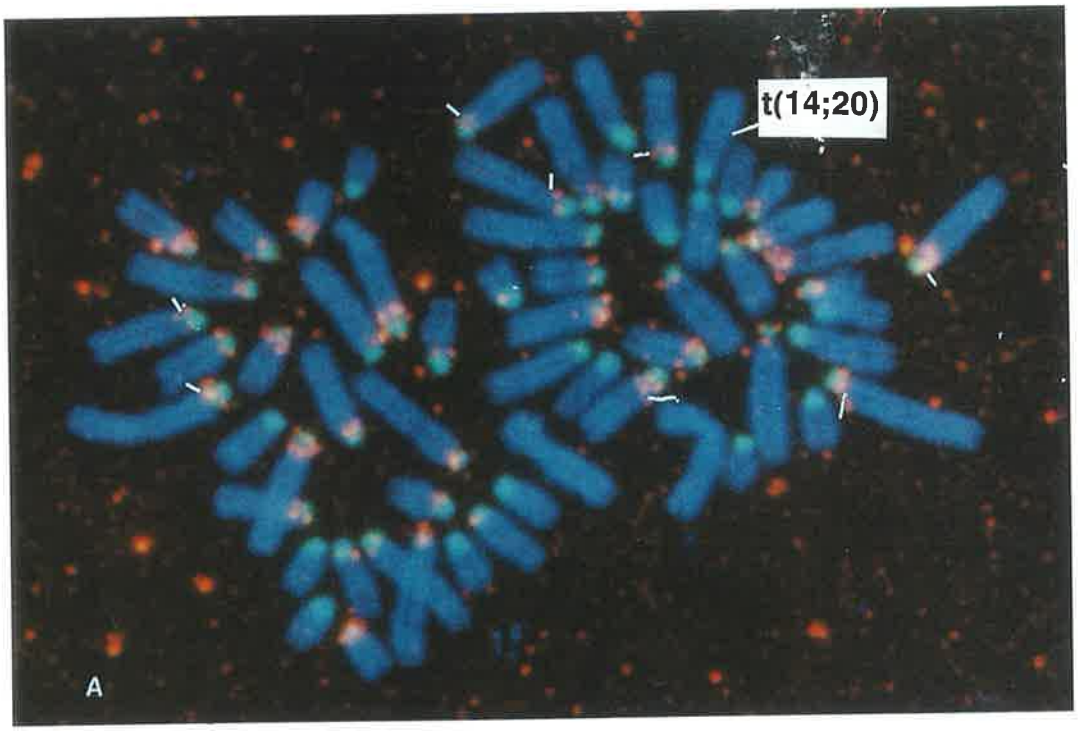

t(1;29)
B

Fig 4.6. Satellite I (green signal) and Satellite III (red signal) hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. When present, Satellite III is on the long arm, distal to Satellite I [**short white lines** in (A)].

(A) The Satellite III signal are too faint to be observed on the t(14;20).

(B) No Satellite I signal is on the t(1;29), but there are two faint separate bands of Satellite III on the long arm.


t(14;20)
A

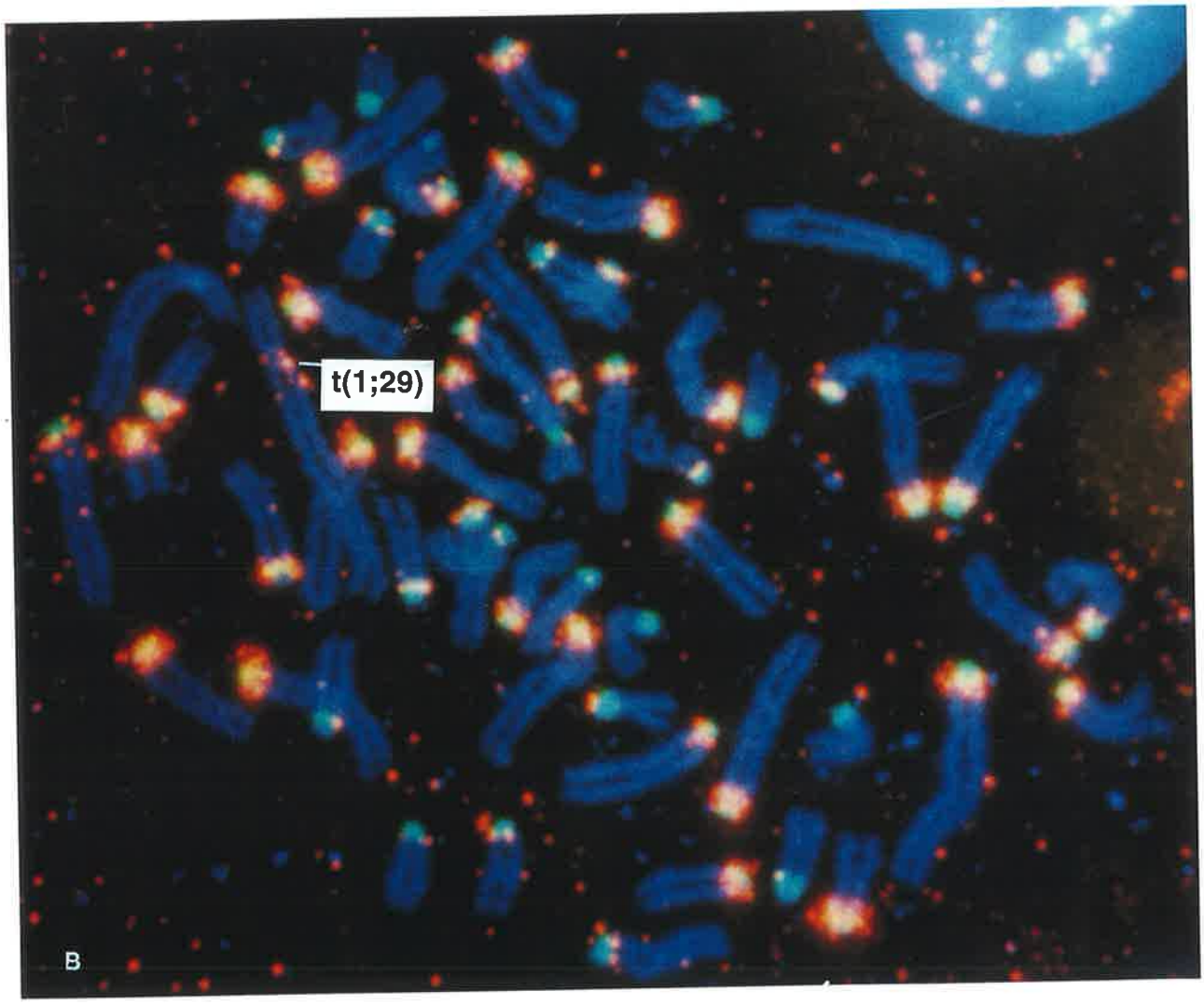
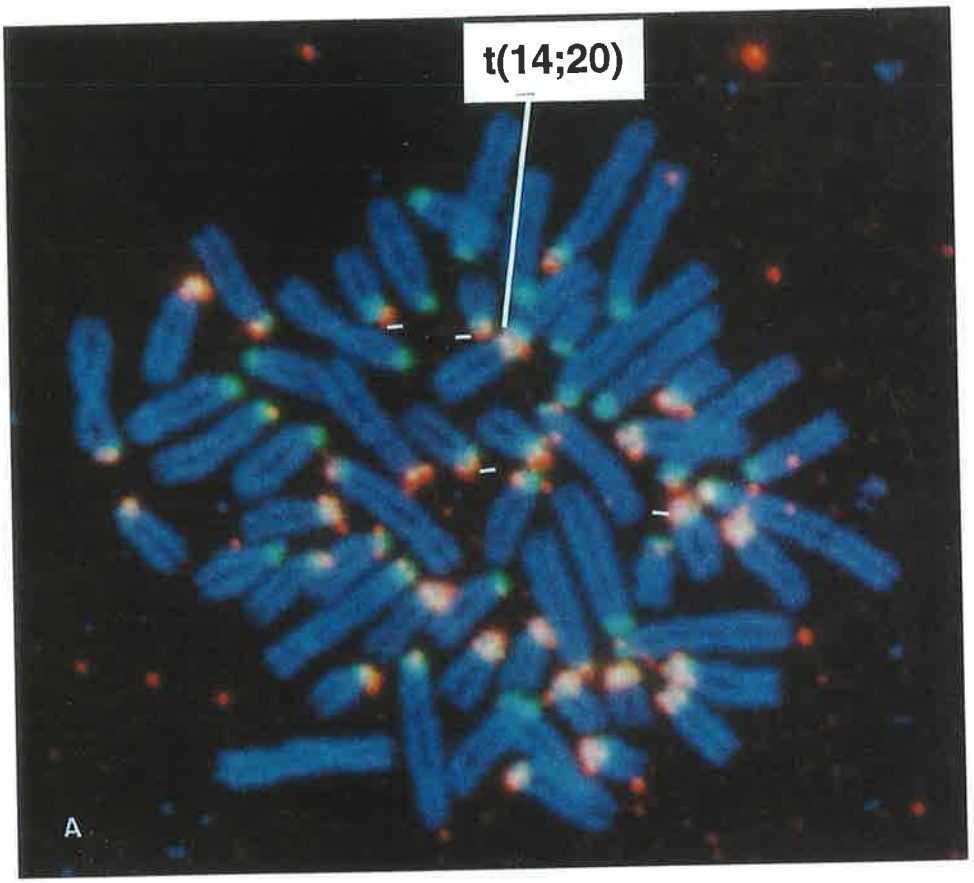

t(1;29)
B

Fig 4.7. Satellite I (green signal) and Satellite IV (red signal) hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite IV is often absent from longer chromosomes, but when present, tends to be closer to the p-terminus than Satellite I [**short white lines** in (A) and (B)].

(A) The Satellite IV signal on the t(14;20) chromosome appears mainly on the short arm.

(B) There is no Satellite I signal on the t(1;29), but there is one block of Satellite IV near the middle of the centromere towards the short arm.


t(14;20)
A

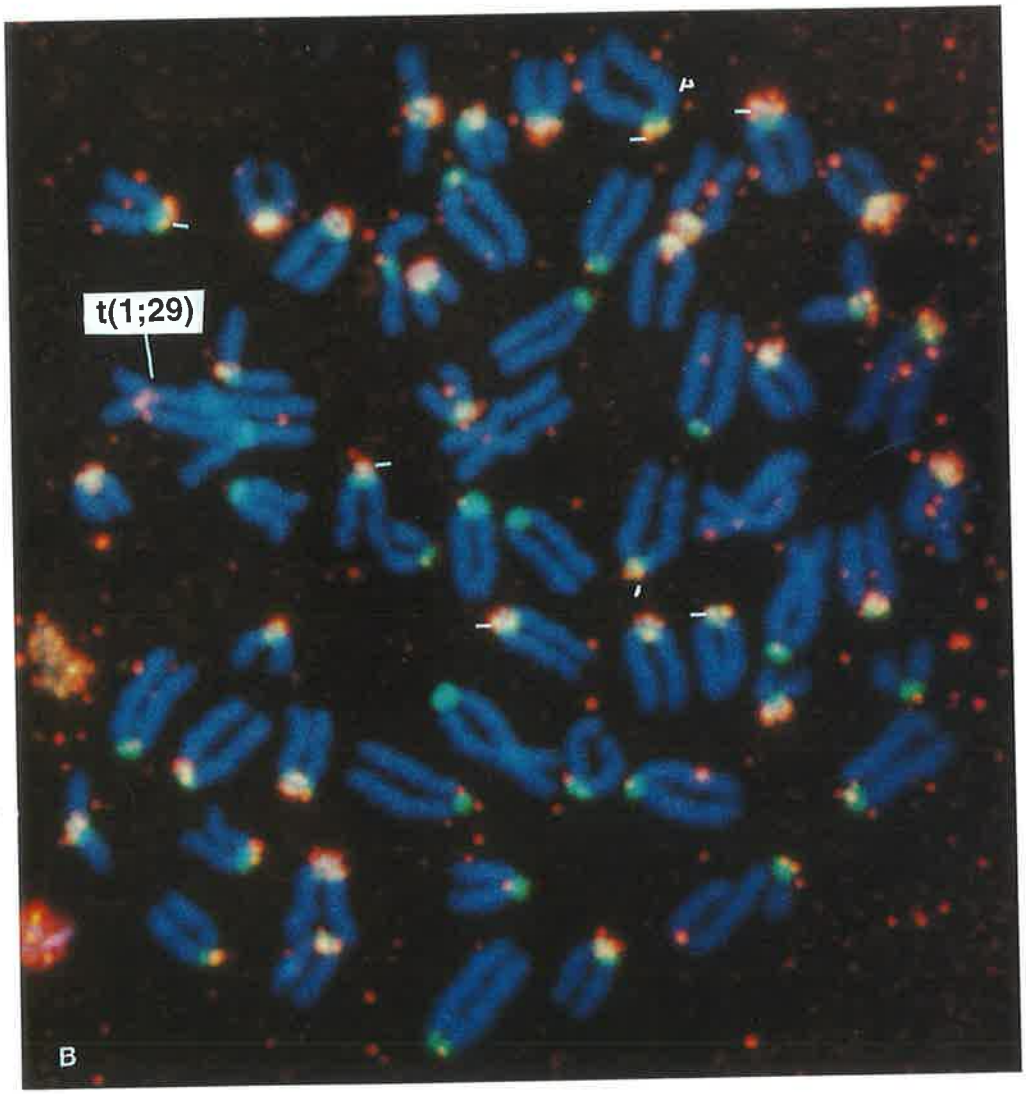
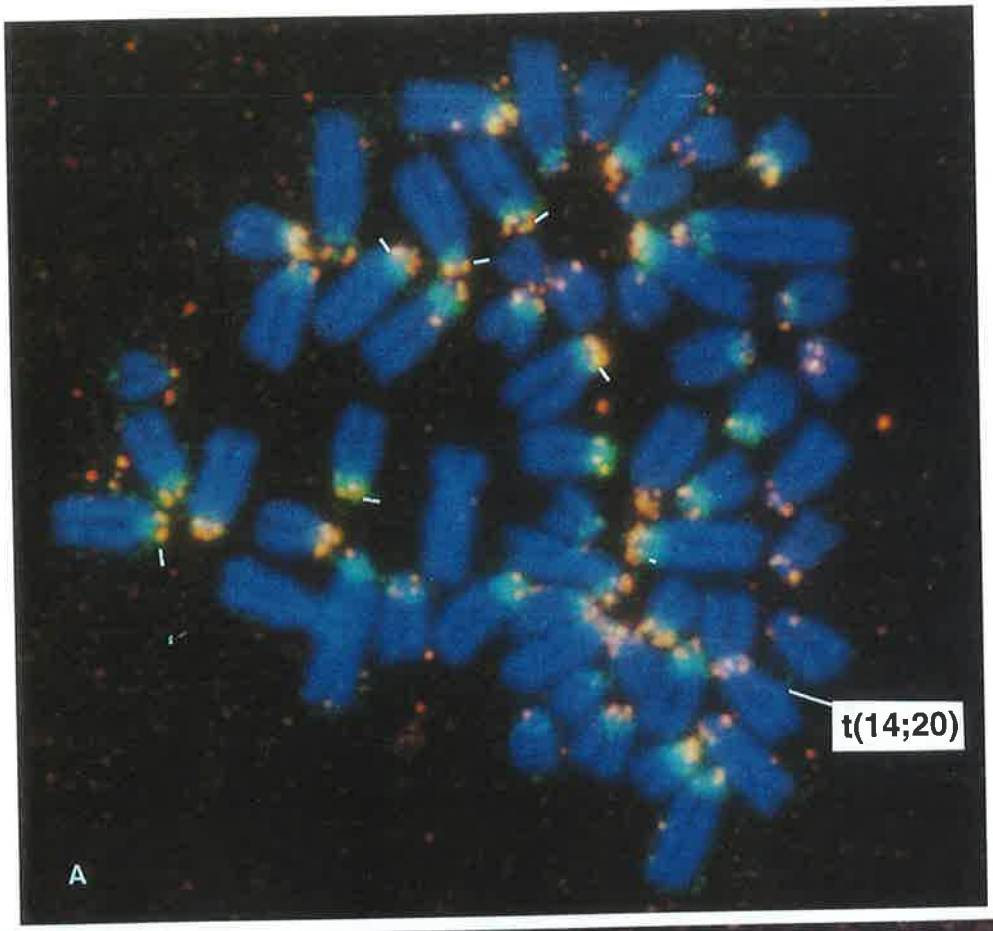

t(1;29)
B

Fig 4.8. Satellite II (red signal) and Satellite III (green signal) hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. The two colour labelling shows that generally Satellite III is on the long arm and Satellite II is closer to p-terminus [**short white lines** in (A) and (B)].

(A) Satellite II is in the middle of centromere of the t(14;20) and gives a much stronger signal than Satellite III.

(B) Satellite II was towards the p-terminus and Satellite III signal towards the q-terminus. Neither Satellite is strongly represented on the t(1;29) chromosome.


t(14;20)
A

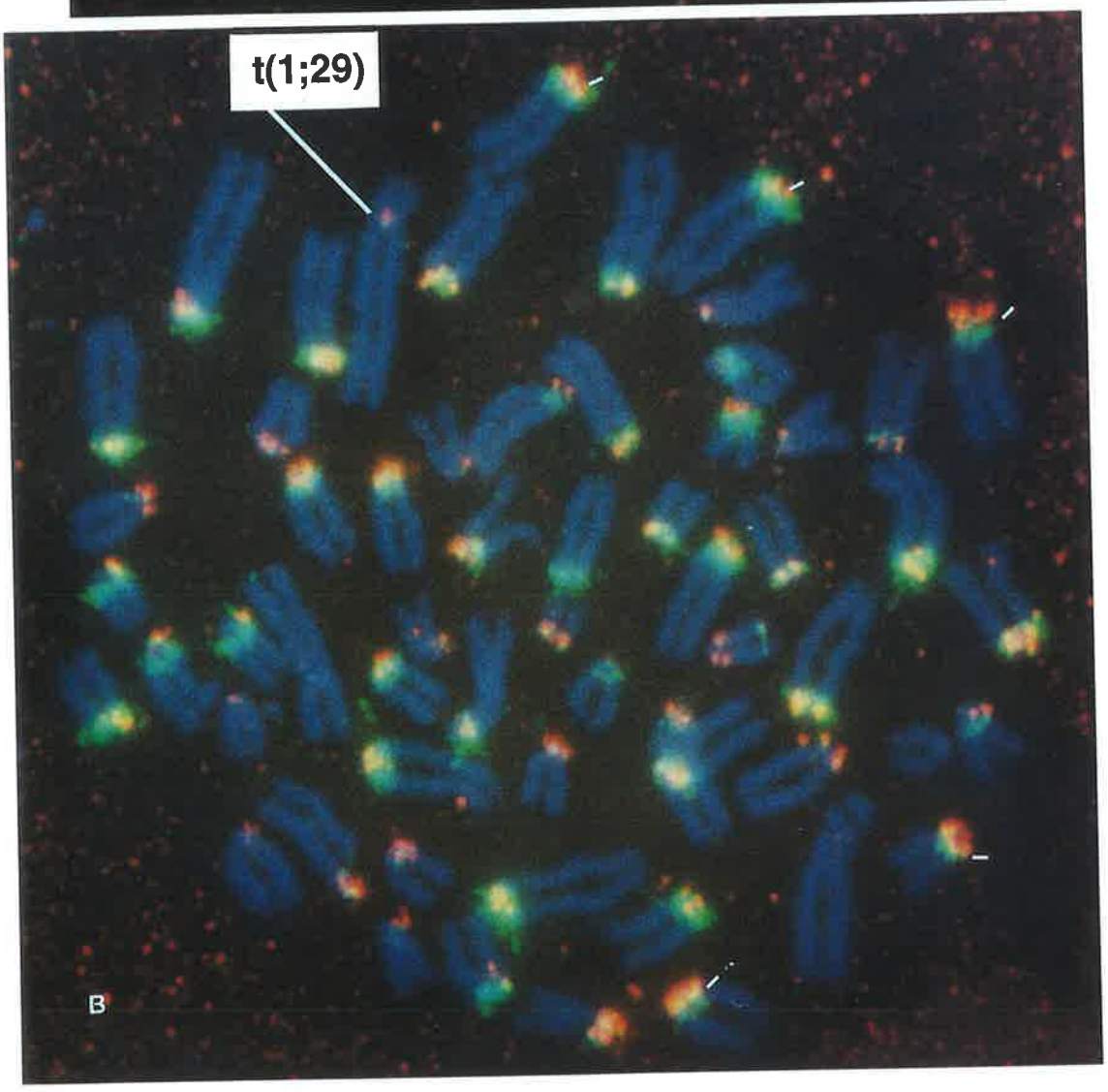
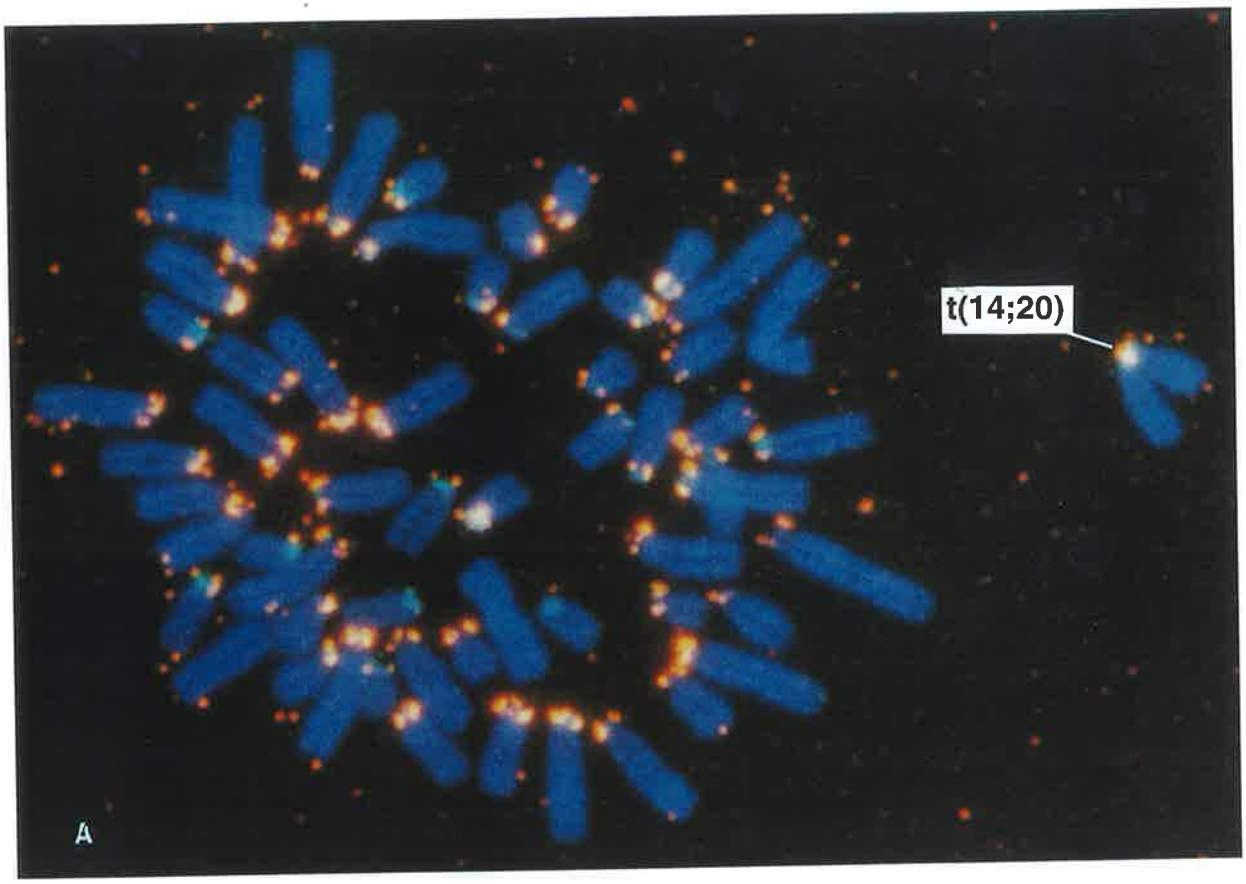

t(1;29)
B

Fig 4.9. Satellite II (red signal) and Satellite IV (green signal) hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite IV is usually closer to the p-terminus than Satellite II [eg. chromosomes marked with a **short white line** in (B)], but not always [eg. the chromosome marked with a **red line** in (B)].

(A) There is an overlap between Satellite II and IV on the t(14;20).

(B) The order of the Satellite II and IV is not clear in the t(1;29) chromosome because their signals appear to overlap.


t(14;20)
A

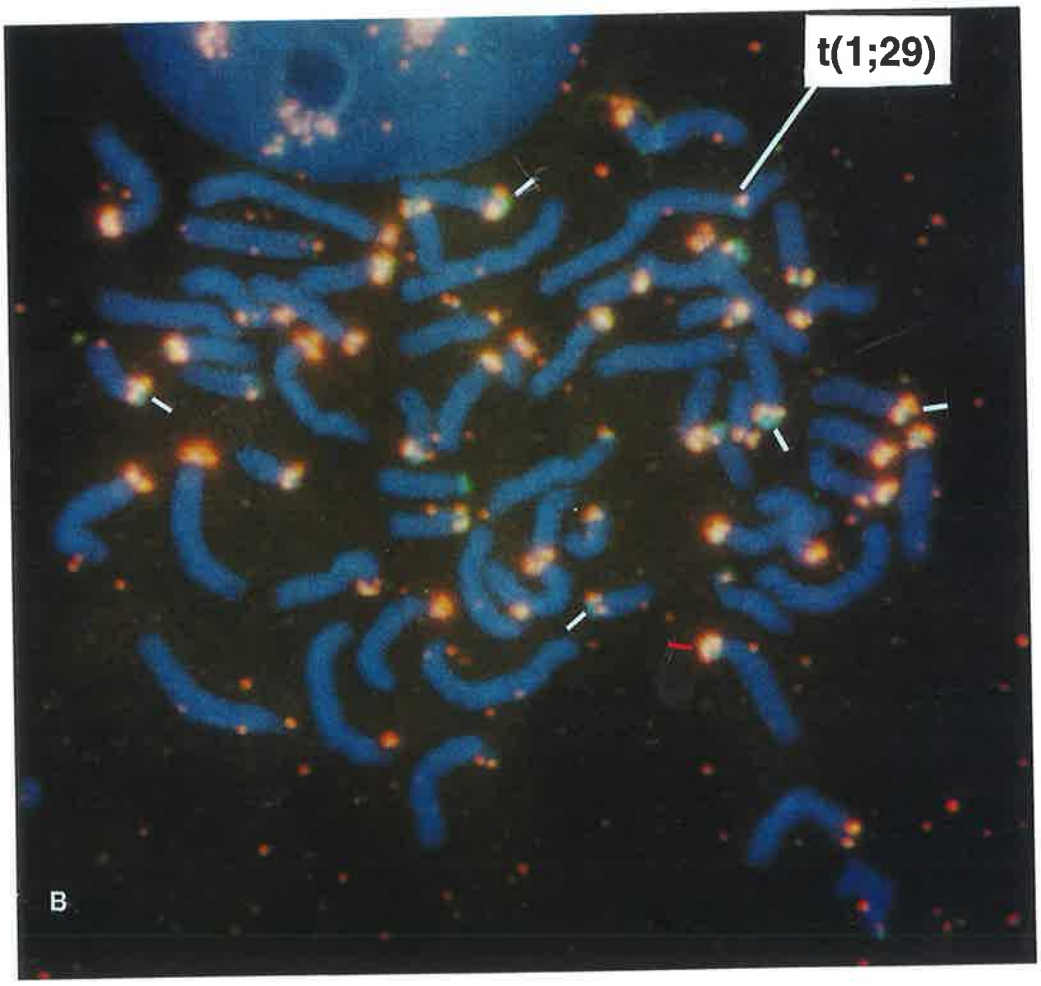
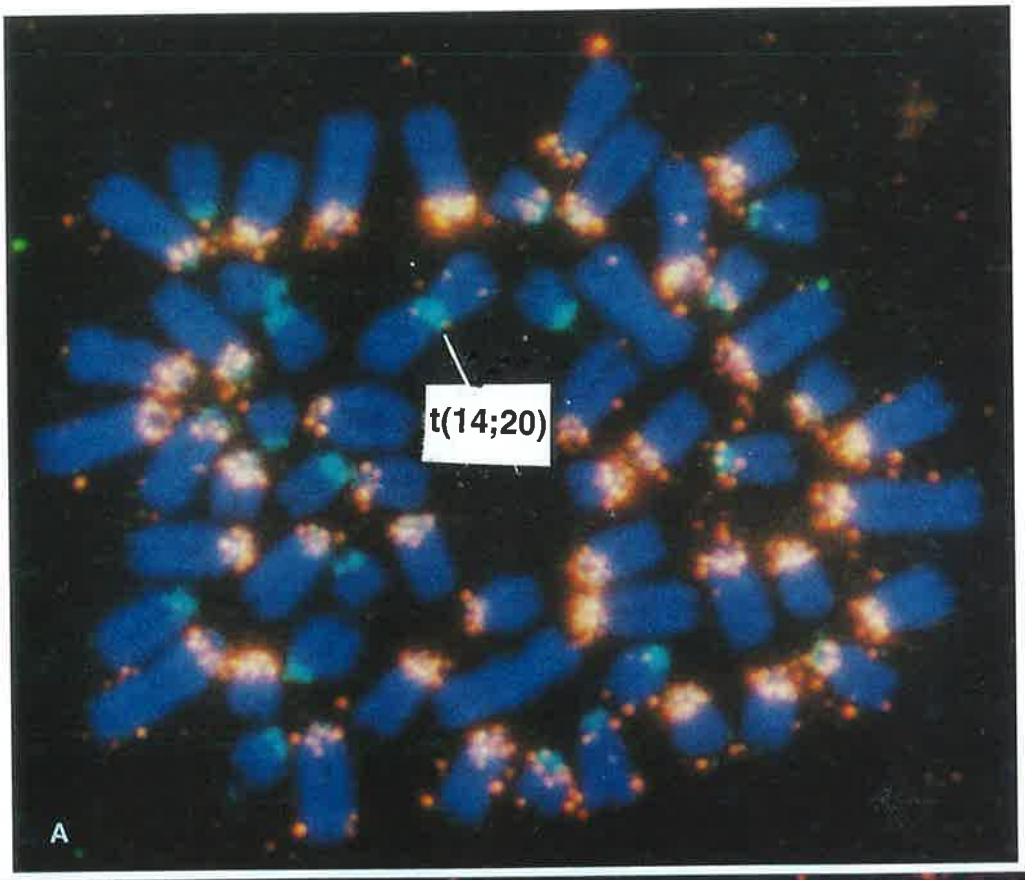

t(1;29)
B

Fig 4.10. Satellite III (red signal) and Satellite IV (green signal) hybridized to the chromosomes of (A) a t(14;20) carrier female and (B) a t(1;29) carrier female. Satellite IV is seen to be generally nearer the p-terminus than Satellite III [**short white lines** in (B)].

(A) There are separate faint signals of Satellite III and one large block of Satellite IV between the two Satellite III signals on the t(14;20).

(B) The Satellite IV signal is in the middle of the t(1;29) chromosome, while the Satellite III signal is on the long arm of the t(1;29) and produced two distal faint bands. A second t(1;29) chromosome is shown to confirm the loss of Satellite III.


t(14;20)
A

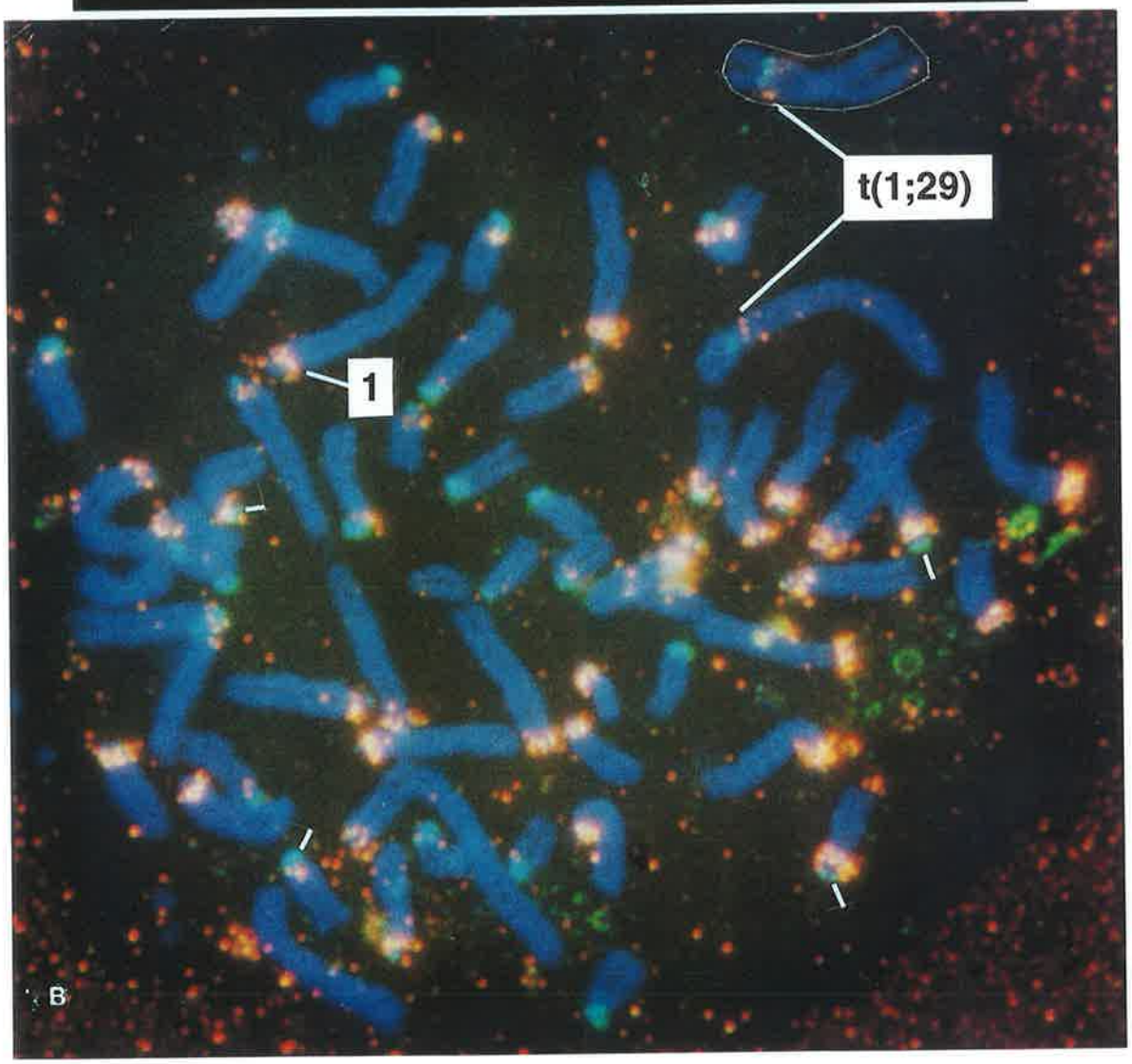
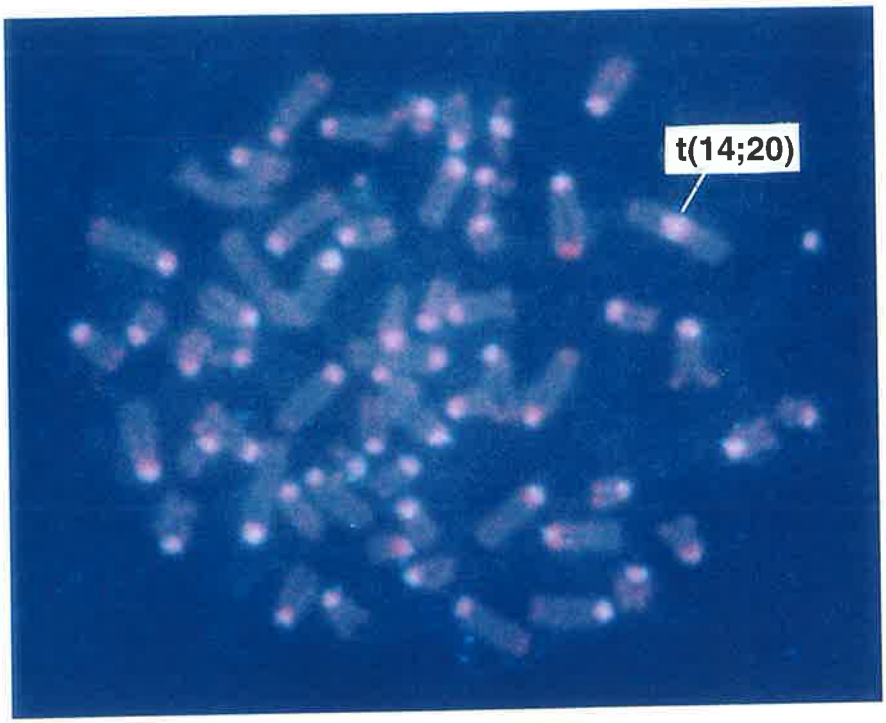

t(1;29)
1
B

Fig 4.11. Satellite I (orange red) and Satellite II (white) hybridized to the chromosomes of a t(14;20) carrier female. On normal autosomes, the Satellite II signal is usually closer to the p-terminus than Satellite I. The AMCA signal (Satellite I) faded very quickly.

Fig 4.12. Model of observed arrangement of Satellites I-IV in the chromosomes of *Bos taurus*. Dotted lines show variation of the boundaries of the Satellites along the chromosome.


t(14;20)

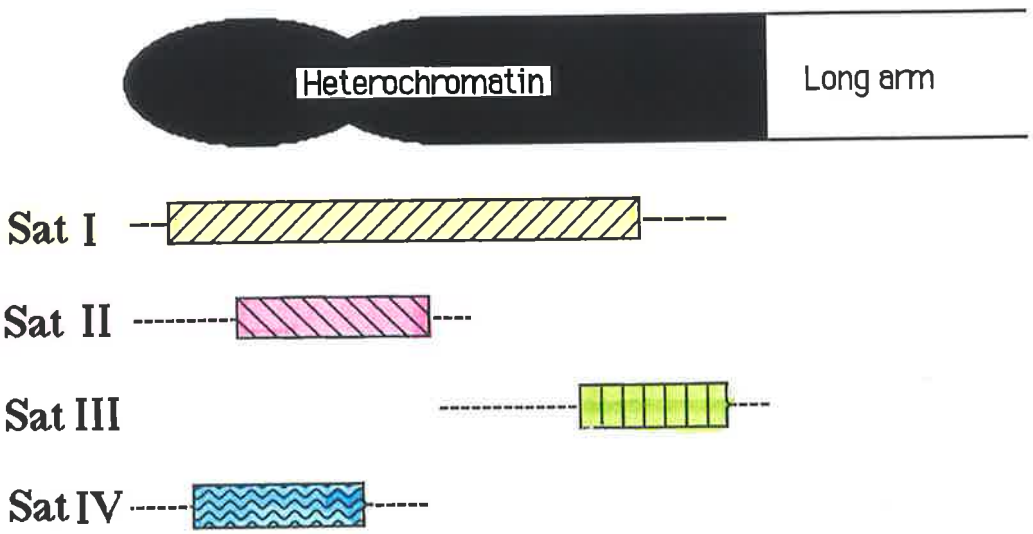
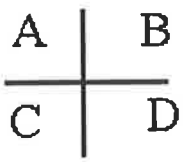
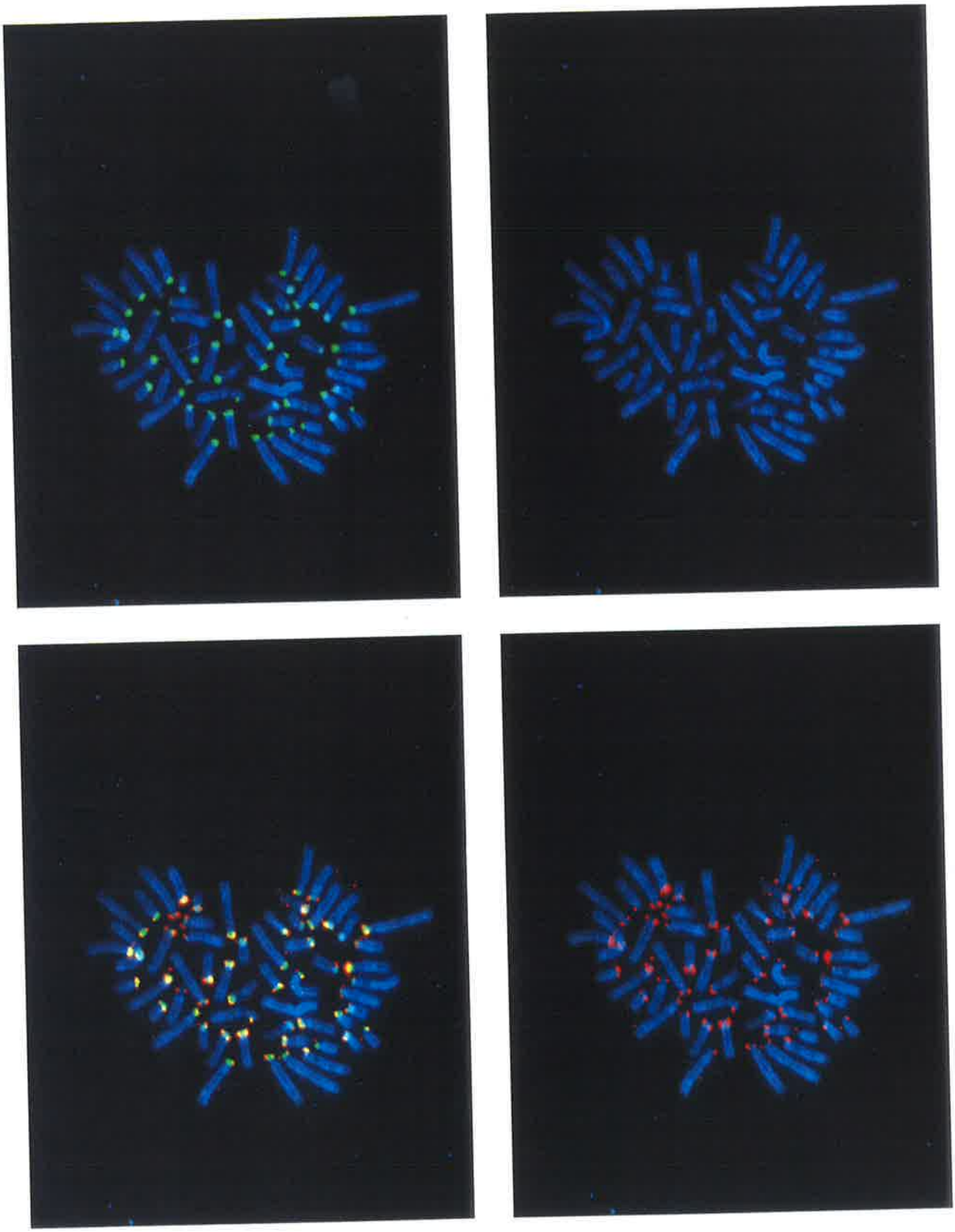

Heterochromatin
Long arm
Sat I
Sat II
Sat III
Sat IV

Fig 4.13. Satellite I (green signal) and Satellite II (red signal) hybridized on the metaphase chromosomes of the t(14;20) carrier. Images of metaphase preparations were captured by a cooled CCD camera using the CytoVision Ultra image collection and enhancement system (Applied Imaging Int Ltd). FISH signals and the Hoescht 33258 banding pattern (using DAPI banding program) were merged for figure preparation. (A) Satellite II (red signal) with metaphase chromosomes; (B) Satellite I (green signal) and Satellite II (red signal) with metaphase chromosomes; (C) G-banded metaphase chromosomes; (D) Satellite I (green signal) with metaphase chromosomes.





Chapter 5

INVESTIGATION OF

STRUCTURE AND ORIGIN OF CENTROMERIC REGIONS

IN ROBERTSONIAN FUSION IN CATTLE

Many of the smaller autosomal pairs have little or no Satellite III, including chromosomes 14 and 20 (Fig 4.3A). There are two faint Satellite III signals on the t(14;20), which presumably came from both of the original 14 and 20 chromosomes.

Chromosome 14 has one Satellite IV signal which appears as a small signal towards the p-terminus (Fig 4.4A). Chromosome 20 has a strong Satellite IV signal which appears as a solid block. There is a stronger Satellite IV signal on the t(14;20) chromosome than chromosome 20, suggesting that the Satellite IV on the t(14;20) is a combination from chromosomes 14 and 20.

5.3.2 Single FISH on t(1;29)

The t(1;29) chromosome does not have any Satellite I (Fig 4.1B). All other autosomes show a strong Satellite I signal, including the unfused chromosome 1 and chromosome 29.

There is one Satellite II signal on t(1;29) (Fig 4.2B). Chromosome 1 shows a much stronger Satellite II signal than the t(1;29) chromosome. Therefore, the fusion has resulted in the loss of some Satellite II.

Chromosome 1 shows a strong Satellite III signal (Fig 4.3A). Since Satellite III is absent from chromosome 29 (Table 4.1), the signal on the t(1;29) must originate from chromosome 1 (Fig 4.3B). The t(1;29) chromosome has Satellite III but less than chromosome 1. The signal has been reduced after fusion and appears to be distributed as two bands (Fig 4.3B).

There is little Satellite IV on the unfused chromosome 1 (Fig 4.4A, 4.4B). Chromosome 29 has a strong signal for Satellite IV (Table 4.1). However, the t(1;29) chromosome has a weak Satellite IV signal.

5.3.3 Two-colour FISH on t(14;20)

When Satelllite I and II are labelled with different fluorescent dyes and co-hybridized, the t(14;20) chromosome is seen to contain two large blocks of Satellite I separated by a narrow gap. The gap is filled by a narrow Satellite II signal (Fig 4.5A).

The Satellite III signals on chromosomes 14, 20 and the t(14;20) are faint (Fig 4.3A). With two-colour labelling of Biotin-I & DIG-III (Fig 4.6A), the Satellite III signal is too faint to be observed. However, Satellite I still appears as two large blocks separated by a narrow gap on t(14;20).

In two-colour FISH, the Satellite IV signal of the t(14;20) overlaps with one block of the Satellite I signal [towards to the middle of t(14;20)] and fills the gap between the two Satellite I blocks (Fig 4.7A). Satellite IV appears mainly on the short arm of the t(14;20) and must be mostly derived from chromosome 20.

Two-colour labelling with Satellites II and III showed a much stronger Satellite II signal than the Satellite III signal on the t(14;20) (Fig 4.8A). Satellite II is in the middle on the centromere of the t(14;20) with Satellite III on the both of the long arms.

A similar pattern was observed with Satellite IV; Satellite III has two separate faint signals flanking the one large block of Satellite IV (Fig 4.10A). On the other hand, there is an overlap between Satellite II and IV on the t(14;20), with Satellite II appearing in the middle of Satellite IV (Fig 4.9A).

5.3.4 Two-colour FISH on t(1;29)

Satellite I alone (Fig 4.1B) or co-hybridized with Satellite II, III or IV showed no Satellite I signal on the t(1;29) chromosome (Figs 4.5B, 4.6B and 4.7B). Satellite III was observed as two faint separate signals on the long arm of the t(1;29) (Fig 4.6B), while Satellites II and IV appeared as one small signal towards the short arm of the chromosome t(1;29) (Figs 4.5B and 4.7B).

With two-colour labelling of Biotin-III & DIG-II, the signals of Satellite II and III were close together on the t(1;29) chromosome, although Satellite II was towards the p-terminus and Satellite III was towards the q-terminus (Fig 4.8B). The two-colour labelling confirms that Satellite III is from chromosome 1 and Satellite II is from chromosome 29.

When Satelllite II and IV are co-hybridized, the t(1;29) chromosome was seen to have Satellite II and IV signals at the same location (Fig 4.9B), so the order of the Satellite II and IV is not clear on the t(1;29) chromosome.

Also with two-colour FISH, the Satellite IV signal was observed on the short arm of the t(1;29) chromosome, while the Satellite III signal was on the long arm of the t(1;29) and produced as two distal faint bands (Fig 4.10B). This suggests that the Satellite IV signal is derived from chromosome 29, while the Satellite III signal is from chromosome 1 but is reduced in the translocation. Evidence of a second distal faint band of Satellite III was also seen with single labelling (Fig 4.3B).

Satellite II, III and IV were all not strongly represented on the t(1;29) chromosome and the overlap of these satellites observed on most of the autosomes was not seen on the t(1;29) (Figs 4.8B, 4.9B and 4.10B).

5.4 Discussion

5.4.1 The centromeric rearrangement of t(14;20)

Chromosomes 14 and 20 both have a strong signal for Satellite I. The translocation chromosome t(14;20) has two strong signals of Satellite I with a clear gap, although the Satellite I signal on the arm from chromosome 14 is less dense than the Satellite I signal from chromosome 20. Consequently, little or no Satellite I appears to have been lost in the fusion of the 14 and 20 chromosomes (Table 5.1).

The t(14;20) has only one Satellite II signal which fills the gap between the two Satellite I signals. The Satellite II signal also appears in the middle of Satellite IV signal, but nearer the q-terminus. The Satellite II signal on the t(14;20) is a little stronger than both the original chromosomes 14 and 20, but less than the combination of the signals from both chromosomes 14 and 20. Thus, some Satellite II may have been lost in the fusion or only one chromosome contributed the Satellite II to the centric fusion.

The t(14;20) contains two faint Satellite III signals on the arms. This coincides with the expected faint Satellite III signals from the original chromosomes 14 and 20.

The Satellite IV signal of the t(14;20) fills the gap between the two Satellite I blocks but overlaps with the Satellite I signal on the short arm. The t(14;20) has a much stronger Satellite IV signal than either of the original chromosomes. The high level of Satellite IV label in the t(14;20) chromosome is presumably due to the combination of Satellite IV sequences from both of the original 14 and 20 chromosomes. However, since more of the Satellite IV signal is on the short arm of the t(14;20), Satellite IV must be mainly derived from chromosome 20.

The 14;20 Robertsonian translocation can be interpreted in simple mechanical terms. The placement of the four Satellite sequences suggests that this rearrangement can be defined in terms of two sub-telocentric breaks and mechanical fusion to form a dicentric chromosome (Fig 5.1, also refer to Fig 1.5C).

5.4.2 The centromere rearrangement of t(1;29)

Satellite I is present on chromosomes 1 and 29 but not the t(1;29) (Table 5.1). Thus, Satellite I has been lost in the centric fusion. Satellite II is present on the t(1;29), but less than expected if both original chromosomes 1 and 29 contributed equally. Satellite II appears to be derived from chromosome 29 because the Satellite II signal is on the short arm of the t(1;29)

(Fig 4.5B). Satellite III is only present on chromosome 1. Satellite IV is strongly represented on chromosome 29, but only faintly on chromosome 1. Two-colour labelling confirms that the Satellite III is from chromosome 1 and Satellite IV is from chromosome 29.

The 1;29 Robertsonian translocation can be interpreted from the distribution of Satellites II-IV (Fig 5.2, refer also to Figs 1.5A, 1.5B) in terms of breakage to produce a monocentric t(1;29) chromosome (Logue 1978). However, the total absence of Satellite I and the partial loss of Satellite II in the t(1;29) chromosome cannot be explained in terms of chromosome breakage. It is possible that Satellite II sequences from the original chromosome 1, incorporated into the Robersonian translocation, may have been present after formation of the t(1;29) but are now lost. Furthermore, the reduction of Satellite III to two faint bands on the t(1;29) chromosome from one original large block on chromosome 1 is not likely to be due to a mechanical rearrangement.

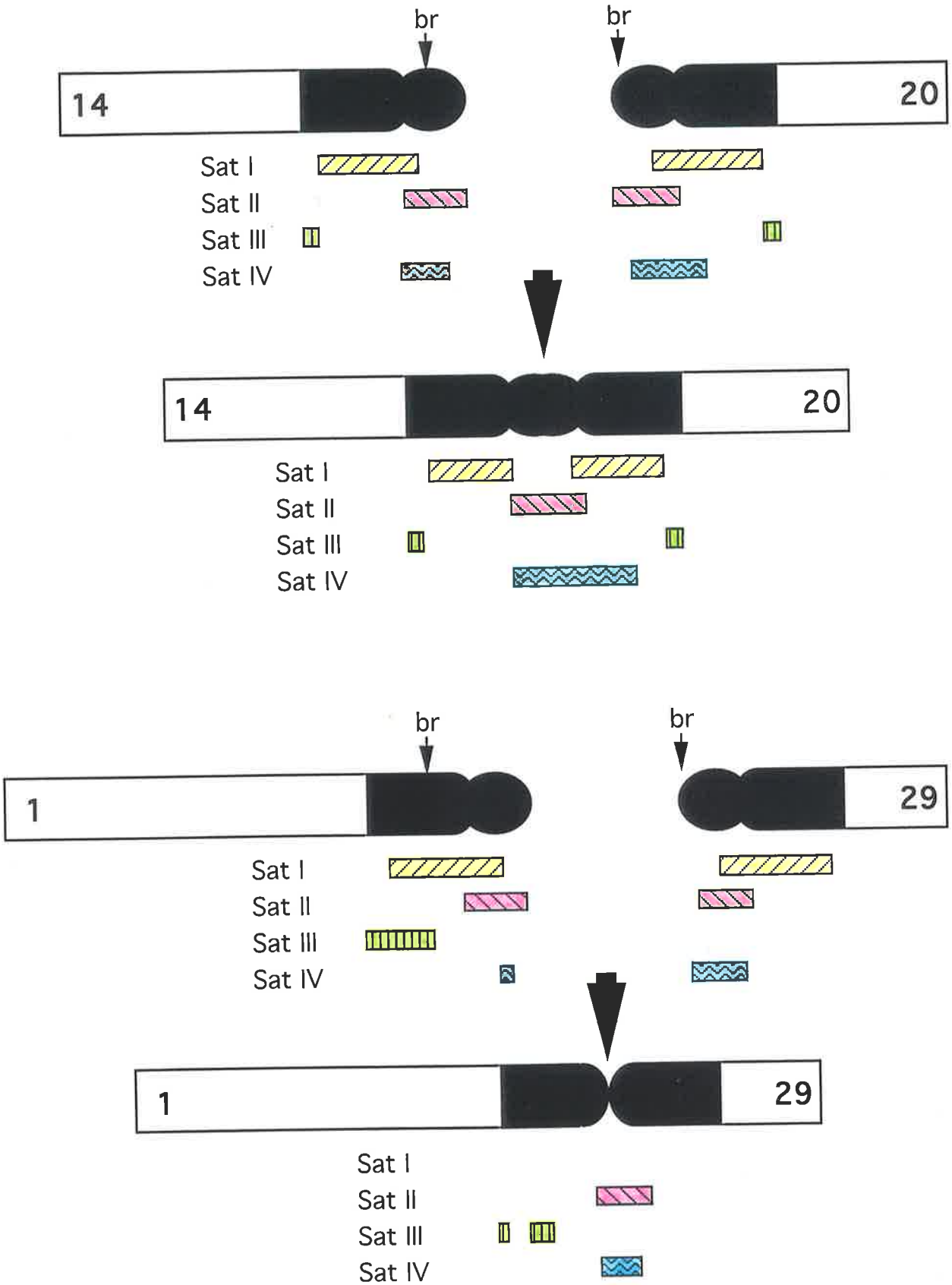
Table 5.1 Summary of Satellite sequences present on the centric fusions and original chromosomes

Chromosomes	Sat I	Sat II	Sat III	Sat IV
14	1, S	1, M	1, W	1, M
20	1, S	1, M	1, W	1, M-S
t(14;20)	2, M with a narrow gap	1, M	2, W 1/arm	1, S
1	1, S	1, M	1, S	0
29	1, S	1, M	0	1, M
t(1;29)	0	1, W	1, W as 2 bands	1, W

S-strong; M-moderate; W-weak; 0-None; 1-One signal; 2-Two signals

Fig 5.1. Model of the 14;20 Robertsonian translocation showing the putative unfused chromosomes and the resultant t(14;20) chromosome. The translocation can be explained by breakage, loss of the small arms and fusion of the two centromeres.

Fig 5.2. Model of the 1;29 Robertsonian translocation showing the putative unfused chromosomes and the resultant t(1;29) chromosome. The rearrangement of Satellites II-IV is explainable in terms of breakage, at the points indicated, and fusion. However, the total loss of Satellite I and partial loss of Satellite III are an enigma in mechanical terms.


br
br
14
20
Sat I
Sat II
Sat III
Sat IV
14
20
Sat I
Sat II
Sat III
Sat IV
br
br
1
29
Sat I
Sat II
Sat III
Sat IV
1
29
Sat I
Sat II
Sat III
Sat IV

Chapter 6

DISCUSSION

FISH is now being used widely in chromosome research. Single FISH can be used to locate a given DNA sequence on metaphase chromosomes. However, to establish the order of two or more adjacent sequences, two or more colours may be necessary. In this study, single and two-colour FISH were used to demonstrate that i) the organization of centromeric satellite sequences varies depending upon the chromosome; ii) the contribution of chromosomes 14 and 20 to the dicentric centric fusion chromosome t(14;20) can be easily explained as both original chromosomes donating their centromeres; and iii) the contribution of chromosomes 1 and 29 to the monocentric centric fusion chromosome t(1;29) can not be easily explained by a simple breakage and fusion mechanism. These results shed light on some questions but raise others.

6.1 Centromere organization in cattle

6.1.1 What is the centromeric DNA sequence in bovine chromosomes?

It is not known which satellite sequence plays the role of centromere in cattle. The known satellite sequences (Satellite I~IV and their sub-classes) are not representative of all centromeres in cattle because none of them are present on the sex chromosomes, X and Y. The theory is that the unbalanced sex pair in animals come from a pair of autosomes (Barch, 1991). During evolution, many rearrangements, additions (O'Neill *et al.*, 1998) and particularly deletions of the Y (O'Neill *et al.*, 1998) must have occurred between this pair of chromosomes because the pair are unbalanced. Their centromeres may also have developed new centromeric sequences differing from the satellite sequences on the autosomes. Eventually, further studies should reveal a bovine sex chromosome-specific centromeric DNA sequence, but whether it will be shared with the autosomes remains an open question.

However, from the present study, it is possible to hypothesize as to which satellite sequences are most likely to be the centromere on the autosomes. Satellites III and IV are not found in all the procentric regions of bovine autosomes, so they are not likely candidates.

Satellite I occurs at the procentric regions of all cattle normal autosomes. However, there is no Satellite I on the monocentric centric fusion chromosome t(1;29). The t(1;29) apparently functions normally through mitosis and meiosis. If Satellite I was the centromere sequence, then t(1;29) should behave as an acentric chromosome.

On the other hand, Satellite II is also on the centromeres of all normal autosomes and the two centric fusion chromosomes, but there is less Satellite II than Satellite I on the autosomes. Small amount satellite DNA is located at the primary constriction of the centromere in mouse acrocentric chromosomes (Kalitsis and Choo, 1997) and bovine Satellite II is similar. This suggests that Satellite II may be the centromeric sequences in bovine autosomes.

6.1.2 What is the better model for organization of Satellites I~IV at cattle procentric region: Block or Interspersed?

Two models are proposed for the organization of Satellite sequences at centromeres: a BLOCK model (Fig 6.1A) where the satellites are present as large consecutive blocks or an INTERSPERSED model where the blocks are intermixed. Two-colour FISH was used to try to resolve which model is more appropriate.

The Satellite sequences were all procentric (that is, near the centromere constriction), but their organization was not readily explained even with two-colour FISH. Satellites I and II, for instance, were observed to overlap one another in two patterns: Satellite II was on the p-terminal side of Satellite I or Satellite II was occasionally in the middle of Satellite I, depending upon which chromosome was examined. Satellites II and IV also appeared to overlap, although Satellite IV was usually closer to the p-terminus than Satellite II. Satellite

IV was observed to have some overlap with Satellite I as well, but not as much as Satellite II. Again, Satellite IV was closer to the p-terminus than Satellite I. Only Satellite III on the long arm of the autosomes did not overlap significantly with the other satellite sequences.

These results suggest that there is some interspersion of some satellites sequences with those of others, supporting an Interspersed model. Satellites II and IV were observed to be overlap each other and with Satellite I. However, side by side blocks of satellite sequences were observed with two-colours without gaps, so blocks must be included in the model. Therefore, a model for the present observations could involve both phenomena, termed herein as a BLOCK/INTERSPERSED model (Fig 6.1B).

Block models have generally been used to illustrate the locations of procentric repeated sequences in human, although recently interspersion has been shown at the junctions of some blocks (Choo, 1997). Moreover, an early investigator in this field has recently confirmed that some interspersion might apply to *Drosophila melanogaster* (Dr. Alan Lowe, pers. comm.). The extreme of interspersion would be single sequences of two or more satellites alternating. However, there is no evidence of this being the case herein. In fact, when characterized, satellite junctions show blocks of many repeats (Kalitsis and Choo, 1997; Bottema, pers. comm.). One well-known case of complete interspersion is at the heterochromatic region of the short arm of the human Y chromosome which is uniformly labelled by four human satellite sequences (Gosden *et al.* 1975).

Confirmation of the Block/Interspersed model will require sequencing or better FISH resolution. In the present work, one serious limitation of resolution was the flare produced by the fluorescent signals. This could have obscured gaps in the signal from Satellite I caused by small blocks of Satellites II or IV. The overlap of the flares from the FITC and TRITC signals was the cause of a white-yellow coloration at the junction of blocks or interspersion of the satellites (Figs 4.5, 4.9).

Another cause of loss of resolution is the imprecision of localization of FISH signals. In single gene work, FISH signals are often staggered on the same chromatid (Kamei *et al.*, 1998), and for a repeated transgene, the strong FISH signal sometimes extend away from the chromosome for about one half of the width of a chromatid (Webb, pers. comm.). In the present study, it was observed that the signal from TRITC was more precise than FITC (Fig 4.5). The precision of localization of a biotin signal might be improved by using FITC-antibodies instead of avidin (Lemieux *et al.*, 1992).

The results from the two-colour FISH of Satellites I~IV on cattle metaphase chromosomes also provided evidence that the organization of cattle satellite sequences near centromeres may vary for different chromosomes. To determine the order of Satellite I~IV using two-colour FISH on individual chromosomes, it would be useful to band the chromosomes with Hoescht 33258 or DAPI. Future work should, therefore, concentrate on obtaining bands with one of these stains. Alternatively, bacterial artificial chromosome (BAC) clones specific for the different chromosomes could be co-hybridized with the satellite probes to identify the chromosomes. When the chromosomes can be identified, the variation of organization of the Satellite I~IV sequences on each cattle autosome can be analysed. However, results herein indicate that there is polymorphism at least in the length of the satellite sequences between individuals, if not the order.

6.2 Robertsonian translocations in cattle

Robertsonian translocations (centric fusions) are one of the most common chromosomal abnormalities in animals. Many chromosomal abnormalities result in obvious disorders or even early death, but usually the only affect in carriers of centric fusions in cattle is reduced fertility. Since the only consequence is sub-fertility, it is possible for these centric fusions to be maintained and become fixed in a population.

The question is whether centric fusions play a role in speciation. Many animals with close evolutionary relationships have close chromosomal relationships. For example, human chromosome 2 is thought to have been formed by Robertsonian translocation from two acrocentric chromosomes of the ancestor (Dutrillaux, 1979). Such is the case in Bovidae where cattle (2n=60,XY, all acrocentric autosomes), goat (2n=60,XY, all acrocentric autosomes) and sheep (2n=54,XY, 3 pairs of metacentric autosomes) have the same number of autosome arms and these arms are equivalent.

To understand how Robertsonian translocations can occur and be maintained, it is necessary to examine examples. For the present study, two cattle examples were available: one representing a dicentric fusion [the t(14;20)] and one representing a monocentric fusion [the t(1;29)]. The approach was to use satellite procentric DNA sequences in FISH experiments to try to resolve the organization of these sequences in both normal and translocated chromosomes and attempt to elucidate the procentric structure of the chromosomes and mechanism(s) of centric fusions.

6.2.1 How are Robertsonian translocations formed?

The centromere structures of the two Robertsonian translocation chromosomes t(14;20) and t(1;29) were studied using FISH to determine which components of the original chromosomes contributed to the new centromere. From the results, the dicentric t(14;20) is easy to interpret in simple mechanical terms as breakage, loss of the p-arms, and fusion of the two original chromosomes. However, chromosome 20 appears to have contributed more to the double-centromere of the t(14;20) than chromosome 14 (Table 5.1 and Fig 5.1).

The centromere of the t(1;29) (Table 5.1 and Fig 5.2) is hard to explain in terms of simple breakage and fusion to produce a monocentric chromosome (Figs 1.5A, 1.5B). Satellite I from both original chromosomes has been totally lost on the t(1;29), Satellites II and IV partially lost, and Satellite III from chromosome 1 reduced to two separate faint bands on the

long arm. Therefore, it would appear that after or during the fusion, some satellite sequences have been deleted. However, the total loss of Satellite I is inexplicable in simple mechanical terms as it has been lost by deletion from both chromosomes.

6.2.2 Can satellite sequences be lost from the centromere in Robertsonian translocations?

A clue to the loss of Satellite I in the cattle t(1;29) might be found in sheep. The metacentric sheep chromosomes 1, 2 and 3 are product of centric fusions. Banding patterns and gene mapping show that sheep chromosome 1, 2 and 3 are derived from the fusions of cattle chromosomes 1;3, 2;8 and 5;11, respectively (Hediger *et al*, 1991). In the sheep metacentric chromosomes, there is only a very small amount of Satellite I in chromosome 1, and a moderate amount in chromosomes 2 and 3 compared to the cattle acrocentric chromosomes (Bottema *et al.*, 1994; Burkin *et al.*, 1996; Daiuto *et al.*, 1997). Centric fusion chromosomes resulting from new Robertsonian translocations of sheep acrocentric chromosomes are dicentric and have substantial ovine Satellite I (Bottema *et al.*, 1994). This suggests that a loss of centromeric satellite sequences can occur after the reduction of chromosome number through centric fusion. Dutrillaux (1979) first described the phenomenon of a loss of 'heterochromatic segment' or at least of its biochemical properties in relation to Robertsonian translocation in Prosimians. Modi *et al.* (1996) made similar observations in other Bovid species by FISH. If true, then the sheep chromosome 1 fusion would be older than chromosomes 2 and 3 fusions as more Satellite I is missing in sheep chromosome 1.

The t(1;29) is the most common Robertsonian translocation in cattle and it has been reported in almost all cattle breeds. This implies that the cattle t(1;29) chromosome may be an ancient centric fusion in the founding *Bos taurus,* and that its centromere may have evolved by losing satellite sequences. On the other hand, the t(14;20) chromosome has only been reported in two cattle breeds, Simmental (Weber *et al.*, 1992) and Dexter (Logue and Harvey,

1978). Thus, the t(14;20) is likely to have arisen relatively recently and might not have had sufficient time to lose satellite sequences. However, this hypothesis assumes that the loss of satellite sequences occurs gradually after centric fusion. This may not necessarily be the case.

6.2.3 How are monocentric fusions formed?

Ancient centric fusion chromosomes (eg, cattle Robertsonian translocation chromosome 1;29, sheep autosomes 1, 2 and 3) are monocentric, while most recent centric fusions (eg. centric fusion chromosomes 14;20, 16;18 in cattle and 9;10 in sheep) are dicentric. The question is how the monocentric Robertsonian translocation chromosomes were formed: did they form directly from the centric fusion or did they evolve from dicentric chromosomes?

6.2.3.1 Hypothesis I: evolution from dicentric chromosomes

In this hypothesis, most of the existing Robertsonian translocations in the Bovidae would be consistently inherited as dicentric fusions at first, and then through centromere evolution become monocentric. The following points support this hypothesis: i) Most centric fusion chromosomes in cattle, including the t(14;20) and a *de novo* t(16;18), are dicentric and occur only in one or two breeds (Table 1.1). In human studies, Niebuhr (1972) found four of five balanced human centric fusion chromosomes were dicentric, the only one 'monocentric' case was thought to be a dicentric with one inactive centromere eventually. Wolff and Schwartz (1992) reported that all of 19 human nonhomologous Robertsonian translocations they found in different families are dicentric by using FISH technique. ii) In sheep, the recent Robertsonian translocation chromosome t(9;10) is dicentric and ovine Satellite I sequences are strongly represented on both arms (Webb, per. comm.).

According to this hypothesis, it would be assumed that the cattle t(1;29) chromosome investigated herein formed as a dicentric, with most satellite sequences from the original

chromosomes, and during its evolution, a "dicentric" t(1;29) lost all Satellite I, part of Satellite III, and perhaps some Satellite IV to become the current monocentric chromosome.

On the other hand, the dicentric t(14;20) chromosome is likely to have arisen relatively recently on the grounds that it has retained Satellite I and is still a dicentric chromosome. Notably, the t(14;20) was also observed to have less Satellite I on the p-arm. It could be postulated that more satellite sequences may be lost in the future and the t(14;20) may evolve to become a monocentric chromosome.

Recently, it has been shown that both centromeres in a stable dicentric chromosome can be active if they are close enough (Page and Shaffer, 1998). Alternatively, in a dicentric chromosome, one of the two centromeres is active, and the other is inactivated (Hsu *et al.*, 1975; Daniel, 1979) or is surppressed (Tyler-Smith *et al.* 1993), perhaps through centromeric sequence evolution, which may eventually lead the disappearance of one centromere.

Despite these points, there is no proof for this hypothesis because such evolutionary events occur over a time scale which cannot be observed. Moreover, *de novo* monocentric fusions have been reported in humans (Hamerton *et al.*, 1975).

6.2.3.2 Hypothesis II: direct formation of monocentric chromosomes

In this hypothesis, satellite sequences from two original chromosomes are reduced rapidly by a non-mechanical mechanism during the formation of a centric fusion and first subsequent cell divisions. The following points support this second hypothesis: i) *De novo* monocentric fusions have been observed in other species (see above) (Hamerton *et al.*, 1975). ii) It has been reported that satellite repeats accumulate instantly in hybrid marsupials (O'Neill *et al.*, 1998), so perhaps similar repeats could be suddenly lost as well.

According to this hypothesis, the cattle t(1;29) investigated herein would have lost all Satellite I and some Satellite III during the direct formation of a monocentric chromosome.

However, there is no model to explain how satellite sequences can be rapidly reduced during centric fusion. Another problem is that there is no evolutionary evidence that mono-metacentric chromosomes evolved from dicentric chromosomes or were formed directly.

It would be of interest to survey a range of independent t(1;29) chromosomes with the prediction that a *de novo* fusion would retain some of the Satellite I sequence from one or both of the parent chromosomes. By following *de novo* fusions through subsequent generations, the hypothesis of loss of satellites might be confirmed, although the time span involved would not be comparable with time spans of evolutionary significance. It would also be interesting to know if independent *de novo* centric fusions of chromosomes 14 and 20 are all dicentric and have the same pattern of satellite sequences as seen in the t(14;20) investigated herein.

6.3 Future studies

To truly determine the structure of bovine centromeres and understand centric fusions, it will be necessary to clone and sequence the entire centromere of all bovine chromosomes as it has been done for some human chromosomes (Kalitsis and Choo, 1997). This will require both substantial time and money.

However, some work could be done practically in the near future. For instance, the centromere structure can be stretched during slide preparation by using a cytospin to make chromosome fibres. Satellites I~IV could then be used as probes (fibre FISH--Claussen *et al.*, 1994; Florijn *et al.*, 1995; Heiskanen *et al.*, 1995; Heiskanen *et al.*, 1996; Florijn *et al.*, 1996; Shiels *et al.*, 1997) to get a better resolution of the order of the bovine satellite sequences at the centromere by using electronic microscopy (Switonski *et al.*, 1987).

In addition, some technical aspects could be examined. For instance, the biotin nick translation gave better incorporation of label into the probes and less background on FISH chromosome spreads than the DIG random priming. It may be worthwhile, therefore, to label

probes with DIG using the nick translation system (Weigant *et al.*, 1991; Verma and Baru, 1995). This might avoid the amplification of the DIG signal and reduce the background of the fluorescent signal from the DIG labelled probes. Also, FITC-avidin might be replaced by FITC-anti-biotin antibodies to improve the resolution of FITC labelling (Lemieux *et al.*, 1992).

Another aspect for improvement is the chromosome banding. Even though chromosomes can be recognized by other methods, such as hybridizing chromosome-specific DNA sequences to metaphase chromosomes by FISH, chromosome banding is still the quickest and the easiest approach. Hoescht 33258 has been used previously to G-band cattle BrdU-labelled chromosomes (Modi *et al.*, 1993), however, it did not work in the present study. Therefore, the next step for banding chromosomes after FISH is to modify the conditions of the Hoescht staining or to test other staining techniques, such as DAPI staining or *Alu*-FISH R-banding (Verma and Baru, 1995).

Alternatively, the probe labelling and detection system of two-colour FISH might be modified to stabilize the signals. The results could then be recorded under the blue or ultraviolet excitation, avoiding the overlap with the propidium iodide colour, which shows R-bands in late BrdU-labelled chromosomes with PPD11 mountant. After modifying the above techniques and/or using an electronic image analysis system, the centromere organization in normal cattle autosomes and centric fusion chromosomes could be re-examined with better resolution.

6.4 Conclusion

In conclusion, the organization of the satellite sequences in cattle chromosomes has been partially delineated. The pattern of the four Satellite sequences on procentric region of chromosomes seems to be better explained by a mixed Block/Interspersed model rather than a

Block model. The centromere organization may be polymorphic and vary between individual chromosomes. Certainly, the amount of particular satellites on specific chromosomes is polymorphic.

The t(14;20) chromosome has been described by Logue and Harvey (1978) as a dicentric chromosome on the basis of C-banding, but it only shows one primary constriction. Presumably, the second centromere is inactive, as has been shown for human dicentric chromosomes (Daniel, 1979). From the order of Satellite I~IV on cattle chromosomes 14, 20 and t(14;20), the formation of the dicentric Robertsonian translocation chromosome t(14;20) can be interpreted by a simple breakage and fusion model.

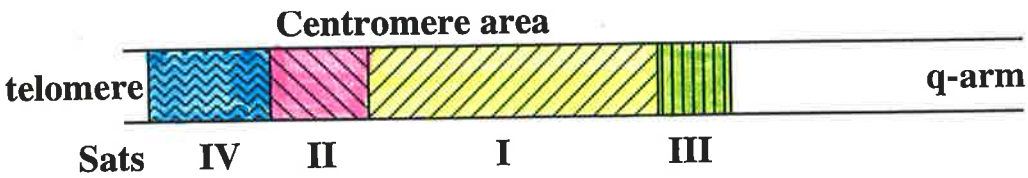
On the other hand, the formation of the monocentric Robertsonian translocation chromosome t(1;29) cannot be interpreted in simple mechanistic terms. The total loss of Satellite I from both original chromosomes 1 and 29, and the partial loss of Satellite III from the original chromosome 1 precludes a breakage and fusion model (Fig 1.5A, 1.5B). It suggests either a further reduction and rearrangement of satellite sequences subsequent to the centric fusion or some other form of instant loss of Satellite I. There is no obvious precedent for this total loss of a major satellite without parallel total loss of other sequences after the centric fusion.

The formation of dicentric Robertsonian translocation chromosomes appears to be straight forward with the donation of both centromeres from the original chromosomes. However, it is not clear which hypothesis best explains the generation of monocentric Robertsonian translocation chromosomes: direct formation, or evolution from dicentric chromosomes.

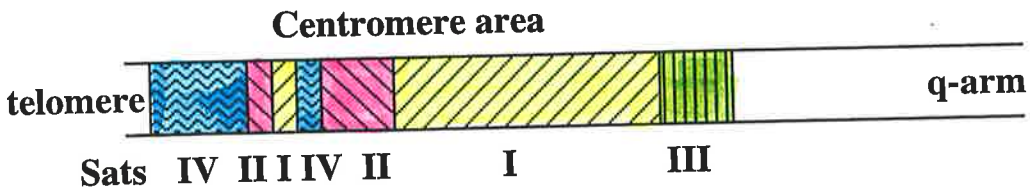
Fig 6.1 Models for organization of cattle procentric sequences

(A) Block model. Example of the organization of the four main cattle Satellite sequences I~IV as tandem blocks.

(B) Block/Interspersed model. Example of the organization of the four main cattle Satellite sequences I~IV as interspersed tandem blocks. Smaller blocks of Satellites I, II and IV are interspersed on the p-terminus of some autosomes.



(A) Block model



(B) Block/Interspersed model

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Appendices

Appendix 1

Media, antibodies, enzymes and main chemical sources

AmnioMax-C100; GibcoBRL: basal 17001-074, supplement 17002.056

avidin-FITC, Fluorescein isothiocyanate conjugated avidin, cell sorter grade (DCS), 2.0 mg/ml, Vector Laboratories, A-2011.

BCS scintillation fluid, Amersham, NBCS104

BioNick kit (kit 1) - BioNick^{TM} Labelling System, GibcoBRL, 18247-015: 10 x dNTP mix, 10 x Enzyme Mix, Stop Buffer, dH_2O

Biotinylated anti-avidin D (goat), 0.5 mg/ml, Vector Laboratories, BA-0300

Blocking reagent, Boehringer Mannheim, 1093657

bovine serum albumin, Sigma, A-6003

5-bromodeoxyuridine, 20 mg/ml,

dextran sulphate, Pharmacia, 17-0340-01

DIG DNA Labeling Kit (kit 2), Boehringer Mannheim, 1175033: 10x hexanucleotide mixture, 10x dNTP Mix and 20x Klenow enzyme. 0.2M EDTA solution (pH 8.0) is used as stop buffer

Dulbecco's Modified Eagle's Medium, CSL, 05172301

foetal bovine serum, CSL, 09702301

gentamycin solution, 10 mg/ml, CSL, 05001301

glutamine, 0.2M, CSL 09871901

goat serum, Sigma Immune Chemicals, Sigma-Alldrich

Gurr buffer tablets, BDH, 1 tablet dissolved in dH_2O and made up to 1 litre produces a solution of pH approximately 6.8 at 20°C

^{3}H-dATP, Amersham, TRK 633 B63

^{3}H-dTTP, Amersham, TRK 576 B95

heparin solution, 25000 unit/ml, Sigma, H-3149

HEPES buffer, 1M, CSL 07391901

Hoechst-33258, Sigma, B-2883

Leishman's stain (eosin-polychrome methylene blue), Sigma, L6254

mouse anti-DIG, 0.1 mg/ml, Boehringer Mannheim, 1333062

nail varnish, colourless, acetone-based solvent

para-phenylenediamine, Sigma P 6001; or p-phenylenediamine HCl, Sigma P 1519

penicillin/streptamycin/fungizone, 10000 unit/ml, 10000 mcg/ml, 25 mcg/ml, CSL, 09291051

phytohaemagglutinin, M form, 10 ml from lyophilized powder, GibcoBRL, 10576-015

poke weed mitogen (lectin), 1.0 mg/ml, Sigma, L-9379

rabbit anti-mouse-TRITC: TRITC-conjugated rabbit antimouse immunoglobulins, DAKO, R270

rabbit serum, Sigma

RNase, 100 µg/ml, Sigma

Roswell Park Memorial Institute medium 1640 with bicarbonate, CSL, 05182301

rubber cement, "Earth" brand, Marumi Ind. Co. Ltd, Japan, 37022; later, art cement, Premier, F/387

Sephadex G-50, Pharmacia, 17-0045-02

sheep anti-DIG-TRITC, Boehringer Mannheim, 1207750

ssDNA - sonicated salmon sperm DNA, 7.7 µg/µl

swine anti-rabbit- TRITC, TRITC- TRITC-conjugated rabbit antimouse immunoglobulins, DAKO, R156

swine serum, Sigma

trypsin/EDTA (1:250) liquid, Surgical & Medical, 21-163-0100

Tween 20 - polyoxyethylenesorbitan monolaurate, Sigma, P-1379

Appendix 2 Solutions

colchicine 100 µg/ml

complete AmnioMax medium

2% v/v PHA and 0.5% v/v PWM mixture of AmnioMax basal and supplement at the ratio of 90:15, with 0.5% v/v glut. and 0.1% hep. 0.5% v/v PSF

complete DMEM with FBS medium

DMEM with bicarbonate, mixed with 10% v/v FBS, 1% v/v glut., 0.2% v/v gent., 2% v/v HEPES buffer, 0.1% v/v hep., 0.5% v/v PSF, 2% v/v PHA, and 0.5% v/v PWM

complete RPMI with FBS medium

RPMI with bicarbonate, mixed with 10-20% v/v FBS, 1% v/v glut., 0.2% v/v gent., 2% v/v HEPES buffer, 0.1% v/v hep., 0.5% v/v PSF, 2% v/v PHA, and 0.5% v/v PWM

complete RPMI with NSS medium

RPMI with bicarbonate, mixed with 15% v/v NSS, 1% v/v glut., 0.2% v/v gent., 2% v/v HEPES buffer, 0.1% v/v hep., 0.5% v/v PSF, 2% v/v PHA, and 0.5% v/v PWM

deionized formamide

Mix formamide for at least 2 hours with 5% ion exchange resin beads (Bio-Rad, AR grade). Store at -20°C.

dextran sulphatesolution in formamide 20% w/v

0.2 g dextran sulphate in 1 ml deionized formamide

fixative

methanol 75%, glacial acetic acid 25% (v/v)

formic acid solution

0.2% v/v HCOOH in dH_2O

high stringency rinsing solution

deionized formamide 50 ml, 20x SSC 10 ml, dH_2O 40 ml, adjust pH to 7.0

hypotonic solution

0.075 M KCl

PBS without Ca^{2+} and Mg^{2+}

One liter contains 8 g NaCl, 0.2 g KCl, 1.15g Na_2HPO_4 , 0.2 g KH_2PO_4, pH 7.4

PPD9

100mg of PPD in 10 ml of PBS; 90 ml of glycerol, adjust to pH 11.0

PPD11

100mg of PPD in 10 ml of PBS; 90 ml of glycerol, adjust to pH 11.0

propidium iodide in PBS

5 µg/ml propidium iodide in PBS

silver nitrate solution

50% w/v $AgNO_3$ in dH_2O

slide denaturation solution

deionized formamide 35 ml, 20x SSC 5 ml, Milli-Q H_2O 10 ml, adjust pH to 7.0

sodiun acetate

Na acetate 3M (pH 5.2)

solid stain

20% Leishman's stain in Gurr buffer (pH 6.8)

20x SSC

3M NaCl, 0.3M sodium citrate, pH 7.0

4 x SSC/BSA

4 x SSC/1.0% w/v bovine serum albumin (BSA)

4x SSC/Tween 20

4x SSC/0.05% Tween 20 (polyoxyethylenesorbitan monolaurate)

10x SSCP

1.2M NaCl, 0.15M Na Citrate, 0.1M Na_2HPO_4, 0.1M NaH_2PO_4, adjust pH to 6.0

$T_{10}E_1$

10mM Tris-HCl, 1mM ethylene diamine tetracetic acid, pH 7.5

Tris-HCl/NaCl

0.1M Tris-HCl/0.15M NaCl (pH7.5)

Tris-HCl/NaCl/BSA

0.1M Tris-HCl/0.15M NaCl/0.5% BSA (pH7.5)

Tris-HCl/NaCl/Tween20

0.1M Tris-HCl/0.15M NaCl/ 0.05%Tween20 (pH7.5)