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THE CHROMOSOMAL LOCALIZATION OF THE BETA GLOBIN GENE CLUSTER IN THE GOAT (*Capra hircus*) By *in situ* HYBRIDIZATION

By

Louis B. Simi

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF REQUIREMENTS FOR THE DEGREE

MASTER OF SCIENCE

IN

EXPERIMENTAL PATHOLOGY

Department of Pathology

Edmonton, Alberta

Spring, 1989

ISBN 0-315-52802-8

Canada

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR LOUIS B. SIMI

TITLE OF THESIS: CHROMOSOME LOCALIZATION OF THE BETA GLOBIN GENE CLUSTER IN THE GOAT (Capra hircus) BY in situ HYBRIDIZATION.

DEGREE: MASTER OF SCIENCE

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ABSTRACT

This study had a two-fold purpose: to characterize the chromosome complement of the goat (*Capra hircus*),(2n=60) and to establish the chromosomal location of the beta globin gene cluster in this species using *in situ* hybridization. Several banding techniques were used to identify chromosomes, prepare karyotypes and in the analysis of some features of chromosome structure. The BrdU G-banding technique was used in the identification of chromosomes both before and after the *in situ* hybridization procedure.

A cloned DNA fragment containing a segment of the beta globin gene cluster was used in this study to determine the chromosomal location of this gene cluster. This was the 1.175 Kb. EcoR 1/BamH 1 DNA fragment which includes a portion of the 5' noncoding region through to the second exon of the goat epsilon IV gene. Using this DNA fragment and the *in situ* hybridization technique, the beta globin gene cluster was localized to region 33-35 on chromosome 7.

ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. C.C. Lin, for providing his expertise and the facilities for performing this study. Secondly, I would like to thank Dr. R. Sasi for his vigilance in instruction and guidance throughout my study in the field of molecular biology. Lastly I would like to thank Dr. J. Kuspira for reviewing this manuscript.

I also thank Tomomi Tainaka for her instruction and in lymphocyte culturing and chromosome banding techniques.

I acknowledge the following people who contributed by assisting in obtaining blood samples from the goat for tissue culturing and DNA extraction: Dean Treichel at the Valley Zoo, Vicki Maitland and Dr. G. Klavano at the Provincial Veterinary Lab., Homer De Domininicus of Symons Valley and Dr. Randy Killian of Sherwood Park.

Lastly and most important, I would like to thank my mom and dad for their total and continuous encouragement and support during the course of these studies.

To my mother and father

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LIST OF ABBREVIATIONS

AA	- Amino Acids
ACO1	- Aconitase 1
ACY1	- Aminoacetylase 1
ADA	- Adenosine Deaminase
AK1	- Adenylate Kinase 1
ALB	- Albumin
BrdU	- Bromodeoxyuridine
CAT	- Catalase
COL1A2	- Collagen, Type 1, Alpha 2
CYP21B	- Cytochrome P450, Steroid 21-Hydrolase (Congenital Adernal Hyperplasia)
dCTP	- Deoxycytosine Triphosphate
EDTA	- Ethylenediamine Tetraacetic Acid, Disodium Salt
ENO1	- Enolase 1
EtBr	- Ethidium Bromide
FCS	- Fetal Calf Serum
GAPD	- Glyceraldehyde-3-Phosphate Dehydrogenase
BGB	- Giemsa BrdU G-Banded
GC	- Group Specific Component (Vitamin D Binding Protein)
GH	- Growth Hormone
GLA	- Galactosidase, Alpha
GLO1	- Glyoxalase 1
GPI	- Glucose Phosphate Isomerase
G6PD	- Glucose-6-Phosphate Dehydrogenase
GSR	- Glutathione Reductase

GUK - Guanylate Kinase

GUSB - Glucuronidase, Beta

^{3}H - Tritium

HBBC - Hemoglobin Beta Globin Complex

HbA - Goat Adult Hemoglobin

HbC - Goat Preadult Hemoglobin

HBSS - Hank's Balanced Salt Solution

HLA-A - Major Histocompatibility Comp'ex A

HPRT - Hypoxanthine Phosphoribosyltransferase

HRAS1 - Harvey Ras Sarcoma 1

IDH1 - Isocitrate Dehydrogenase 1

IFREC - Interferon B Cell Surface Receptor

IGF2 - Insulin-Like Growth Factor 2

INS - Insulin

ITP(A) - Inosine Triphophatase (nucleoside triphosphate pyrophosphatase)

LDHA - Lactate Dehydrogenase A

LDH1 - Lactate Dehydrogenase A-Like Sequence 1

LDHB - Lactate Dehydrogenase B

MDH2 - Malate Dehydrogenase 2

ME1 - Malic Enzyme 1, (Soluble)

MP1 - Mannose Phosphate Isomerase

NOR - Nucleolar Organizer Region

NP - Nucleoside Phosphorylase

PEPB - Peptidase B

PEPC - Peptidase C

PGC	- Phosphogluconate Dehydrogenase
PGM1,2,3	- Phosphoglucomutase 1, 2, 3
PGY-P	- Glycoprotein/Multiple Drug Resistance 1
PHA	- Phytohemagglutinin
PKM1	- Pyruvate Kinase Muscle 1
PKM2	- Pyruvate Kinase Muscle 2
PTH	- Parathyroid Hormone
RT	- Room Temperature
SDS	- Sodium Dodecyl Sulfate
SOD1	- Superoxide Dismutase 1, soluble
SOD2	- Superoxide Dismutase 2, mitochondrial
SSC	- Salt/Sodium Citrate
TE	- Tris/EDTA
TP11	- Triosephosphate Isomerase 1
U	- Unassigned
(w/v)	- weight/volume

INTRODUCTION

Genetic mapping involves the localization of genes to specific regions of chromosomes that carry them (Reiger 1980). This has become an important area of investigation in mammalian cytogenetics. The ever growing genetic map of these species is of importance in comparative gene mapping which involves the study of the maintenance, gain or loss of both linkage and syntenic relationships in divergent mammalian species. The main aim of such comparative gene map analysis is in the study of evolution through genome organization and gene expression.

The members of the Family Bovidae represent a commercially important group of animals. Comparative genetic mapping in these species has gained momentum due to the recent interest in the potential application of molecular genetics techniques to the improvement of breeds of cattle, sheep and goats.

Thus far, gene mapping in cattle has involved the establishment of syntenic group using mainly somatic cell hybrids and biochemical markers. These studies have resulted in the establishment of 25 syntenic groups each containing one or more genes as well as the provisional assignment of genes to several autosomes (Dain et al. 1984). The syntenic group (G6PD-HPRT-PGY-GLA) was assigned to the X chromosome (Heuertz and Hors-Cayla 1978; Shimizu et al. 1981). More recently, the <u>in situ</u> hybridization techniques has been used to localize genes to chromosomes in these animals. For example, Popescu et al. (1988) assigned a specific repetitive DNA sequence to the Y

chromosome, while Fries et al. (1986) localized the BoLA (bovine lymphocyte antigen) complex to chromosome 23 (23q13-23q23) using this procedure.

This study had two objectives: one was to identify the chromosomes of the goat. This involved the construction of a banded karyotype and idiogram of the goat. The second objective was to localize the beta globin gene cluster to a goat chromosome using the in situ hybridization technique.

CHAPTER ONE

LITERATURE REVIEW

[I] CHROMOSOME IDENTIFICATION AND BANDING TECHNIQUES

All of the early studies on chromosomes of different mammalian species used standard (non-banding) procedures to determine their chromosome number and general morphology. For example, in man it was not until 1956 that Tjio and Levan showed that the somatic (diploid) chromosome number was 46 instead of 48. Using standard staining procedures, chromosomes in the complement of most if not all eukaryotic species could be characterized with respect to overall arm length, position of centromeres, secondary constrictions and the presence or absence of satellites which assist in the accurate identification of chromosomes and pairing of homologous chromosomes. Yet, in many cases individual chromosomes in the complement cannot be identified.

The possibility of chromosome banding techniques to aid in the identification of chromosomes was initially suggested by both Levan (1964) and Stubbelfield (1964). Several different types of banding techniques have been developed since 1968. Caspersson et al. (1969) first discovered that chromosome banding can be produced by quinacrine mustard (Q-banding). This facilitated the identification of individual chromosomes and chromosome segments involved in chromosomal aberrations. Another technique was developed to produce banding patterns similar to Q-bands without the need for fluorescent microscopy. The trypsin G-banding technique was first published by

Seabright (1971) as well as Wang and Fedoroff (1972). This banding technique gives a more permanent banding pattern then the Q-banding technique. Another banding technique, the R-banding method results in a banding pattern that is the reverse of the G-banding techniques. R-banding can be achieved by either a non-fluorescent (Giemsa the stain method) (Dutrillaux and Lejeune 1971) or fluorescent (acridine-orange method) Bobrow et al. (1972). An advantage of the R-banding procedure is that it allows visualization of the terminal regions of chromosomes in some cases. The rRNA genes associate with nucleolar organizing regions often presented as "secondary constrictions" in metaphase chromosomes. The ammoniacal silver staining technique of Goodpasture and Bloom (1975) has been used to identify the location of these regions in many mammalian species. Another banding technique was developed around this time (Arrighi and Hsu 1971) removes most of the DNA from the chromosomes except the highly resistant repetitive DNA sequences next to the centromeres and in the long arm of the human Y chromosome. This technique was called the C-banding technique.

(A) THE G-BANDING TECHNIQUE

Staining of chromosomes with Giemsa following many different means of pretreatment have been developed which result in G-banded chromosomes. These include the incubation of slide preparations in organic or inorganic salt solutions; basic solutions, chelating agents, buffers, detergent, proteolytic enzymes or oxidizing agents (Dutrillaux and Lejeune 1971; Kato and Moriwaki 1972; Kato and Yosida 1972; Wang and Fedoroff 1972; Utakoji 1972; Lee et al. 1973). What

ever the method of pretreatment used the results are the with G-positive (dark staining regions) and G-negative (light staining regions). The G-position region has been found to be late replicating regions of the chromosome through autoradiography study (Ganner and Evans 1971).

The G-banding technique has been used in the goat by several researchers to identify the chromosomes of this species (Evans et al. 1973; Hageltorn and Gustavsson 1974; Schnedl and Czaker 1974; Buckland and Evans 1978a and Bunch and Nadler 1980). The procedure of Wang and Fedoroff (1972) was modified and used in this study to produce G-banded chromosomes in the goat. The results of these studies will be discussed later.

(B) THE DEVELOPMENT OF CHROMOSOME AND CHROMOSOME BANDING NOMENCLATURE IN MAN.

During the first conference on human chromosomes in Denver in 1960 (Book et al. 1960) it was established that the autosomal chromosome pairs would be numbered from 1 to 22 and as nearly as possible arranged in order of descending length. At the 1963 conference in London the 23 chromosome pairs were classified into 7 groups (A to G). The X chromosome was included in the C-group containing chromosome pairs 6-12 (Patau 1960).

At the 1971 conference in Paris the banding nomenclature for human chromosomes was established. Rules regarding chromosome band nomenclature established in man have subsequently been applied to the study of all mammalian species and many other organisms as well.

The Reading Conference (1980) led to the acceptance of world-wide standard G-banded karyotypes for cattle, sheep, and goats.

Another method of G-banding is important in this study because of its use in goat chromosome identification for the in situ hybridization technique. This involves the incubation of cells in culture in 5-BrdU during DNA replication.

BrdU (bromodeoxyuridine) incorporation into DNA was first used in human cytogenetic studies by Kaback et al. (1963) to accentuate secondary constrictions of chromosomes 1, 9 and 16. Palmer and Funderburk (1965) and Palmer (1968; 1970) showed that BrdU incorporation increased the number of secondary constrictions within the genome and that certain chromatid segments could increase in length which was assumed to be due to a delay in the spiralization of chromatin associated with chromosome condensation in that region.

In 1972, Zakharov and Egolina allowed two cycles of DNA replication to take place in the presence of bromodeoxyuridine. This results in BrdU being substituted for thymidine in one DNA polynucleotide strand of one chromatid (stains darkly) while the BrdU was incorporated into both polynucleotide strands of the other sister chromatid (stains lightly). In part, this difference in staining has been attributed to a delay or decrease in mitotic spiralization of the chromosomal DNA which has undergone two rounds of DNA replication in the presence of BrdU. This suggests that in creating the BrdU G-banded metaphase chromosome preparatives that cells were harvested prior to two full DNA replication cycles.

The BrdU G-banding technique was initially used by Perry and Wolff (1974). This method of G-banding is utilized in this study to identify the goat chromosome complement. It is considerably more detailed and time consuming than the other methods of G-banding. An explanation of the advantages of this procedure as well as an evaluation of its' effectiveness in the study of goat chromosomes are discussed later.

(C) THE C-BANDING TECHNIQUE

The C-banding technique began as an offshoot of the in situ hybridization technique of Pardue and Gall (1969b) and Jones (1970). It involves the extraction of large amounts of non C-band chromatin while leaving constitutive heterochromatin intact (Comings et al. 1973; Pathak and Arrighi 1973; Burkholder 1975). It has been suggested that resistance to denaturation of the DNA is due to some unique feature of chromosomal protein-DNA interactions. However, this has not been confirmed.

This procedure has previously been used by Arrighi and Hsu (1971); Evans et al. (1973); Schnedl and Czaker (1974); Buckland and Evans (1978b) in studies of the chromosome complement of the goat. C-banding in bovine species is discussed later in this report

(D) THE AMMONIACAL SILVER STAINING TECHNIQUE

Many techniques have been used to localize 18S and 28S rRNA genes. These include: (i) in situ hybridization (Gall and Pardue 1969b); (ii) silver staining techniques (Howell 1982; Hubbell 1985)

and (iii) the N-banding technique (Matsui and Sasaki 1973; Matsui 1974; Funaki et al. 1975). Silver-staining occurs in NOR regions that were actively transcribing in the preceding interphase (Miller et al. 1976a,b). All these procedures stain the acidic protein(s) unique to NOR regions as indicated by the studies of Das (1962); Howell et al. (1975); Goodpasture and Bloom (1975); Schwarzacher et al. (1978); Olert et al. (1979).

The NOR staining technique have been used by Henderson and Bruere (1979) and Mayr and Czaker (1981) to identify NORs in the goat. These and other studies including those in cattle will be discussed later.

[II] BRIEF OVERVIEW OF THE HUMAN AND GOAT BETA GLOBIN GENE CLUSTERS

(A) STRUCTURE AND FUNCTION OF THE HUMAN HEMOGLOBIN MOLECULE

The human hemoglobin molecule is a tetramer composed of two alpha polypeptides each 141 amino acids long which are coded for by genes located in the alpha globin gene cluster and two beta polypeptides each 146 amino acids long specified by genes in the beta globin gene cluster. Each molecule has an approximate weight of 64,500 daltons and a total of 574 amino acids. The four polypeptides are ionically bound to a haem $(Fe)^{2+}$ group. These proteins are associated with iron within the red blood cell and function to bind oxygen obtained from gas exchange in the alveoli of the lung and deliver it to tissues of low oxygen partial pressures.

(B) ONTOGENY OF CAPRINE HEMOGLOBINS

The sequence in which the beta globin gene products appear is important in understanding the time of onset of clinical manifestations of hemoglobinopathies and thalassemias (Philips and Kazazian 1983). The use of the goat as a model for the study of the switching mechanism is advantageous for two reasons. The first being the reversibility of the B^c (pre-adult) - B^a (adult) switch. The switch back to B^c can be induced by the induction of hypoxia or the injection of the erythropoietin. The second being that the B^c - B^a switch occurs exo utero so it can easily be studied. The ontogeny of the goat hemoglobin biosynthesis is illustrated in Figure 1.

(C) THE GENETICS AND EVOLUTION OF THE HUMAN AND GOAT BETA-GLOBIN GENE CLUSTERS

The human beta globin cluster contains 7 gene loci of which 5 are functional and called genes while 2 are non-functional and called pseudogenes. The gene cluster is localized in the 11p15 region of the short arm of chromosome 11 (Deisseroth et al. 1978). The sequence of the genes in the cluster in the 5' to 3' directon are illustrated in Figure 2. These genes are also known to follow the same order of developmental expression as the 5' to 3' physical gene order within species of primates as well as rabbits and mice (Jahn et al. 1980).

The human globin genes envolved as shown in Figure 3. The ancestral globin gene codes for a 150 AA globin duplicated some 500 million years ago. This was followed by a transposition and a series of gene mutations which resulted in the formation of the alpha and

FIGURE 1. Expression of the beta globin gene cluster during the stages of development in goats.

$\varepsilon_2 E_2^{I}$ and $\varepsilon_2 E_2^{II}$ are expressed during the embryonic stage of development in the goat.

The expression of the beta globin gene B^F peaking around three months prenatally.

The B^A reachs its maximum level of expression twelve months after birth which is maintained throughout life.

The B^C preadult gene product of the beta globin gene starts to be taken over by the B^A adult form at one year of age.

ΩΔΙ

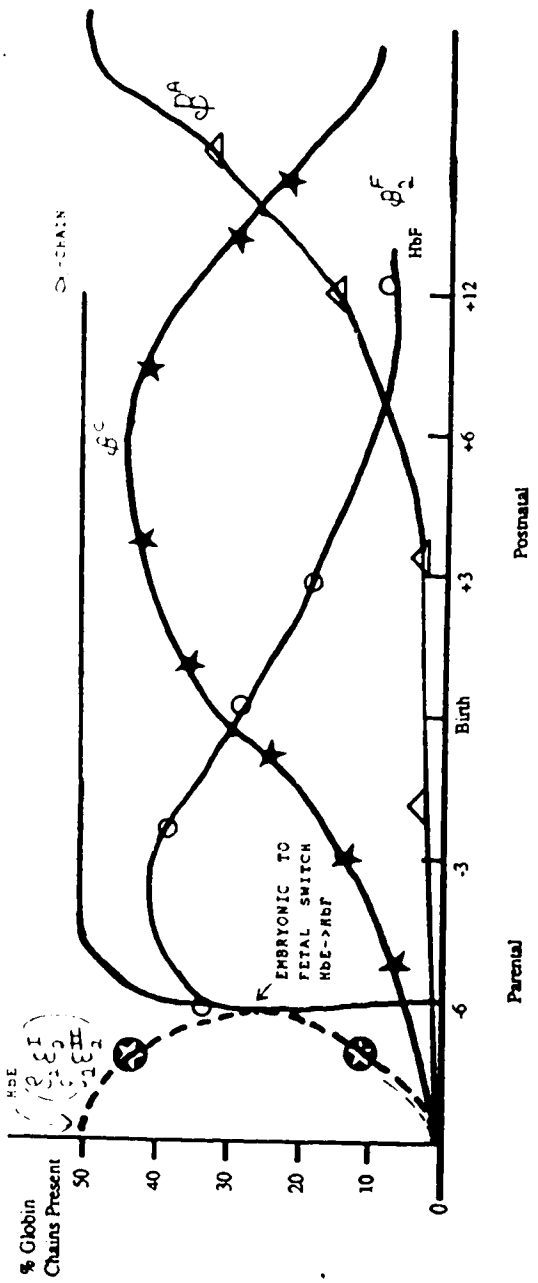

% Globin Chains Present
50
40
30
20
10
0
HbE
EMBRYONIC TO FETAL SWITCH HbE->HbF
HbF
-6
-3
Birth
+3
+6
+12
Parental
Postnatal

FIGURE 2. Goat Beta Globin Gene Cluster.

ε^{I}-ε^{VI} are genes of embryonic origin.

$\psi\beta^{X,Y,Z}$ are pseudogenes and do not produce functional gene products.

β^{A}, β^{C} and β^{F} are genes expressed during fetal, preadult and adult stages of development in the goat.

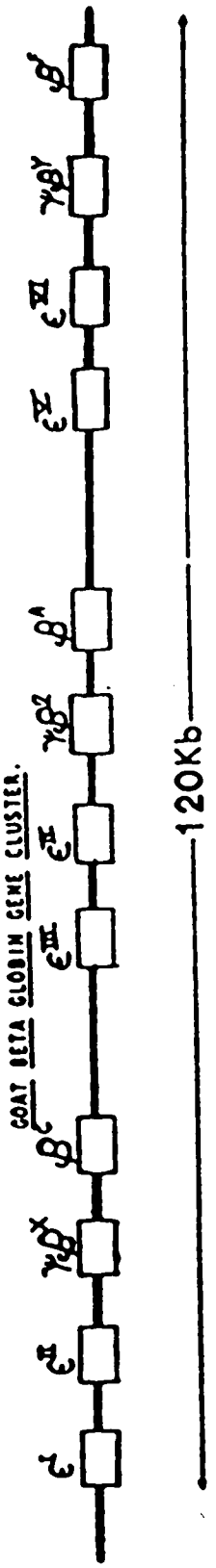

GOAT BETA GLOBIN GENE CLUSTER.
120Kb

FIGURE 3. Schematic diagram showing the evolution of the ancestral beta globin gene cluster. Jeffreys (1982)

beta genes. This was followed by a duplication that gave rise to the gamma globin gene. During primate evolution still another duplication and several mutations resulted in the formation of the delta gene in man (Jeffreys 1982).

The goat beta globin gene cluster contains 12 linked genes that span more than > 120 Kb. of DNA (Lingrel et al. 1983). These genes are arranged as shown in Figure 2. The gene cluster can be broken down into three blocks of four genes each. Each such block includes two epsilon globin genes, a pseudogene and a beta globin gene. Unlike the genes in the human beta globin gene cluster, those of the goat are not expressed during development in the same 5' - 3' physical order of the genes.

The evolution of this globin gene cluster initially began with a single ancestral beta globin gene. This gene was duplicated to produce the embryonic progenitor (epsilon gene) which in turn was duplicated. Then another duplication occurred to produce the basic block of four genes currently present in the goat. The DNA sequence homology existing among the genes within each block of four genes and the higher degree of homology between genes in corresponding positions in each of the other two blocks of four genes suggests triplication of this ancestral block of four genes to form the goat beta globin cluster (Townes et al. 1984b). Sheep also have been assessed a similar triplication of the block of four beta genes (Bonsignore et al. 1967). The triplication therefore very likely preceded the divergence of goats and sheep.

Globin gene DNA sequences in the goat and other mammals are highly homologous. This was initially suspected by Robbins et al. (1979) and used in the initial isolation and cloning of the cluster of beta globin genes of the goat. A DNA sequence of the epsilon IV gene will be used in this study to cytologically localize the beta globin gene cluster in goat chromosomes.

[III] CLONING AND ISOLATION OF DNA PROBES: EMPHASIS ON THE GOAT BETA GLOBIN GENES

Gene cloning allows individual genes to be selected and purified in large amounts. The following standard procedures are used in cloning the beta genes including the one used in this study. This involves a series of steps to produce recombinant DNA molecules, introduce them into bacteria or viruses in a form that promotes their replication and the selection of DNA fragments containing certain genes or portions of those genes. The first involves the restriction enzyme digestion of genomic DNA. Next, a plasmid or bacteriophage is selected with several features to ensure the formation of the genetically engineered vector, the transport of that vector into the bacterium and the selection of those bacteria which contain the engineered vector. In plasmids, for example, this includes a single unique restriction enzyme site for the insertion of a DNA fragment within a region coding for resistance to a specific antibiotic. This serves as a method of selection between those bacterium which contain the recombinant plasmid from those that do not contain the plasmid or contain it without an inserted DNA fragment. Further selection for

bacteria harboring the recombinant plasmid is gained by a second antibiotic resistance site to select those bacterium containing no plasmid from those containing the recombinant plasmid. An important aspect of the plasmid is that it be small in size so that shearing of the plasmid DNA will be minimized when harvested (Davis, 1986).

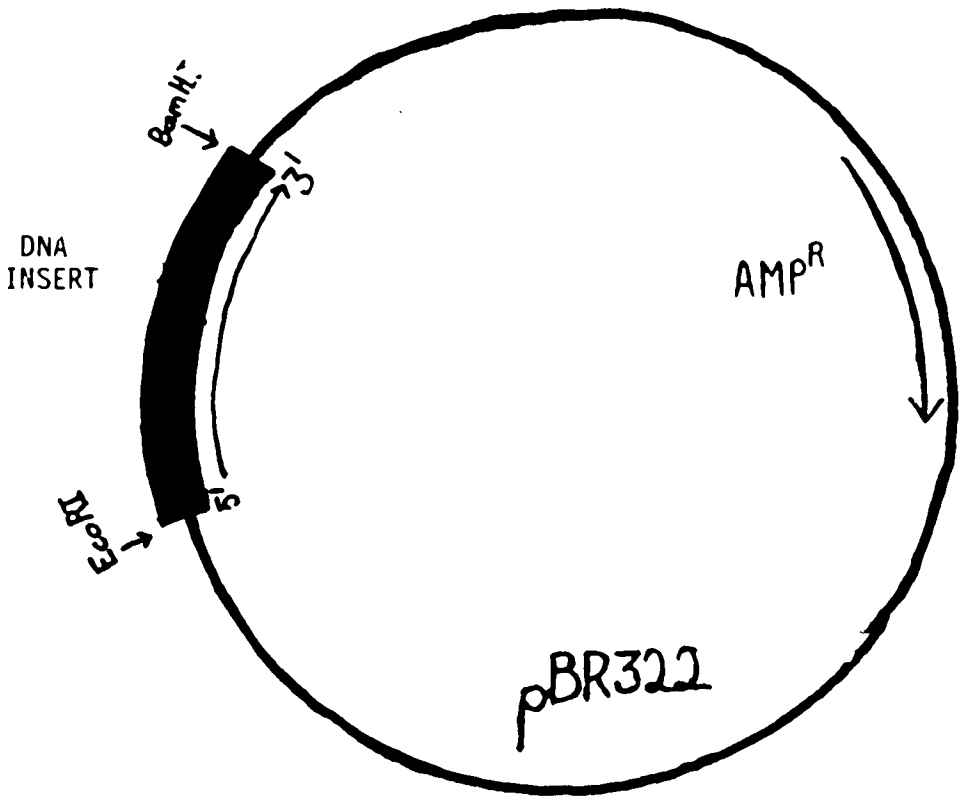
The above procedure was followed by Robbins et al. (1979) in the cloning of the goat beta globin gene cluster.

Specifically, the experiment began with a partial digestion of the goat genomic DNA. The resulting DNA fragments were then cloned and introduced directly into lambda phage Charon 4A. Recombinant phage were plated on DP50 sup F E. coli. These were then transferred to nitrocellulose. Human globin cDNA sequences were used to screen for those colonies which may harbor goat globin DNA sequences. Having accomplished this, those plaques of interest were propagated. These goat genomic DNA fragments were then isolated and hybridized against human structural beta globin DNA sequences to discover structural goat globin DNA sequences. This basic procedure was also followed by Townes et al. (1984a,b) to discover that the goat beta globin gene cluster actually consisted of 12 genes. One of the DNA fragments isolated by Townes et al. (1984a) was sent to Dr. Lin by Dr. J. Lingrel. The plasmid used in this work is illustrated and described in Figure 4. The DNA insert of this plasmid was isolated and used to determine the chromosomal location of the beta globin gene cluster in the goat genome using the in situ hybridization technique.

FIGURE 4.

Description of the Plasmid pG16Ec3Bm2

The plasmid pG16Ec3Bm2 contains 1.2 kb. EcoR 1/BamH 1 goat genomic DNA insert which includes a portion of the 5' flanking region through to the second exon of the goat epsilon IV gene. The vector is pBR322. This recombinant DNA plasmid was maintained in the host E. coli strain HB101. An EcoR 1/BamH 1 double digest should yield a 4kb pBR322 DNA fragment and the desired 1.2 kb genomic DNA insert.



Adapted from Townes et al. Journal of Biological Chemistry 259: 1896-1900 (1984).

[IV] GENE MAPPING IN CATTLE, SHEEP AND GOATS

The chromosome complement of cattle, sheep and goats has been identified and characterized through the use of several established banding techniques as indicated by (Hageltorn and Gustavsson 1974). Chromosomal mapping studies in cattle has developed slowly, but a number of syntenic relationships have been established. Several of the techniques used in genetic mapping in man have been applied to the mapping of chromosomes of members of the Family Bovidae. These include the use of linkage analysis in appropriate pedigrees (Larson 1977), analysis of selected clones derived from somatic cell hybrids, and in situ hybridization.

Somatic cell hybridization has been used in several studies to establish syntenic relationships. The first such relationship was established for the X-linked genes GGPD, PGK, GALA and HPRT in cattle by Heuertz and Hors-Cayla (1979) who also established three autosomal syntenic groups: (1) LDHB-PEPB-TPI; (2) PGM3-ME1-SOD2; (3) PGD-ENO1. They also identified nine other genes: LDHA, MOH2, LOH1, SOD1, PKM2, PGM1, PGM2, AK1 and MPI to be nonsyntenic. Shimizu et al. (1981) confirmed the syntenic relation of the genes G6PD-PGK-GALA-HPRT on the bovine X chromosome. Echard et al. (1984) confirmed the existence of the three autosomal syntenic groups reported by Heuertz and Hors-Cayla above and reported nonsynteny from six other markers that were studied. Womack and Cummins (1984) showed that the genes IFREC and SOD1, ENO1 and PGD, and TPI-LDHB showed concordance in

somatic cell hybrids whereas the genes LDHA, PEP C, PGM1, PGM2, MPI, MDH2, IDH1, GPI, ACY1, GSR, ME1 and PKM1 were found to be nonsyntenic with the other genes under study. The first assignment of genes to autosomal chromosomes in cattle was made by Dain et al. 1984) using cattle/mouse somatic cell hybrids (Gustavsson et al. 1976). Their study resulted in a provisional assignment of the PEPC gene to bovine chromosome 5, genes LDHB and PEPB to chromosome 19 and perhaps gene SOD1 on chromosome 13. They also indicated that the LDHA gene may be located on one of the small bovine chromosome pairs 23-29. Their finding that genes LDHB and PEPB were syntenic was later confirmed by other studies (Heuertz and Hors-Cayla 1979; 1981). Moreover these two genes are also carried by the same chromosome in sheep (Saidi Mehtar et al. 1981) and in men (Chen et al. 1973). Saidi Mehtar et al. (1981) also showed that the TPI gene was syntenic to the LDHB-PEPB syntenic group as previously identified by Heuertz and Hors-Cayla (1979).

The study by Womack and Moll (1986) using somatic cell hybrids have mapped 28 genes in cattle to 21 of a possible 31 syntenic groups if the Y chromosome was included. The analysis of relationships between loci had resulted in the provisional assignment of eight loci into different syntenic groups (GAPD, ITPA, ADA, ACO1, GDH, BUK, CAT and GLO1). The confirmation that three other loci (ACY1, GSR and NP) are syntenic was also been obtained.

[v] THE *in situ* HYBRIDIZATION TECHNIQUE

This procedure for localizing genes was first developed by Gall and Pardue (1969b) and John et al. (1969). Initially, the technique permitted the localization of repetitive sequences to chromosome regions. Later, it was refined to permit localization of single-copy genes (Harper and Saunders 1981; Gerhard et al. 1981). There were two technical improvements that allowed the cytologically localization of single-copy genes. The first involved obtaining sufficient amounts of pure probe DNA (gene). This has been achieved in many cases by amplification of recombinant plasmids in *E. coli*. The second involved obtaining probe DNA of sufficiently high radioactivity to produce an interpretable signal in a reasonable amount of time. The radioisotope used in the *in situ* hybridization procedure has been tritium which is incorporated into nucleotides. The tritium radioisotope emits low energy beta particles (0.0181 MeV). These beta particles travel less than 1 um through the nuclear track emulsion film exposing silver grains which upon developing of the film results in dark silver grains well approximating their emission source on the chromosome.

The major factors that affect the outcome of *in situ* hybridization experiments, include:

(1) FIXATION OF CHROMOSOMES:

The fixative used in this study is Carnoys' I which is a mixture of methanol and glacial acetic acid (3:1, v:v). It is thought that concentrations of glacial acetic acid greater than 45% degrade DNA and adversely effect chromosomal proteins. The use of acetic acid:

methanol fixatives appear to remove some of the basic proteins which appears to assist in hybridization (Steffersen 1973).

(2) ENZYMATIC PRETREATMENT:

According to Szabo et al. (1975) and Steffensen et al. (1977) RNase is used to remove endogenous RNA so that hybridization efficiency will be improved in either RNA-DNA or DNA-DNA hybridization.

(3) CHROMOSOMAL DNA DENATURATION:

The denaturation of metaphase chromosome preparations can take one of the three forms. These include: (1) the use of 0.07 N sodium hydroxide for thirty seconds to two minutes (Gall and Pardue 1971); (2) 0.2 N HCI, for 10 to 30 minutes at 25 to 37^{o}C; and (3) high temperatures (ie: 60^{o}C) in 2X SSC. The temperature can be dramatically lowered in 2X SSC solutions if mixed with deionized formamide (Steffensen and Wimber 1972). Every 1% formamide in the solution allows for the chromosomal DNA denaturation temperature to be lowered 0.7^{o}C. The chromosome preparation must then be quickly cooled and dehydrated to prevent reannealing of chromosomal DNA (Steffensen and Wimber 1972; Szabo 1974). It is thought by some researchers that the use of acidic conditions during chromosomal DNA denaturations maintains the best chromosomal morphology but results in less efficient probe DNA hybridization (Gall and Pardue 1971; Jones 1972). Singh et al. (1977) found that heat denaturation of chromosomal DNA at 100^{o}C in 0.1X SSC gave excellent probe DNA hybridization, yet resulted

in significant degeneration of chromosome morphology. Once the denaturation of chromosomal DNA has been achieved, it is important to know how complete the process of denaturation has been. Several studies support the suggestion that highly denatured chromosomal DNA is indeed achieved by the above denaturation procedures by placing the preparation under reannealing conditions for five hours before the addition of the probe were found to have the same probe hybridization results as those to which the hybridization-probe mixture was added immediately (Alonso et al. 1974; Szabo et al. 1975, 977; Barbera et al. 1979).

(4) HYBRIDIZATION MIXTURE AND HYBRIDIZATION CONDITIONS:

McConnaughty et al. (1969) was the first to point out some of the advantages of using formamide in the hybridization mixture which include: (1) hybridization can take place at lower temperatures; (2) 50% formamide deactivates RNase such that RNA-DNA hybridization can take place; (3) the percentage formamide can be changed to lower the Tm (melting point) and thus prevent interstrand scissions; (4) high formamide concentrations promote the maintenance of ssDNA in environments closer to room temperature (RT) to make working conditions easy on the researcher. The addition of dextran sulphate to the hybridization mixture has been shown to accelerate the rate of nucleic acid hybridization to immobilized nucleic acids (Wahl et al. 1979). Variables such as temperature during the hybridization process, length of probe DNA and probe concentration can alter the

efficiency of hybridization (Szabo et al. 1977; Singh et al. 1977; Yu et al. 1978). Serious problems with high background hybridization levels can occur when probe DNA concentrations are in excess of 100 ng/ml in the hybridization mixture. The probe length also appears to have an effect on the hybridization rate. Brahic and Haase (1978) studied the hybridization rate for probes from 50-500 bp in length and showed that the shortest 50 bp DNA probe resulted in a 3-fold increase in hybridization rate over the 500 bp probe. Suggestions have also been made by other investigators which may result in the reduction of nonspecific binding of radiolabelled DNA probe in *in situ* hybridization studies. Gerhard et al. (1981) suggested that slides be incubated for a minimum of 5 h. in 10X Denhardt's solution (1X is 0.02% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone) at 60°C to reduce binding of the probe to the glass slide.

Guidelines for the localization of gene loci by *in situ* hybridization have been outlined by Pardue et al. (1987).

[VI] *in situ* HYBRIDIZATION IN CATTLE, SHEEP AND GOATS

Prior to this study, *in situ* hybridization has been used only twice in the Family Bovidae to cytologically map a gene. Fries et al. (1986) tentatively mapped the bovine lymphocyte antigen (BoLA) gene to region q13-23 of chromosome 23 in cattle. Popescu et al. (1988) reported *in situ* hybridization of a 1.2 kb repetitive DNA sequence specific for the Y chromosome.

This study reports the first attempt in gene mapping in the goat; specifically the beta globin gene cluster to a specific chromosome region of the goat genome.

CHAPTER TWO

MATERIALS AND METHODS

[I] LYMPHOCYTE CULTURING AND PREPARATION OF SLIDES FOR STAINING CHROMOSOMES

Blood was drawn on several occasions from goats of the nubian breed by Dr. Randy (J.R.) Killian of Sherwood Park as well as by Dean Treichel of the Valley Zoo. For chromosome preparation, 10 drops of whole blood were added to ten ml of F10 media containing 15% FCS; 1% Penicillin/Streptomycin and 1% glutamine. The lymphocytes were stimulated to grow and divide with 0.1 ml of phytohemagglutinin (PHA)(Nowell 1960). Cultures were incubated in 5% CO_2 at 37^{o}C for 72 h. Then 0.1 ml of colcemid [final conc. 0.05 ug/ml] was added for 30 minutes (Tjio and Levan 1956; Ford and Hamerton 1956). Cultures were then centrifuged at 900-1000 rpm for 8 min. The supernatant was then removed and 10 ml of 0.075 M KCl, prewarmed to 37^{o}C, was added for 10 minutes (Hsu and Pomerat 1953; Hungerford 1965). The cells were centrifuged and fixed for thirty minutes in 3:1 methanol/glacial acetic acid. Cells were again centrifuged at 900 rpm for 8 min and then fixed overnight in 3:1 methanol/glacial acetic acid (Saksela and Moorhead 1962). The following day, cells were centrifuged, supernatant was removed and cells fixed. Enough fresh fixative was then added to the cell pellet to create a milky white suspension. Slides were kept at a 45 degree angle, and two to three drops of cell suspension was applied dropwise to the slide from a height of 15 cm. Slides were then placed cell side up about 5 cm above a 65^{o}C steam

bath to allow greater spreading of the chromosome after the cells containing them has been ruptured.

[II] PROCEDURES USED IN KARYOTYPING

(A) THE TRYPSIN G-BANDING TECHNIQUE

Prepared slides, seven to ten days old, were baked at 65°C overnight. The slides were placed in 70% ETOH for one minute and then in 95% ETOH for two to three minutes to remove any grease present on them. The slides were then rinsed in Gurrs' buffer to remove any excess ethanol to prevent the inhibitory effects of ethanol on trypsin. A mixture of 50 ml Hanks' balanced salt (HBSS) [Gibco] (pH 6.8) and 0.009 gm. of Bactotrypsin [Difco Corp.] was made. Slides were placed in this solution for 20-25 seconds and quickly transferred to a 70% ETOH wash (Wang and Fedoroff 1972). This was then followed by a wash in 95% ETOH. Slides were then rinsed in two changes of phosphate buffer solution (pH 6.8) for 90-150 seconds. Slides were then rinsed in two changes of Gurrs' buffer (pH 6.8) and one change of Gurrs'/H_2O [50:50]. Slides were then air dried and ready for microscopy.

(B) THE BrdU SUBSTITUTED G-BANDING TECHNIQUE

Whole blood was cultured in F10 media containing 0.1 ml PHA and 1% Penicillin/Streptomycin in 5% CO_2 at 37°C for 72 h. After 72 h., 0.2 ml of 5-Bromodeoxyuridine [Sigma B5002] (0.01 gm/ml) was added to a final conc. of 0.001 gm/ml and incubated for 17 h. Cultures were then centrifuged and washed twice in 1X HBSS. The third wash was

carried out in F10 media lacking FCS. Cultures were then centrifuged at 800-900 rpm for 8 min and the supernatant was removed. Cells were then placed in 10 ml of F10 medium containing 15% FCS. Subsequently, 0.1 ml of PHA and 0.1 ml of 10^{-3} M Thymidine and followed by the quick addition of 3.3 ul of 1ug/ml Hoechst 33258 and the medium incubated at $37^{o}C$ and 5% CO_2 for 4 h. 55 mins. Then 0.1 ml of Colcemid (Wellcome) was added for 10 min and the cultures were again centrifuged at 900-1000 rpm for 8 min. The supernatant was then removed and 10 ml of 0.075 M KCl prewarmed to $37^{o}C$ was added for 10 min. The cells were centrifuged, supernatant removed, and fixed in 3:1 methanol/glacial acetic acid overnight at $4^{o}C$. The next day, cells were centrifuged, supernatant again removed and fixed again. The supernatant was then removed and enough fixative was added to create a milky white solution. Slides were made as previously described in lymphocyte culturing and slide preparation and then subjected to the following steps to produce BrdU substituted G-banded chromosomes.

Slides were stained in 1 ug/ml Hoechst 33258/2X SSC solution for 15 min and then flooded with 2X SSC and a coverslip was placed overtop. The slides were then placed on filter paper moistened with 2X SSC and exposed to a lamp containing a super pressure Mercury Phillips SP 500 bulb 7.5 x 10^{6} J/m^{2} for 1 h. at a distance of 2.5 cm. Slides were then rinsed in distilled H_2O, air-dried and stained in a 3% Giemsa/Gurrs' solution for 3 min. This procedure includes some modifications to the procedure of Perry and Wolff (1974).

(C) THE C-BANDING TECHNIQUE

Slides were aged for 7 days prior to C-banding. They were then treated for 45 min - 1 h. with 0.1 N HCl at room temperature. Slides were then rinsed in deionized H_2O and air-dried. This was then followed by incubation of slides in a 5% solution of Barium Hydroxide $Ba(OH)_2$ for 10-20 seconds at 60^0C. The slides were again rinsed in deionized H_2O, air-dried and incubated for 20 min in 2X SSC at 60^0C. This was followed by a third rinse in deionized distilled water. This procedure is a slight modification of the procedure used by Sumner et al. (1972). Slides were then stained in (1:50) Giemsa/Gurrs' solution, dipped in deionized distilled water and air-dried.

(D) THE AMMONIACAL SILVER STAINING PROCEDURE

The ammoniacal silver staining method of Verma and Babu (1981) was used to stain nucleolus organizing regions. The protocol is as follows:

Two drops of solution 1 is placed on the slide. Solution 1 consists of 2 g. Knots gelatin in a mixture of 99 ml double distilled H_2O and 1 ml pure formic acid (pH 3.4). Then four drops of Solution 2 (50% (w/v) silver nitrate solution) was added to the solution on the slide, coverslipped and placed in a plastic dish which is floated in a water bath of 60^0C until development of the slide was complete. Slides were then rinsed in distilled H_2O and counterstained in a 3% Giemsa/Gurrs' buffer solution.

[II] ISOLATION OF THE DNA PROBE AND CONFIRMATION FOR *in situ* HYBRIDIZATION

(A) ISOLATION OF HIGH MOLECULAR WEIGHT GOAT GENOMIC DNA

Blood from a five month old male goat of the nubian breed was collected in tubes containing EDTA by Dr. J.R. Killian. These samples were centrifuged at 700 rpm for ten minutes separating the blood into plasma, buffy coat and red cell layers. The plasma layer was aspirated away while the lymphocyte rich buffy coat layer (approx. 3 ml) was retained and added dropwise to a 35 ml solution of [NH_4Cl:Tris]. This was gently mixed for 10 minutes and then centrifuged at 2000 rpm for 10 minutes. The resultant supernatant was aspirated leaving a white pellet of lymphocytes. 20 ml of saline was used to resuspend this pellet; then centrifuged at 2000 rpm for ten minutes and the supernatant aspirated. This resuspension was again carried out. The cells were then resuspended in 2 ml of high TE. Lymphocytes were then lysed quickly by injecting 2 ml of lysis mixture from a 5 ml syringe with a 16-18 gauge needle. To this solution Proteinase K is added to a final conc. of 100 ug/ml and incubated overnight at 37^{o}C. Next day, an equal volume of TE-saturated phenol was added to the cell lysate mixture and the milky white emulsion was agitated gently for 10 minutes. This was followed by centrifugation at 2000 rpm for 10 minutes which separated the solution into organic and aqueous phases. The upper aqueous phase and the interface were removed and pooled separately using a large bore pipette. The white interface containing trapped DNA was re-extracted several times with low TE saturated phenol. The pooled aqueous layers from the phenol

extractions were then extracted twice with [24:1] chloroform:isoamyl alcohol. Between extractions, the solution was mixed gently for ten minutes. The upper aqueous layers were pooled and RNase A added to a final conc. of 100 ug/ml and incubated overnight at 37°C. The mixture was then reextracted once with phenol. This aqueous layer was again extracted, this time with a 50:50 (phenolchloroform:isoamyl alcohol) solution and then the aqueous layer extracted twice with [24:1] chloroform:isoamyl alcohol. The aqueous phase was then extracted once with diethyl ether. The diethyl ether layer was aspirated and the remaining diethyl ether was evaporated by passing a stream of compressed air over the remaining solution. The mixture was then dialysed against two changes of low TE. To this aqueous layer, a 1/40 volume of 10 M ammonium acetate and subsequently an equal volume of isopropanol was added. The solution was gently mixed until long strands of high molecular weight DNA precipitated out of solution. The strands of DNA were spooled up and washed with 70% ethanol. The DNA was then redissolved in 1 ml of low TE buffer overnight.

(B) AMPLIFICATION AND ISOLATION OF THE PROBE pG16Ec3Bm2

The cloned 1.2 kb EcoR 1-BamH 1 fragment from the 5' non-coding through the first two exons of the goat epsilon IV gene was obtained from Dr. J.B. Lingrel. The characterization and subcloning of this fragment was described by Townes et al. (1984a,b). A loopful of the 50% glycerol solution containing the transformed bacteria was plated on LB agar plates. The next day, bacteria from a single colony were used to inoculate a tube containing 10 ml of incomplete Casamino Acids

media (pH 7.4) containing 50 ug/ml sodium-free ampicillin. This was aerated and incubated overnight at 37°C. The following day the pure culture was used to inoculate 1 L of incomplete Casamino Acids (pH 7.4) containing 50 ug/ml sodium-free ampicillin. The growth of the transformed bacteria was monitored through spectrophotometric readings at the 600 nm wavelength. When the optical density of the solution containing the growing bacterial population reached a value of 0.600: 170 mg of chloramphenicol was added to the culture and incubated for an additional 12-16 h.

Cells were then harvested by centrifugation at 3200 rpm for 30 minutes, supernatant aspirated and cell pellet washed with 20 ml normal saline. Cells were then resuspended in 24 ml SET buffer. This suspension was subsequently treated with 5 ml of 1 mM EDTA 10 mM Tris-HCl (pH 8) and centrifuged at 6,000 rpm at 4°C for 5 min. The supernatant was then decanted and pellets were stored at 4°C at this step. Cells were then suspended in 2 ml of solution containing 25% sucrose 50 mM Tris, 40 mM EDTA (pH 8). To this, 0.5 ml of freshly prepared lysozyme (10 mg/ml) was added and mixed gently. This mixture was then kept on ice for 5 min. To this, 1 ml of 0.5 M EDTA (pH 8) was added and the solution was kept on ice for 10 min. Proteins were then digested by the addition of 0.2 ml of predigested pronase (20 mg/ml) and kept on ice for 5 min. The E coli cells within the mixture were then lysed by the vigorous injection of 8.5 ml lysing buffer (3 ml 10% (v/v) Triton X-100; 12.5 ml 0.5 M EDTA (pH 8); 5 ml 1M Tris (pH 8)) made to 100 ml with distilled water. This mixture was then mixed

every 2-3 minutes by inversion for 15 min and centrifuged at 17,000 rpm for 60 minutes. The supernatant was removed leaving a viscous solution behind. The volume of this solution was measured and 0.92 g/ml cesium chloride (CsCl) ultra-pure reagent was added. To this solution, ethidium bromide was added to a final concentration of 300 ug/ml and mixed. Centrifuge tubes (polyallomer size 5/8" diameter 0.3" capacity 13 ml) for the Beckman Ti80 rotor were filled with this solution and heat sealed. This was then centrifuged at 55,000 rpm for 24 h. The next day, the lower plasmid band was removed and ethidium bromide was extracted from the solution with 3-5 changes of isopropanol (CsCl saturated H_2O). Isopropanol was then removed using ether extraction. The remaining DNA containing solution was then dialyzed against 10 mM Tris-HCl (pH 8) 1 mM EDTA at 4^oC overnight. The solution was then extracted with phenol and chloroform-isoamyl alcohol 2-3 times each. The DNA containing solution was then ether extracted several times. The remaining DNA containing solution was then precipitated by adding 1/10 volume of 4 M ammonium acetate and one volume of isopropanol. This was then left overnight at -20^oC and spun at 17,000 rpm for 1 h. at $+4^oC$, the supernatant was then removed and the pellet dissolved in 200 ul of 5 mM Tris-HCl (pH 8.0) 0.1 mM EDTA.

(C) HIGH-SPEED CsCl CENTRIFUGATION

The cleared lysate method developed by Kunkel et al. (1977) was used to isolate plasmids of sizes up to 20 kb. Gentle lysis of E. coli bacteria allows the release of plasmid, chromosomal DNA and

cellular debris. The use of ultracentrifugation in a CsCl density gradient allows the sedimentation of cell debris and cellular RNA. Much of the chromosomal DNA was also pelleted to the bottom of the tube following centrifugation. The presence of ethidium bromide (EtBr) in the CsCl serves to decrease the buoyant density of linear and nicked DNA as well as assist in the visualization of DNA. Following the butanol extraction of EtBr, the plasmid containing solution was dialyzed overnight to remove CsCl.

(D) ISOLATION OF THE PROBE DNA FRAGMENT

An overnight EcoR 1/BamH 1 double digest was performed on the 10 ug of plasmid DNA at 37^{o}C. The digest was then electrophoresed on 0.8% agarose gel (Seakem-LE) resulting in DNA fragments represented by a 1.2 kb (DNA insert) and a 4.0 kb band (plasmid DNA fragment). The band of the gel containing the desired 1.2 kb DNA insert was then excised from the gel and the DNA extracted from the agarose gel by electroelution. Again EtBr was removed and the DNA fragment was precipitated. An analytical agarose gel was carried out to determine the purity of the fragment isolated. A total of 90 ng DNA was used to tritium-label the DNA fragment in the in situ hybridization procedure.

(E) RADIOACTIVE LABELLING OF THE DNA PROBE

The DNA fragment was radiolabelled with ^{3}H-dCTP for in situ hybridization and alpha [^{32}P] dTTP (3,000 Ci/mmol) for Southern hybridization according to the procedure of Feinberg and Vogelstein (1983; 1984). The DNA probe was labelled to a specific activity of

($3.3x10^8$ cpm/ug) for in situ hybridization and $2.5x10^8$ cpm/ug for Southern hybridization. For the in situ hybridization procedure approximately $1x10^5$ cpm/slide was added.

(F) PHOTOGRAPHY OF GOAT GENOMIC DNA AGAROSE GEL

Gels were photographed using Kodak type 57 film (3000 ASA) with an F-stop of 4.5 and exposure of one second. The DNA was made visible using longwave UV transillumination.

(G) GEL TREATMENT IN PREPARATION FOR SOUTHERN TRANSFER

Agarose gel DNA was depurinated in 0.25 N HCl solution twice for periods of 15 min each then denatured twice in 0.5 N NaOH-1M NaCl solution each for 15 min. The alkaline condition created was then neutralized in two washes of 0.4 M Tris-0.3 M NaCl (pH 7.5), each for 15 min.

(H) SOUTHERN TRANSFER AND HYBRIDIZATION

Southern transfer was carried out according to the procedure of Southern (1975) except that Gene Screen Plus (Du Pont) was substituted for nitrocellulose as a filter membrane. The filter was then baked at 80°C for 2 h. The filter was then placed in a Seal-A-Meal bag to which 30 ml of pretreatment solution (Denhardt 1966) was added. The minimum amount of prehybridization solution used was > 0.2 ml/cm^2 of filter membrane to minimize nonspecific binding of the DNA probe. The bag was then sealed, being careful not to leave any bubbles, and incubated at 65°C for 15 h. The prehybridization mixture was then

removed and replaced with 20 ml of hybridization mixture containing the denatured ^{32}P labelled probe ($2.0x10^5$ cpm/ml hybridization mix). The minimum amount of hybridization mixture used was suggested at > 70 ul/cm^2 of filter membrane. This was then incubated at 70°C for 15 h. The hybridization mixture differs from the prehybridization mixture in that it contains dextran sulphate and the denatured labelled probe. Dextran sulphate serves to greatly increase filter hybridization kinetics (Wahl et al. 1979; Jeffreys et al. 1980). The hybridization and washing was carried out in 1X SSC at 70°C. Under these conditions at least 87% homology should be present for hybridization to take place with the radiolabelled probe. Perfectly matched hybrids only begin to melt from the filter when washed in 0.1X SSC at 65°C for an extended period of time until background was minimized.

The filters were then washed under gentle agitation in two washes of each: 2X SET-0.1% SDS; 1X SET-0.1% SDS; 0.4X SET-0.1% SDS; 0.1X SET-0.1% SDS for 15 min/wash at 65°C. The last wash was extended until background hybridization was reduced to the minimum level.

(I) AUTORADIOGRAPHY

Filters were placed in autoradiographic cassettes with Kodak XR-5 autoradiographic film and a fast tungstate intensifier at -80°C for 12 h.

[IV] In situ HYBRIDIZATION: PREPARATION OF SLIDES AND LOCALIZATION OF THE BETA GLOBIN GENE CLUSTER

(A) PREPARATION OF SLIDES FOR in situ HYBRIDIZATION

The method of Viegas-Pequigot and Dutrillaux (1981) was used to obtain high resolution BrdU G-banding of goat chromosomes. Slides were prepared by the indicated in the BrdU substituted G-banding technique.

(B) CHROMOSOME IDENTIFICATION

BrdU G-banded chromosome preparations were obtained using the technique developed by Perry and Wolff (1974). Slides were stained in freshly prepared Hoechst 33258 (Sigma) solution (1 ug/ml in 2X SSC) for 15 min. They were then flooded with 2X SSC and placed under a long-wave UV lamp (Sylvania GTE, black light blue, 15W) for 1 h. at a distance of 2.5 cm rinsed in H_2O and air dried. Each slide was then flooded with 1 ml 0.25% Wright's stain in 3 ml phosphate buffer for 3 min and air dried. Photographs were then taken of the metaphase chromosome spreads using the Zeiss Photomicroscope II with Zeiss Epiplan 80/95 Pol dry lens (oil immersion need not be used).

(C) PROBE LABELLING

The 1.2 kb EcoR I/BamH I genomic DNA fragment containing the 5' noncoding region through the first two exons of the epsilon IV gene was tritium-labelled using ^{3}H-CTP according to the random primer method of Feinberg and Vogelstein (1983, 1984).

(D) PREPARATION OF PROBE-10% DEXTRAN SULFATE HYBRIDIZATION MIXTURE

A 10 ml hybridization solution (1.25X) was prepared containing 6.35 ml of 100% formamide (deionized, pH 7.2 MCB), 0.625 ml of 1M Hepes (pH 7.0, Na^+), 0.125 ml of Denhardt's, 1.875 ml of 20X SSC (1X SSC = 0.15 M Sodium Chloride and 0.015 M Sodium Citrate); 0.525 ml of denatured E coli DNA (10 mg/ml in H_2O); 0.188 ml of yeast RNA (10 mg/ml) in high TE and 0.312 ml of H_2O. A 10% dextran sulfate hybridization mixture was then obtained by adding 1.0 ml of the 50% dextran sulfate (in H_2O) to 4 ml of the 1.25X hybridization solution. The denatured radiolabelled DNA at a conc. of 25 ng/ml was quickly added to the 10% dextran sulphate - 1.25X hybridization solution and placed in an ethanol-ice bath until used in the in situ hybridization procedure.

(E) In situ HYBRIDIZATION

In situ hybridization was carried out using a modification of the method of Harper and Saunders (1981) and Zabel et al. (1983). Slides that were approximately three months old were treated with 200 ul of RNase/slide (100 ug/ml in 2X SCC, Worthington), covered with coverslips and placed in a moist chamber for 1 h. at 37°C. They were washed in four changes of 2X SSC at RT, 2 min each; and dehydrated in 70%, 80%, 90% and absolute ethanol solutions for 2 min each. The slides were then denatured in 70% ethanol baths of 70%, 80%, 90% and absolute ethanol for 2 minutes respectively and air dried. DNA hybridization began with the layering of 100 ul of denatured probe

hybridization sulphate mixture onto prepared slides. This mixture contains 2.5 ng of tritium-labelled DNA with an approximate level of radioactivity of $3X10^5$ cpm/slide. Slides were then covered with a coverslip and incubated in a moist chamber at 37°C for 12-16 h. The slides were then washed in five changes of 50% formamide-1X SSC at 41°C each for 2 min, followed by five changes in 2X SSC at 40°C for 2 min each. Lastly, slides were washed in three changes of 1X SSC for a total of 1 min at 40°C and subsequently dehydrated in 70%, 80%, 90% and absolute ethanol, each for two minutes at RT.

(F) AUTORADIOGRAPHY

Slides were dipped in Kodak nuclear emulsion (diluted 1:1 with H_2O, at 45°C) and dried in a light tight box for 2 to 3 h. They were then transferred into light tight boxes containing drierite and kept at 4°C for 9 days. The slides were then developed in Kodak Dektol developer, diluted 1:1 with H_2O for 2 min at 15°C (gently agitated throughout), dipped in H_2O, and transferred to Kodak fixer for 5 min. Following fixation, slides were rinsed in running water for 10-20 minutes and dried quickly using a fan. Slides were then stained for 10 min as previously described in the BrdU substituted G-banding procedure and air dried.

(G) SCORING OF SLIDES FOR THE NUMBER AND LOCATION OF GRAINS

Good metaphase chromosome spreads that had been previously photographed following BrdU G-banding were located following in situ hybridization and BrdU G-banded once again. Those metaphase spreads

that were found to have silver grains on or closely associated with their chromosomes were considered informative and photographed again. The information gained from these metaphase spreads were compiled into a histogram (Figure 14) and presented in the following Results section.

CHAPTER THREE

RESULTS

[I] STUDY OF THE NUMBER, MORPHOLOGY AND BANDING CHARACTERISTICS OF GOAT CHROMOSOMES

(A) THE UNIFORMLY GIEMSA STAINED GOAT KARYOTYPE

Chromosome spreads were stained in a uniform fashion with Giemsa. A standard karyotype was obtained from metaphase chromosome spreads and presented in Figure 5. Chromosomes in the karyotype were arranged in a standard manner as previously described. This figure shows that the somatic chromosome complement of the goat consists of 60 chromosomes. This includes 29 pairs of acrocentric autosomes as well as two sex chromosomes: an X chromosome and a small submetacentric Y chromosome in males (Figure 5b) and two X chromosomes in the female. Photographs of uniformly stained metaphase spreads were also used to measure the length of chromosomes. Chromosome pairs were identified using the trypsin G-banding procedure which will be discussed later. The results of these measurements are given in Table 1. The length of goat metaphase chromosomes decreased fro 7 um for the longest autosome to 2 um for the shortest autosome in the female goat studied. The total length of the metaphase chromosomes in one goat genome is approximately 120 um. The estimated length of the goat X chromosome which is the third longest chromosome in the complement was 5.9 um. This chromosome represents approximately 4.9% of the total goat genome length.

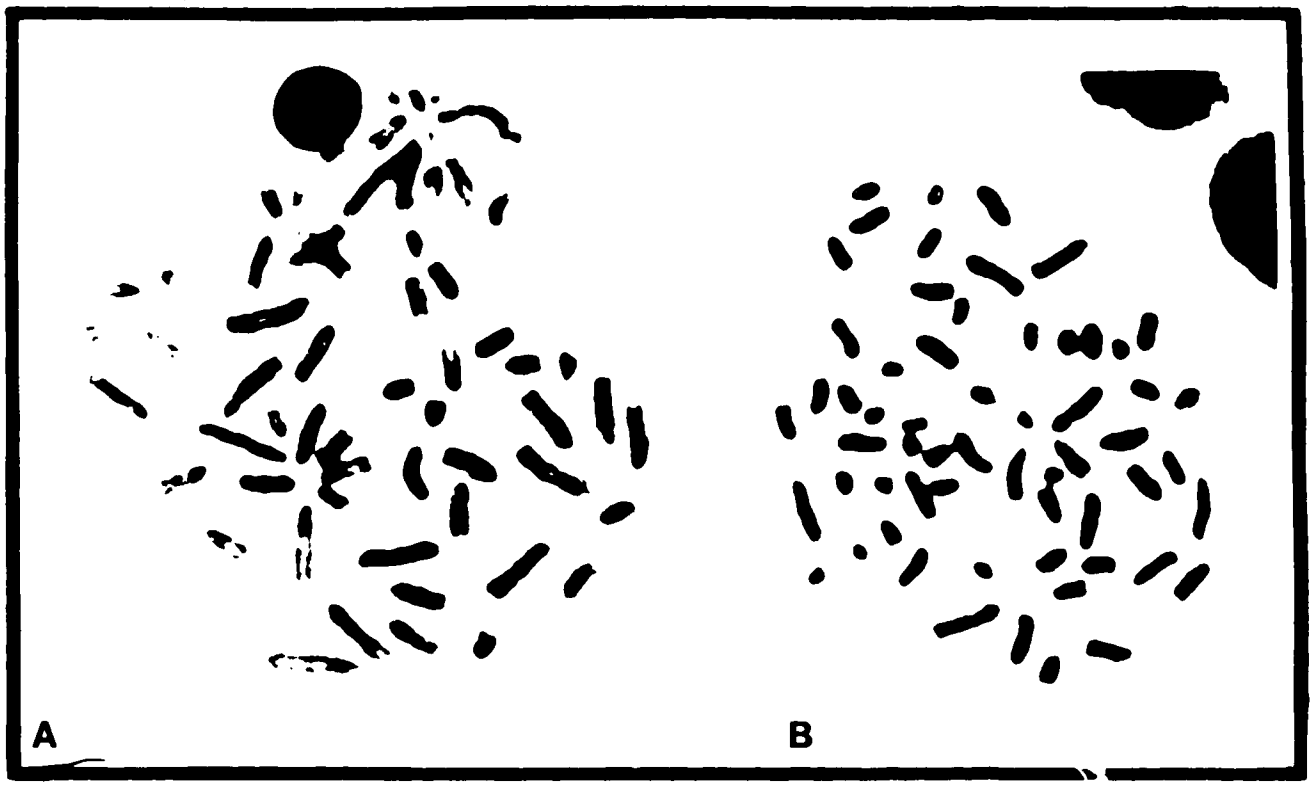
FIGURE 5. Uniform Giemsa staining of male and female goat metaphase chromosomes. Magnification approx. 2800x.

(A) Uniformly stained metaphase chromosome spread of female goat (Capra hircus).

(B) Uniformly stained metaphase chromosome spread of male goat (Capra hircus).

(C) Uniformly stained karyotype of the female goat (Capra hircus). Arranged from (A).

(D) Uniformly stained karyotype of the male goat (Capra hircus). Arranged from (B).


A
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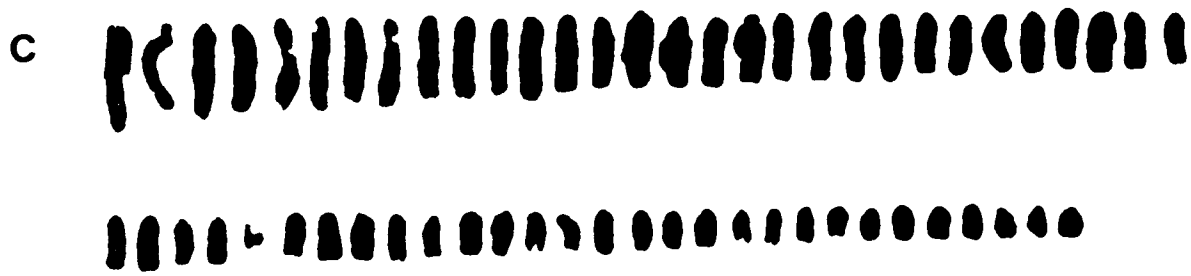
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FEMALE

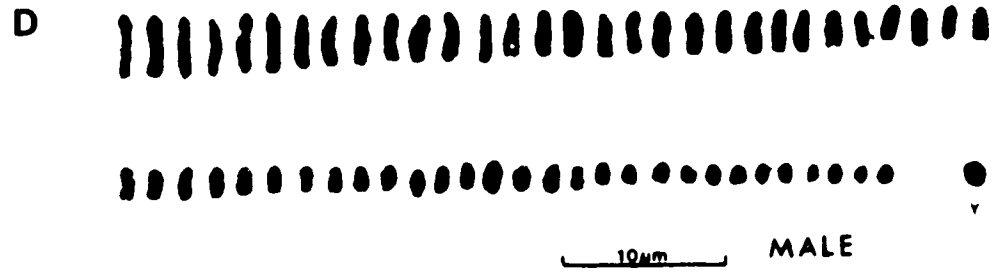

D
Y
10µm
MALE

TABLE 1. Chromosome Measurements of Female Caprine Prometaphase-Metaphase Chromosomes.

Photographs were taken of metaphase chromosome spreads. Following printing, this resulted in an image amplification of approximately 2800X. Homologues of each chromosome pair spread were measured from the print and an average was taken. This was done for ten chromosome spreads and the overall average is recorded in the column ($um. \times 10^6$).

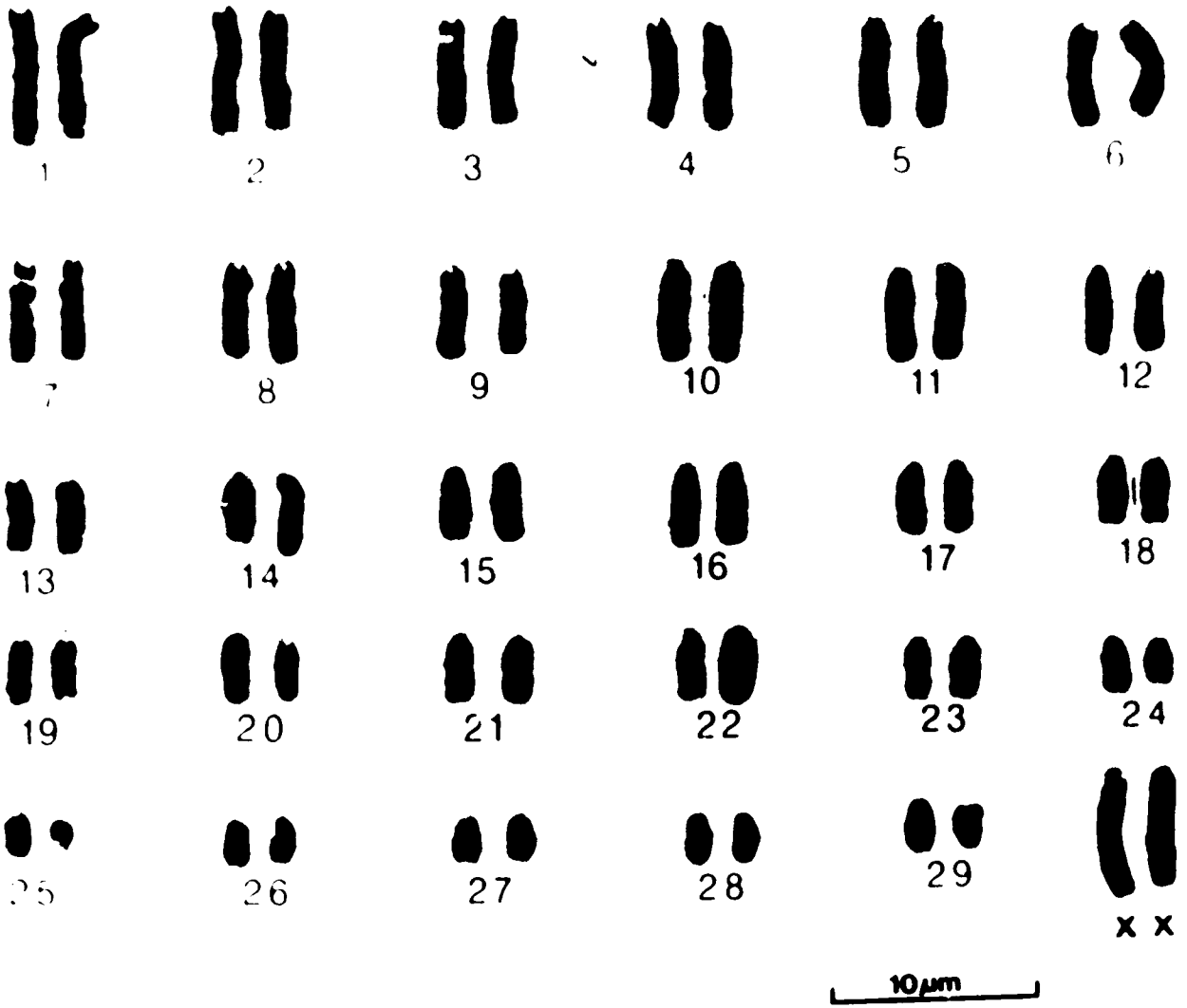
CHROMOSOME	COLUMN 1 ESTIMATED LENGTH FROM PHOTOGRAPH (um.) 1 x 10^6	COLUMN 2 STANDARD DEVIATION	COLUMN 3 APPROX. LENGTH OF CHROMOSOME (um.)	COLUMN 4 STANDARD DEVIATION
1	1.93	1.93 +/- .24	6.9	6.9 +/- 0.93
2	1.79	1.79 +/- .20	6.4	6.4 +/- 0.78
3	1.63	1.63 +/- .19	5.8	5.8 +/- 0.76
4	1.56	1.56 +/- .20	5.6	5.6 +/- 0.75
5	1.57	1.57 +/- .20	5.6	5.6 +/- 0.75
6	1.48	1.48 +/- .18	5.3	5.3 +/- 0.68
7	1.46	1.46 +/- .17	5.2	5.2 +/- 0.71
8	1.39	1.39 +/- .19	4.9	4.9 +/- 0.76
9	1.33	1.33 +/- .15	4.7	4.7 +/- 0.65
10	1.37	1.37 +/- .14	4.9	4.9 +/- 0.53
11	1.31	1.31 +/- .12	4.6	4.6 +/- 0.54
12	1.20	1.20 +/- .13	4.3	4.3 +/- 0.49
13	1.22	1.22 +/- .14	4.3	4.3 +/- 0.60
14	1.17	1.17 +/- .12	4.2	4.2 +/- 0.47
15	1.13	1.13 +/- .13	4.0	4.0 +/- 0.52
16	1.09	1.09 +/- .11	3.9	3.9 +/- 0.14
17	1.03	1.03 +/- .09	3.6	3.6 +/- 0.44
18	1.04	1.04 +/- .08	3.7	3.7 +/- 0.35
19	1.01	1.01 +/- .07	3.6	3.6 +/- 0.32
20	0.97	0.97 +/- .10	3.4	3.4 +/- 0.48
21	0.96	0.96 +/- .10	3.4	3.4 +/- 0.38

CHROMOSOME	COLUMN 1 ESTIMATED LENGTH FROM PHOTOGRAPH (um.) 1 x 10^6	COLUMN 2 STANDARD DEVIATION	COLUMN 3 APPROX. LENGTH OF CHROMOSOME (um.)	COLUMN 4 STANDARD DEVIATION
22	0.87	0.87 +/- .09	3.1	3.1 +/- 0.38
23	0.78	0.78 +/- .08	2.8	2.8 +/- 0.30
24	0.77	0.77 +/- .08	2.7	2.7 +/- 0.33
25	0.76	0.76 +/- .08	2.7	2.7 +/- 0.33
26	0.68	0.68 +/- .09	2.4	2.4 +/- 0.37
27	0.65	0.65 +/- .01	2.3	2.3 +/- 0.41
28	0.61	0.61 +/- .07	2.1	2.1 +/- 0.33
29	0.55	0.55 +/- .07	2.0	2.0 +/- 0.25

(B) THE TRYPSIN GIEMSA AND THE BrdU G-Banded GOAT KARYOTYPE

The trypsin Giemsa banding pattern of the goat chromosomes (Figure 6) shows a great deal of similarity to the chromosomes of cattle (Lin et al. 1977) and other bovine and caprine species. The cattle karyotype of Lin et al. (1977) as well as the recently published reverse banded karyotype of Di Berardino et al. (1987) were used as a basis to form the karyotype of Figure 6 because their quality was much better than previously published G-banded goat karyotypes (Evans et al. 1973; Hageltorn and Gustavsson 1974; Schnedl and Czaker 1974).

Several observations were made regarding the G-banded chromosomes. Firstly, near centromeric regions of all chromosomes except the X were G-band positive. Secondly, smaller chromosomes, particularly chromosomes 23 through 29 were difficult to identify due to the few bands they possess. Figure 6 displayed chromosomes which show a low degree of contrast between G-positive and G-negative band regions. The BrdU substituted G-banding technique was also used to identify chromosomes and later used for the in situ hybridization procedure. The BrdU G-banded goat karyotype is presented in Figure 7. An attempt has been made to identify the chromosome regions of each chromosome pair and a diagrammatic representation of each pair is shown in Figure 7. This is followed by a detailed description of the BrdU G-banding pattern of the chromosomes (Table 2). In general, the chromosome banding patterns obtained through the trypsin G-banding and the BrdU G-banding technique are quite similar. A sample of BrdU G-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X X
10µm

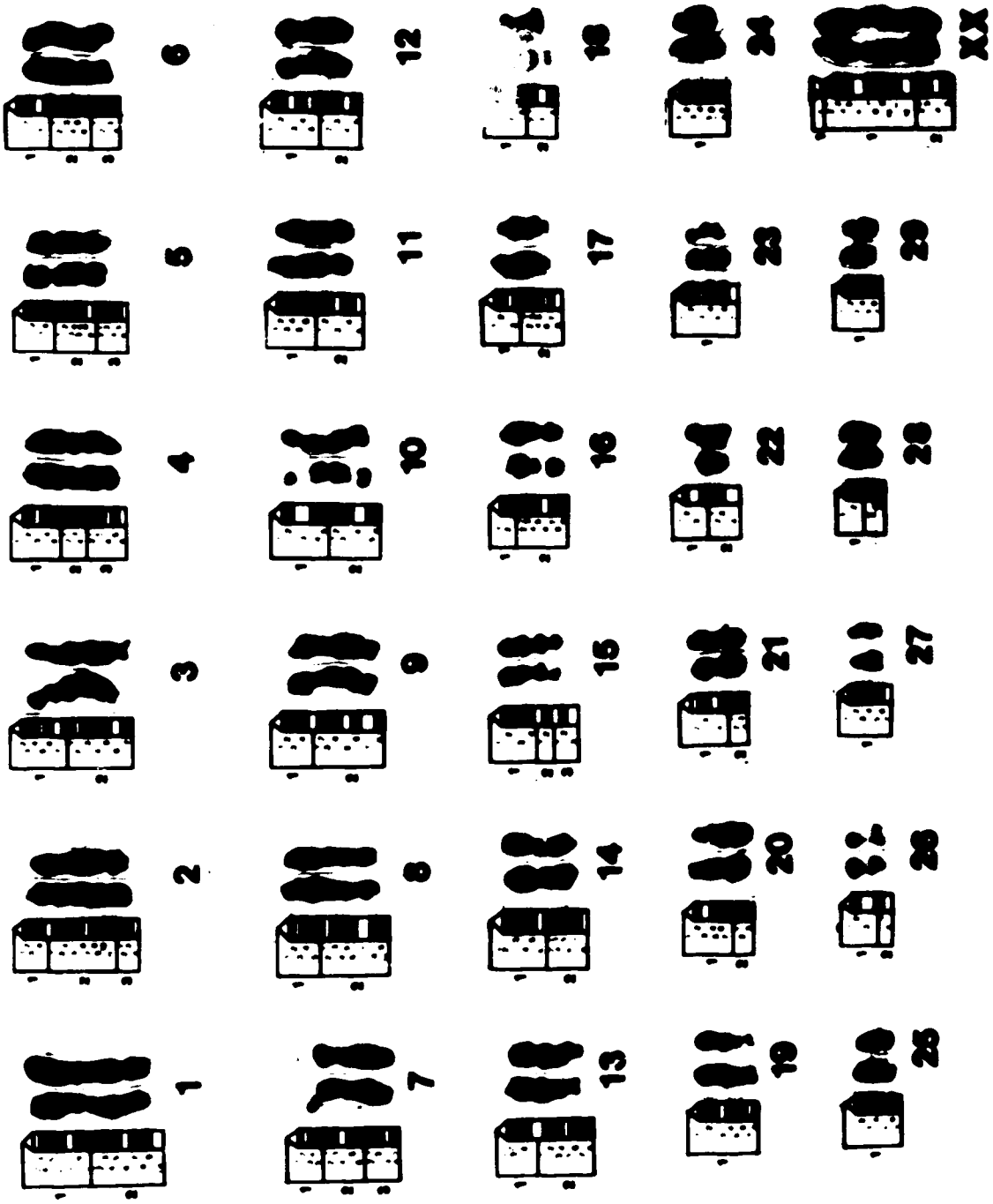


TABLE II. Description of the BrdU G-Banding patterns of the long arms of goat chromosomes. Bands were not observed in the short arms.

Chromosome:	
1	This chromosome has a narrow light positive band in a large negative band in the central region of the chromosome. Two dark bands are distinguishable in the distal region.
2	Two distinct negative banding regions occur on the chromosome. One being the same distance from the centromere as the other is from the distal region.
3	This chromosome is easily distinguished by having a large negative band in the proximal 1/3 except for its' interruption by one light positive band. There is also a large positive band near the center of the chromosome followed by a small negative band. There is also a large distinct light terminal band.
4	This chromosome has a symmetrical banding pattern between proximal and distal portions of this. There is also a distinct positive band flanked by two negative bands.
5	Similar to chromosome 2; except for the central area being smaller and there being a distinct negative band in the terminal region.
6	Generally a darkly stained chromosome with the distinct landmark band being a very broad dark band about 2/3 of the way down the chromosome. This band is surrounded by a darker region.
7	The proximal 1/3 of the chromosome distinguishes it from most other chromosomes. The region near the centromere contains a distinct positive band region 2.1 which is followed by another negative band region.
8	This chromosome is easily distinguished by the distal 2/3 of the chromosome is fairly lightly staining with a dark band near the terminus.
9	This chromosome contains a large negative band (2.2) which is followed by three bands of approximately equal size including a terminal band 2.6 which helps in the identification of this chromosome.

10 This chromosome has large negative bands occurring on both sides of two dark distinct positive bands. There is also a characteristic dark terminal band.

11 Four bands of various widths are distributed nearly evenly throughout the chromosome with 2.2 being a landmark band.

12 The banding pattern of this chromosome is similar to that of chromosome 9, but can be distinguished from the latter by the absence of a large separate and distinct terminal band.

13 This chromosome has two dark proximal bands. The distal third is lightly staining which is a distinguishing characteristic of this chromosome.

14 There are two broad positive proximal bands near the centromer of this chromosome followed by a broad negative band region and two large dark terminal bands near the terminus which aid in chromosome identification.

15 This chromosome is easily divided into the dark staining proximal half and a lightly staining distal half. A broad dark band bordering the centromere is accomplished by a second dark band 1.4 in the proximal half of the chromosome. The distal half of the chromosome is considerably lighter staining with two lighter positive bands 2.2 and 3.2 demarcating the rest of the chromosome.

16 There is a broad dark band bordering the centromere of this chromosome which has a distinctive light band at 2.5. Two near terminal bands 2.7 and 2.9 are separated by a large negative band 2.6.

17 This chromosome possesses one dark proximal band with three central bands at 2.1, 2.3 and 2.5.

18 This is a symmetrically banded chromosome. It is easily distinguished by a centrally located dark band 2.1 bordered by two lighter bands 1.4 and 2.2. Terminally there is a narrow light positive band.

19 This chromosome is characterized by a large band bordering the centromere. It also has a landmark band 1.4 in an area that is distinctive for its general light staining.

20 Bands 2.1 and 2.3 give this chromosome the appearance of having a single large terminal positive band. The proximal 2/3 of the chromosome is fairly light staining with a dark band bordering the centromere.

21 There are two well-defined positive bands in the distal half of the chromosome. There is a large positive band 1.3 near the centromere, and a centrally located negative band area 1.4.

22 The identifying landmark of this chromosome are two dark centrally located bands 2.1 and 2.3 which can easily merge.

23 This chromosome possesses a large positive band bordering the centromere with two clearly defined dark bands.

24 This chromosome is distinguished by the broad dark bands 1.4 and 1.6 which sometimes merge together. There is also a narrow negative band near the terminus of these chromosomes.

25 Two positive proximal bands and a positive band near the terminus characterize this chromosome.

26 This chromosome possesses a positive band bordering the centromere. The rest of the chromosome is lightly staining except for a broad light band near the terminus.

27 A positive proximal band and a large positive central band distinguish this chromosome from others. The chromosome is morphologically similar to chromosome 26, except the distance between the two bands is much shorter.

28 A uniformly dark staining chromosome with a dark band bordering the centromere and a dark terminal band.

29 This chromosome has a positive proximal band near the centromere. Chromosome 29 has a similar banding pattern to chromosome 26.

X The long arm of this chromosome has a large negative band area at the distal border of the proximal 1/3 of the chromosome. Distally the chromosome is divided by six bands. After the first three bands there is another negative band that can be easily seen. The late replicating X is easily recognized by the lack of definition between positive and negative banding regions.

banding and the variation in the banding pattern and quality is shown in Figure 8.

(C) THE C-BANDED GOAT KARYOTYPE

The constitutive heterochromatic regions of goat chromosome were identified by the C-banding technique. A C-banded karyotype of the species is shown in Figure 9. The centromeric regions of nearly all chromosomes in this species appear to possess constitutive heterochromatin. The arms of some of the chromosomes also appear to possess such chromatin. This was not a consistent finding. The presence of C-bands on chromosome arms depends on the length of treatment of the metaphase chromosome spreads to $Ba(OH)_2$. The shorter the treatment the more chance of C-bands. The X chromosome (identified on the basis of chromosome length) appears to contain very little centromeric heterochromatin, while the Y chromosome centromeric area appears to contain a very large amount of centromeric heterochromatin.

(D) THE AMMONIACAL SILVER STAINING OF GOAT CHROMOSOMES NOR's

The NOR's of the goat appear to occur terminally on the long arms of the chromosomes that have NOR's in cattle and sheep (Figure 10). Some goat metaphase spreads have shown the association of 2 to 3 NOR bearing chromosomes which could indicate that these chromosomes were closely associated with the nucleolus during the previous metaphase. The modal number of NOR bearing chromosomes in the three goats studied was 7 based on a study of 111 cells at metaphase, 37 metaphase spreads from each goat. This indicates that at least four

FIGURE 8. The BrdU G-banding pattern of goat chromosome 7s' with BrdU idiogram of the chromosome on the left.

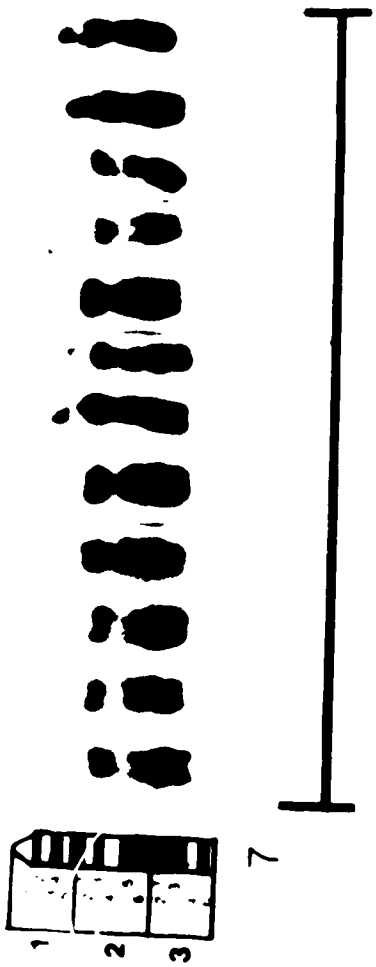

7
1
2
3

FIGURE 9. C-banded karyotype of the male goat (Capra hircus, 2n=60, XY). Magnification Approx. 2800x.

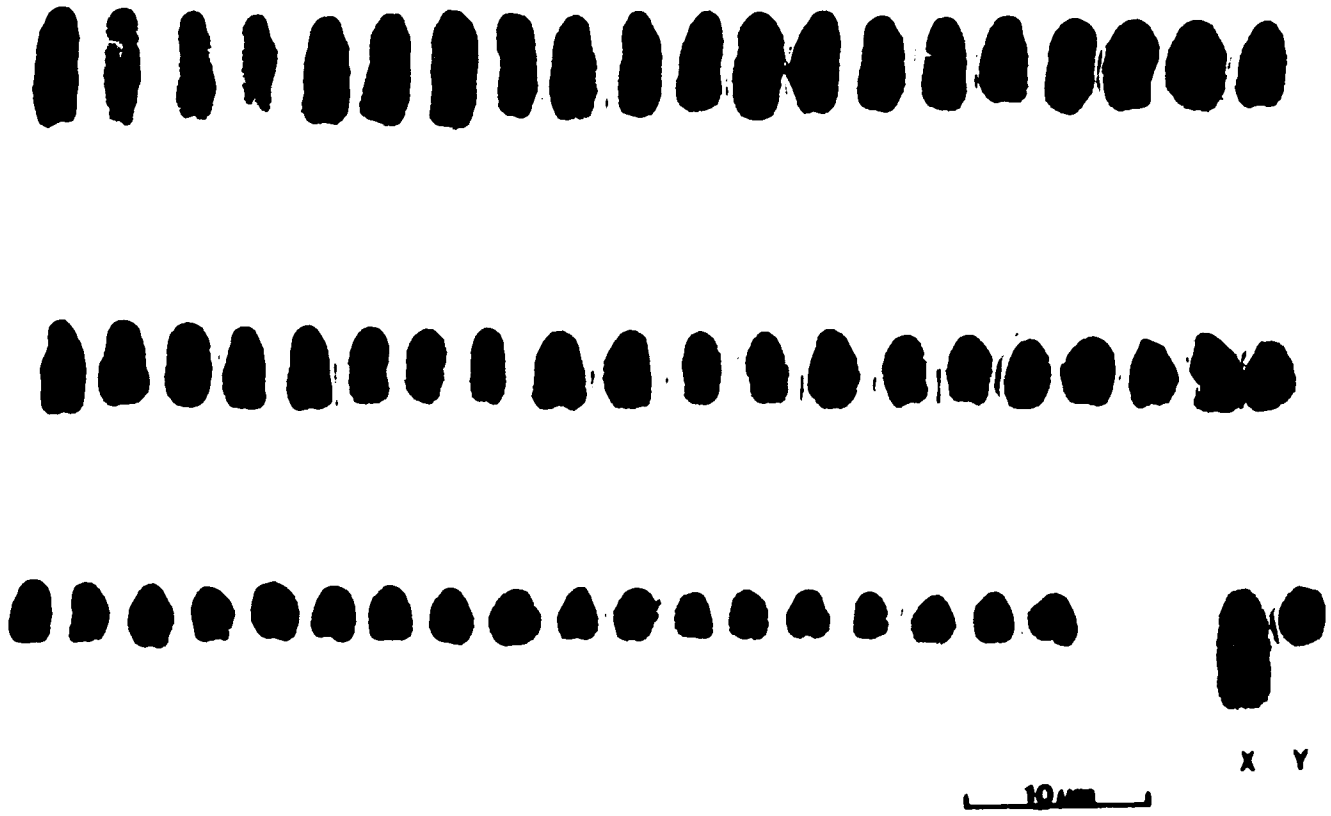
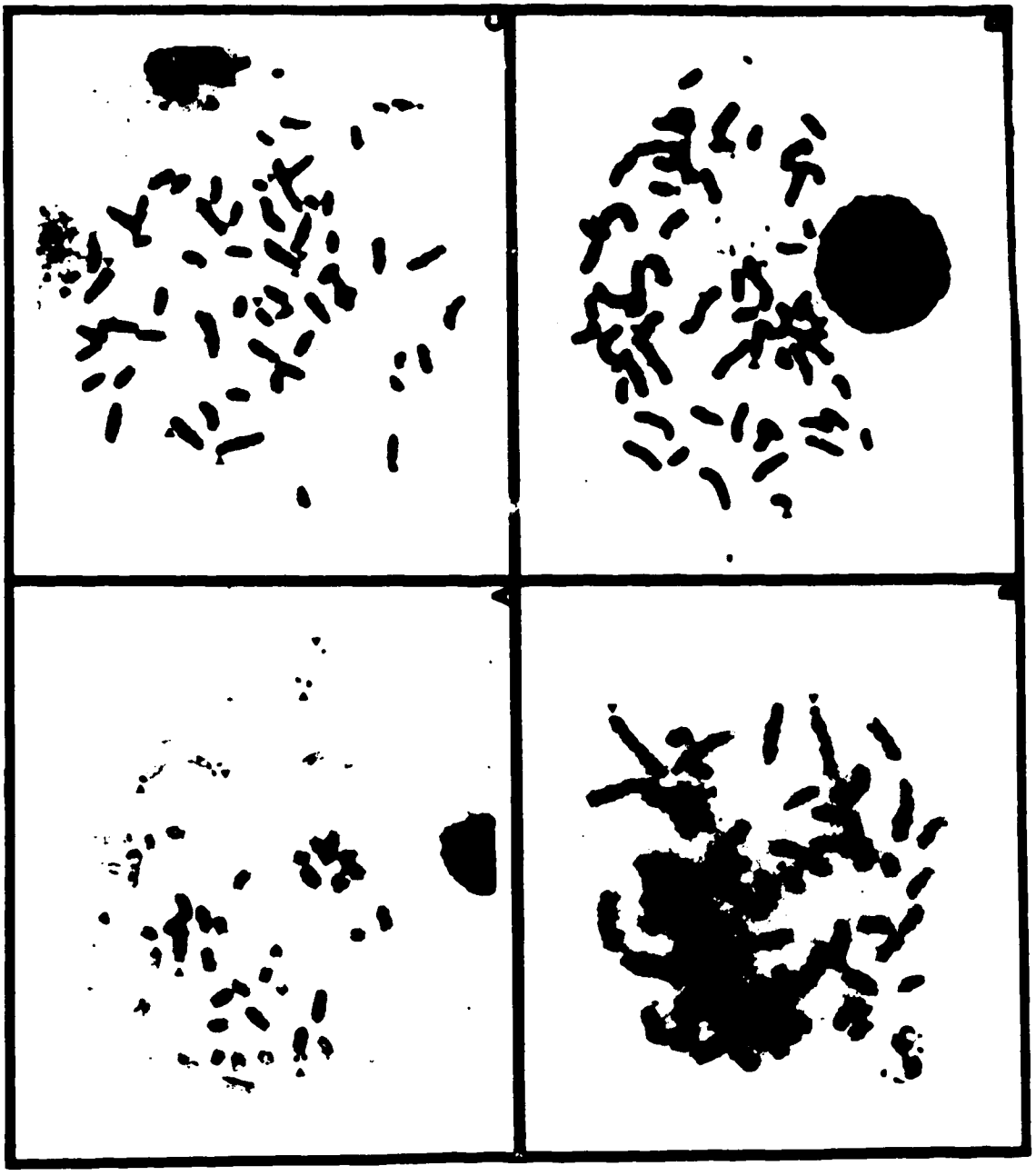

X Y
10 μm

FIGURE 10. Ammoniacal silver staining of chromosomes at metaphase in male and female goats (Capra hircus). Magnification Approx. 2800x.

(A) and (B) Metaphase chromosome spreads of the male goat showing seven and six chromosomes respectively with silver staining.

(C) and (D) Metaphase chromosome spreads of the female goat showing seven and six chromosomes respectively with silver staining.



chromosome pairs (or maximum 8 chromosomes) were associated with active NOR's during the previous interphase. Identification of which chromosomes contain the NOR's was not possible because sequential banding and photography of the metaphase spreads was not carried out in this case.

[III] SOUTHERN TRANSFER AND HYBRIDIZATION OF EPSILON IV DNA PROBE TO GOAT GENOMIC DNA

The Southern transfer and hybridization procedure confirmed that the male possesses a goat genomic DNA fragment carrying the epsilon IV gene as the probe. The initial restriction enzyme digestions (EcoR I, EcoR I/BamH I, Pst I and Sst I) of this probe resulted in the characteristic smear of DNA following agarose gel electrophoresis and EtBr staining of the gel (Figure 11). Following transfer to the "Gene Screen Plus" membrane, the filter membrane was hybridized with the ^{32}P radiolabelled epsilon IV probe DNA fragment. Two hybridization bands in the form of a doublet were obtained with an estimated length of 2.5-2.6 kb from the EcoR I goat genomic DNA digest (Figure 12 Lane 2). A 1.2 kb band and a 6.3 kb band were obtained from an EcoR I/BamH I digest (Figure 12 Lane 3). The 1.2 kb band represents that DNA fragment which was initially derived by Townes et al. (1984b) and inserted into pBR322. This DNA fragment was used as the probe in the Southern and <u>in situ</u> hybridizations. The plasmid which contains the inserted epsilon IV DNA fragment was shown previously in Figure 4. Three major bands (4.5 kb., 8.5 kb. and 18 kb.) were obtained from the Pst I digestion of genomic DNA (Figure 12 Lane 4) other bands of

FIGURE 11. Restriction enzyme digested goat genomic DNA electrophoresed on agarose gel and stained with ethidium bromide.

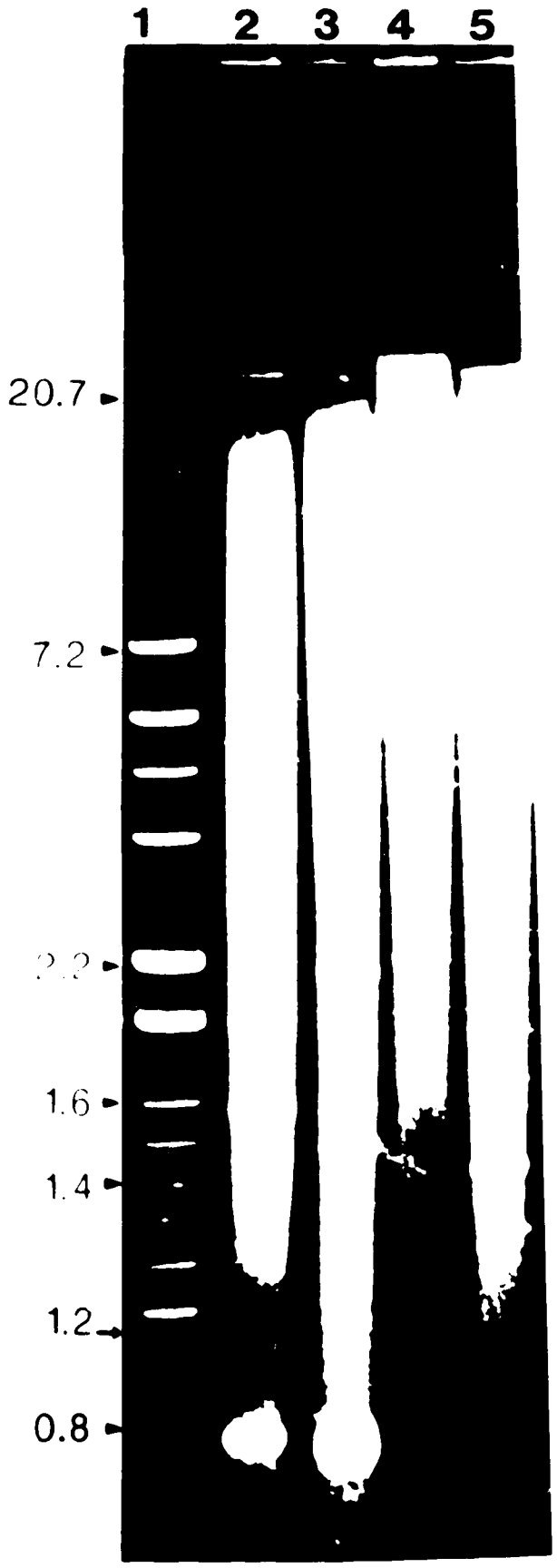
Lane 1: EcoR I and Hind II/Hind III digested lambda phage used as a size marker.

Lane 2: EcoR I digested male goat genomic DNA.

Lane 3: EcoR I/BamH I double digested male goat genomic DNA.

Lane 4: Pst I digested male goat genomic DNA.

Lane 5: Sst I digested male goat genomic DNA.


1
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3
4
5
20.7
7.2
2.3
1.6
1.4
1.2
0.8

FIGURE 12. Southern hybridization of goat genomic DNA fragment carrying a portion of the epsilon IV gene to digested goat genomic DNA.

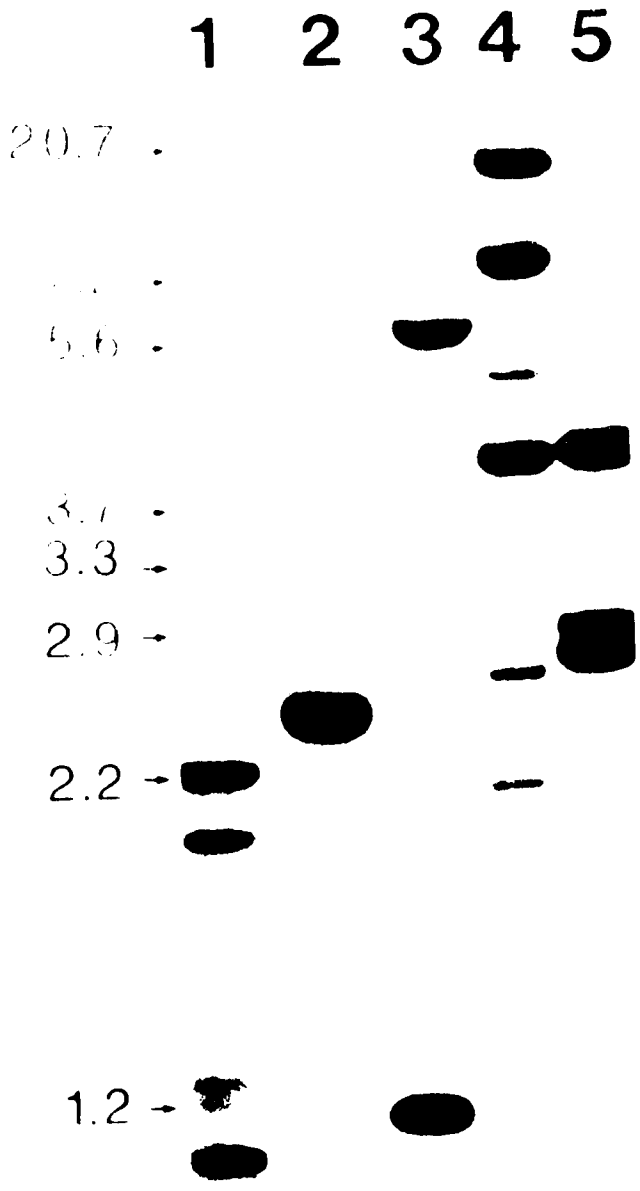
Lane 1: EcoR I and Hind II/III digested lambda phage DNA.

Lane 2: EcoR I digested goat genomic DNA.

Lane 3: EcoR I/BamH I double digested goat genomic DNA.

Lane 4: Pst I digested male goat genomic DNA.

Lane 5: Sst I digested male goat genomic DNA.


1 2 3 4 5
20.7
5.6
3.3
2.9
2.2
1.2

lesser intensity (Figure 12 Lanes 4 & 5) represent portions of other genes within the cluster which have a high degree of sequence homology due to the triplication of the ancestral block of four genes which helped to form the gene cluster. A total of three bands from a Sst I digest a doublet of 2.8-2.9 kb as well as a band of approximately 4.5 kb (Figure 12 Lane 5). The results obtained from Figure 12 are compared with those of Townes et al. (1984b) and presented in Table III. Only the EcoR I and EcoR I/BamH I double digests can truly be compared with those of Townes et al (1984b). The Pst I and Sst I digests show new restriction enzyme sites of the goat beta globin gene cluster. They indicate that some differences may exist between individual goats in the case of the EcoR I digest while the EcoR I/ BamH I double digest resulted in a 6.0 kb band which occurred as a result of partial digestion of some of the goat genomic DNa in the double digest. The 1.2 kb band in this double digest served to confirm that the probe sent by Dr. J.B. Lingrel was actually the 1.2 kb EcoR I/BamH I fragment of the goat epsilon IV gene.

[III] THE CHROMOSOME LOCALIZATION OF THE GOAT BETA GLOBIN GENE CLUSTER BY *in situ* HYBRIDIZATION

To facilitate chromosomal mapping of the goat beta globin gene a goat karyotype was prepared using the BrdU substituted G-banding (GbG-banding) procedure (Perry and Wolff 1974). This technique appears to show a more defined banding pattern then the trypsin G-banding procedure especially after *in situ* hybridization. It also served to assist in the detection of the X chromosome in the female goat. This

TABLE III. Comparison of the restriction enzyme fragments obtained with previously published work.

RESTRICTION ENZYME DIGEST.	# OF BANDS	*APPROX. LENGTH (kb.)	APPROX. EXPERIMENTAL LENGTH (kb.)
(1) MARKER DNA			
(2) EcoR I	2	1.2	2.3; 2.5
(3) EcoR I/BamH I	2	1.2; 14.5	1.2; 6.0
(4) Sst I	3	--	6.0; 9.5; 18
(5) Pst I	3	--	2.8; 2.9; 6.0

*Approx. Length of restriction enzyme fragments of the beta globin gene cluster from goat genomic DNA were obtained from Towner et al. (1984b).

banding technique served to establish chromosome band nomenclature (number the bands) and was the basis for precise detection recording and communication of hybridization grains for chromosomal localization of the gene.

The *in situ* hybridization procedure was performed on goat metaphase chromosome preparations resulting in an average of 1-2 informative hybridization grains per spread were scored. Informative silver grains are those which are [illegible] ly associated with a chromosome. The presence of [illegible] informative silver grains per metaphase spread indicates that a high background wash has taken place during the *in situ* hybridization procedure. Several partial metaphase spreads with grains located in corresponding bands of chromosome no. 7s are shown in Figure 13. The location of 144 grains was determined in 103 metaphase spreads. 21 of these grains (14.6%) were found to be located on chromosome 7 (Figure 14). Of these 21 grains, 17 (11.8%) were located in region 33-35 of chromosome 7. The next highest number of grains were found on chromosome 12 with five grains (3.5%) in region 24-25 and four grains (2.8%) were found in region 15-16 of chromosome 19. This indicates that the epsilon IV gene of the goat beta globin gene cluster and therefore the cluster itself, is located in the region 33-35 of chromosome 7.

FIGURE 13. Partial metaphase spreads showed hybridization grains located on chromosome No. 7s'.

(A) A partial metaphase spread with 2 chromosomes having hybridization grains indicated by broad arrows.

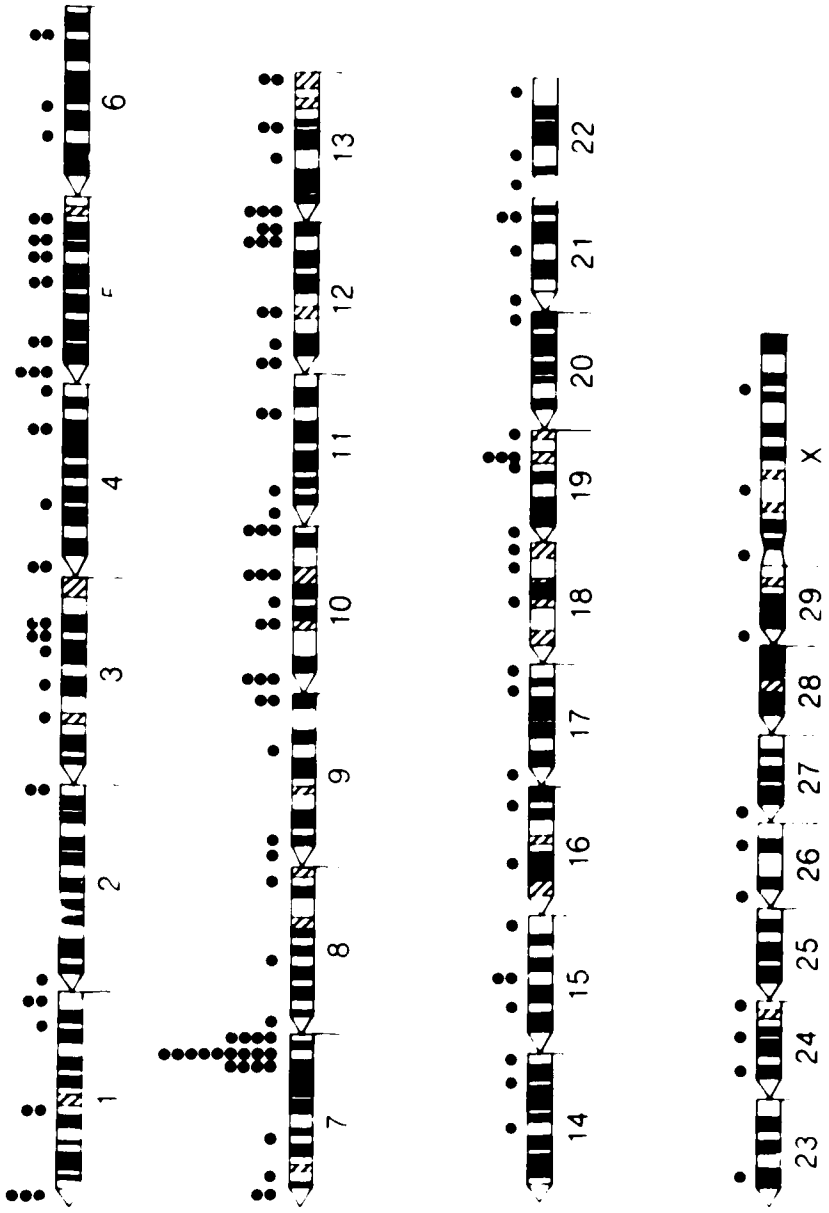
(B) Same partial metaphase spread as in (A) which was GBG-banded prior to in situ hybridization. Chromosome with hybridization grains were identified as chromosome 7s' (indicated).

(C, E) Two partial metaphase spreads each showing a hybridization grain on a chromosome.

(D, F) Same partial metaphase as C and E respectively. These metaphase were G-banded prior to the in situ hybridization and the chromosome with hybridization grain was identified as chromosome 7s' (indicated).


a
b
c
d

Table 4. Chromosomal distribution of 144 silver grains from 103 GBG-banded metaphase chromosome spreads from the female goat (Capra hircus 2n=60, XX). 21 grains where localized to chromosome 7. Of these, 17 grains were located in the regions of bands 33 to 35 of this chromosome.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CHAPTER FOUR

DISCUSSION

[I] CHROMOSOME IDENTIFICATION AND BANDING TECHNIQUES: EMPHASIS ON IDENTIFICATION OF GOAT CHROMOSOMES

Sokolov (1930) was the first to report the correct diploid number of chromosomes to be 60 in the goat. Dain (1970; 1972) confirmed the number and measured the length of chromosomes in this species. Dain compared lengths of homologous chromosomes in five male and five female goats and 13 male and 13 female sheep all solid stained with orcein. Chromosomes were paired in order of decreasing length. Differences were found between male and female sheep for three large autosome pairs. The long arms of chromosomes 1 and 2 as well as the acrocentric chromosome 4 were found to be longer in males than females. Six other smaller chromosome pairs were suspected to be larger in female than male sheep. In the goat, no major differences in chromosome length were found between the two sexes. Therefore it is likely that the difference in chromosome length between the 2 sexes in sheep is an artifact of the preparation of metaphase chromosome spreads.

(A) THE GIEMSA G-BANDING TECHNIQUE AND ITS' USE IN BOVINE CHROMOSOME IDENTIFICATION

Prior to this study, there have been several G-banding studies carried out on goat metaphase chromosomes (Evans et al. 1973; Hageltorn and Gustavsson 1974 and Schnedl and Czaker 1974). In this study, the trypsin G-banding technique was used to establish a G-

banded karyotype of the female goat using chromosome compleme (see Figure 6). The G-banded karyotypes have also been used in comparing the karyotypes of species of the Superfamily Bovoidea. For example, (Evans et al (1973) found G-banding pattern similarities among sheep, goat and cattle chromosomes. The long arms of sheep, goats and cattle chromosome 1 show a similar banding pattern. The short arm of sheep chromosome 1 has the same banding pattern as goat and cattle chromosome 3. The long arms of sheep, goats and cattle chromosome 2 have a similar banding pattern while the short arm of chromosome 2 has a similar banding pattern to goat and cattle chromosomes 8. The long arm of sheep chromosome 3 has the same banding pattern as goat and cattle chromosome 4 whereas the short arm of this chromosome has a similar banding pattern to chromosome 9 of the goat and cattle.

The high degree of similarity in the banding patterns and morphology of cattle and goat chromosomes suggests that speciation has taken place with very little chromosomal rearrangement. Buckland et al. (1978a) found that chromosome pairs 11 and 12 of the Ox had a different banding pattern from that of chromosome 11 and 12 of goats. Members of the Hippotraginae subfamily have chromosome banding patterns for chromosome pairs 11 and 12 like those of the goat. The X chromosome of species of the subfamilies Caprinae and Hippotraginae are large acrocentric chromosomes with minute but distinct short arms. The ox and bison are members of the subfamily Bovinae and their X-chromosome is submetacentric with the short arm being 1/3 the length of the long arm while in the Kudu the same chromosome is acroc c.

According to Buckland and Evans (1978a), the long arm of the bovine X-chromosome differs from the long arm of the caprine X because it has an extra positive band. Moreover the bovine X is a submetacentric chromosome whereas the X in the goat is an acrocentric chromosome. The difference between the two chromosomes probably arose as a result of a pericentric inversion (Buckland and Evans 1978a). Our results confirm the findings of Buckland and Evans (1978a).

(B) THE USE OF THE BrdU G-BANDING TECHNIQUE IN BOVINE SPECIES

The BrdU substituted G-banding technique was used to confirm the karyotype of the goat and to determine the chromosomal location of the beta globin gene cluster in this species. This technique was selected over other G-banding methods because of the greater contrast between G-positive and G-negative band regions which assists in chromosome identification particularly prior to in situ hybridization. However, the BrdU technique has other advantages over other G-banding methods.

(1) It allows for the identification of the late replicating X chromosome in female goats. The late replicating X displaying less contrast between G-positive and G-negative regions than the other early replicating X chromosome;

(2) It represents a non-radioactive means of identifying the late replicating X chromosome.

The further development of the BrdU G-banding technique in goats and cattle will allow for quick localization of genes by in situ hybridization. This will occur by enabling chromosomes to be identified directly after in situ hybridization as described by Lin et

al. (1985) as opposed to the banding and photography procedure to localize genes to specific chromosome regions.

It was concluded from the inspection of Figures 6 and 7 that the BrdU G-banding technique would give a G-banding pattern much like that of the trypsin G banding technique yet would be more advantageous for use in the localization of the goat beta globin gene cluster by in situ hybridization.

(C) THE C-BANDING TECHNIQUE AND ITS' USE IN DOMESTIC BOVINE SPECIES

The C-banding procedure was used to detect the location of constitutive heterochromatin within the goat chromosome complement. C-banding studies have been carried out previously in cattle, sheep and goats by Schnedl (1972a), Evans et al. (1973) and Schnedl et al. (1974). Schnedl et al. (1974) showed that the centromeres of the X-chromosomes of cattle, goats and sheep appeared to contain very little centromeric heterochromatin. The cattle Y-chromosome contains a considerable amount of constitutive heterochromatin and this accounts for the difference in size between it and the Y-chromosome of sheep and goats. Constitutive heterochromatin was found to be present as a single block of material, next to the centromeres of all chromosomes. There was a great deal of variation in the darkness (banding) of the chromosome arms. This appears to be due to the relative exposure of chromosomes to $Ba(OH)_2$ and is therefore an artifact of the experimental procedure.

(D) THE AMMONIACAL SILVER STAINING OF NOR REGIONS OF BOVINE CHROMOSOMES

The nucleolar organizer regions (NORs') are areas of chromosomes which possess genes coding for rRNA. The number of NOR's is species-specific. The reason for a certain number of chromosomes and different combinations of those chromosomes showing Ag-As positive staining in each cell is that the interphase nucleolus reassembles on specific chromosomes after each mitosis. Many cells and individuals within a species must be studied and their chromosomes identified to determine the species-specific number of chromosomes harboring NORs'.

Studies of these have been limited to a small number of domestic species which include sheep, swamp buffaloes, goats and cattle (Henderson and Bruere 1977, 1979, 1980; Mayr and Czaker 1981), rabbits (De Leon et al. 1978), buffaloes, goats and cattle (Di Berardino et al. 1979; 1981). NOR's have been found to be located on the same arms of homologous chromosomes in sheep and goats (Mayr and Czaker 1981).

Di Berardino et al. (1979) found that the number of chromosomes showing NORs' per metaphase spread in cattle ranged from 4-10 with a mean of 7.7 +/- 1.2 using the Ag-NOR technique. Through sequential banding and silver staining techniques they found that chromosomes 2, 3, 4, 11 and 29 possessed NORs. None of the metaphase spreads showed all ten chromosomes to have NOR regions by the Ag-NOR staining method. For example, chromosome 2 could be found to lack staining on both homologs in any cell. Ninety-three percent of the cells examined seemed to show staining of chromosome 29, thirty percent of which showed staining on only one homolog. The finding of an Ag-NOR

staining on chromosome 29 is in conflict with that of Henderson and Bruere (1979) who suggested that the NOR's in cattle and goats were located in the telomeric regions of chromosome 2, 3, 4, 5 and 28. The likely explanation for this discrepancy in NOR chromosome assignment was likely due to the difficulty in identifying the smaller chromosomes in the complement. Mayr and Czaker (1981) have shown that at least one more pair of chromosomes possesses NOR regions in goat and sheep. The NOR regions are on chromosomes 2, 3, 4, 11, 29 in cattle, chromosomes 1, 2, 3, 4, 25, 28 in sheep, and goat chromosomes 2, 3, 4, 5, 11, 29.

In this study at least 4 pairs of chromosomes appeared to carry the Ag-NOR sites instead of six pairs observed by other investigators. There are several possible reasons for these discrepancies. These include: (1) the small number of individuals used in our study; (2) sequential banding to identify the chromosomes was not used in this study while it was in that of Mayr and Czaker (1981); (3) potential differences in the way the technique was used in the two laboratories represent some of the potential reasons for the differences obtained in the number of chromosomes which contained NOR's that were active during the previous interphase.

[II] THE LOCALIZATION OF THE BETA GLOBIN GENE CLUSTER IN THE GOAT

The precise mapping of genes requires a banded karyotype. The nomenclature f the chromosome bands in each chromosome of the goat was modified for the BrdU G-Banded (GBG) karyotype in this study (Table

II). This karyotype (Figure 7) and the nomenclature of the chromosome bands served as a basis for recording the localization of hybridization grains on chromosomes. The GBG banding technique appears to show a more defined banding pattern than the routine GTG banding procedure in caprine metaphase spreads especially after in situ hybridization.

There were several problems that I attempted to overcome in arriving at the suggested localization of the goat beta globin gene cluster to chromosome 7. Firstly there was difficulty in establishing a standard BrdU G-banded karyotype for the goat. Previously published G-banded karyotypes of this species were not clear. As a consequence we used a very clear cattle G-banded karyotype from Lin et al (1977) as a reference to establish our goat karyotype for in situ work. Secondly, the in situ hybridization procedure was followed by chromosome banding and photography such that metaphase chromosome spreads are only photographed once. This proved to be unsuccessful. The G-banding technique was attempted so that easily identified banded chromosomes could be obtained following the in situ hybridization procedure of Cannizzaro et al. (1984). This resulted in the peeling of nuclear track emulsion from the slides. In addition, the technique destroyed the morphology of the chromosomes causing them to appear as rectangles with no distinction between sister chromatids. The banding patterns on these chromosomes was also faint; rendering these slides noninformative for our purposes. The next method attempted to identify and localize the gene cluster by in situ hybridization was

the one developed by Lin et al. (1985) for localizing genes in man. This included the use of the BrdU G-banding procedure of Perry and Wolff (1974). Several attempts were made to identify the goat chromosomes and localize the beta globin gene cluster in using this procedure and photographing metaphase chromosome spreads once without success. This resulted in the need to band and photograph goat metaphase chromosome spreads both prior to and following the in situ hybridization process. This resulted in the process being highly labour intensive.

[III] COMPARATIVE GENE MAPPING BETWEEN MAN AND MEMBERS OF THE BOVINE SPECIES

The committee on comparative gene mapping (Lalley et al. 1987) reported that 50 genes in cattle have been assigned to 26 chromosomes. Comparison of this data with that in man shows that at least nine human linkage groups have undergone considerable rearrangement in cattle. For the easy understanding in the following text let it be noted the location of genes in the human genome and whether the gene is located on the short arm designated (p) or the long arm designated (q) will be indicated in brackets. These include the genes SOD2 (6p) and ME1-PGM3 (6q) which have undergone rearrangement to be located on an acrocentric cattle chromosome. The second linkage group involves genes present on human chromosome 6p. The gene SOD2 has been separated from the other human 6p genes with the genes HLA-A, GLO-1 and GYP21B which has been assigned to cattle chromosome 23. The third involves the previously unassigned linkage group U3 which has now been

assigned to cattle chromosome 19. This group includes the genes GAPD-LDHB-TPL (12p) and PEPB (12q). The fourth rearrangement (pericentric inversion) involves the cattle syntenic group U11 containing the genes ITPA (20p) and ADA (20q). The fifth involves the unassigned syntenic group U24 which includes the linkage group GH-ALB-GC. In man, the genes ALB and GC are located on human 4q, while the GH gene is located on chromosome 17. There are also several examples which show reorganization among genes in cattle which are found to be syntenic in man. And finally, human chromosome 1p genes ENO1 and PGD have been found to be nonsyntenic to PGM1 (1q) in cattle; the gene PEPC has become nonsyntenic with the GUK in cattle; and the human chromosome 11p genes have divided into at least three different syntenic groups in cattle separating LDHA, CAT, and PTH.

The human beta globin gene is located on chromosome 11. Some of the genes located on the short arm of this chromosome include CAT, LDHA, HRAS1, PTH, INS and HBBC. In the mouse the beta globin gene cluster and the parathyroid hormone gene also syntenic appear to be located on chromosome 7. These two genes also may be syntenic in cattle (Womack personal communication). Since the parathyroid hormone gene (PTH) is on chromosome 15 (Hediger et al. 1987), then the beta gene cluster could also be on this chromosome. It would then appear that the region of the mammalian chromosomes possessing the parathyroid and beta globin genes has been conserved.

My study of chromosomes in the goat and comparison to published banded karyotypes of cattle (Lin et al. 1976) indicated that the

banding patterns of cattle and goat chromosomes 15 in these species are similar. Progress on chromosomal mapping in sheep (considered to be more closely related to the goat in an evolutionary sense) has been very slow since there is only one researcher presently working in this field. Saidi Mehtar and others have established 15 syntenic groups in this species as of 1987. However, genes have only been assigned to the sheep X chromosome and to chromosome 3 (Saidi-Mehtar et al. 1981; Jones et al. 1985). Of relevance to my work one of the most recent findings is that the genes LDHA (11p) and the beta globin gene cluster were found to be nonsyntenic (Saidi-Mehtar et al. 1987). This further suggests that there is considerable reorganization of genes in the bovine species. It also suggests that more studies into the genetic map of bovine species is needed for comparative gene mapping in the bovidae and particularly species more closely related to the goat must be made before accurate parallels can be drawn between bovine species.

CHAPTER FIVE

SUMMARY AND GENERAL CONCLUSIONS

The chromosome complement of the goat was studied using several banding techniques. The C-banding technique showed constitutive heterochromatin to be located in the centromeric regions of all autosomes which are acrocentric while the X chromosome showed little or no heterochromatin. The Ag-NOR staining technique showed that at least four pairs of chromosomes in the goat possessed nucleolar organizing regions.

The chromosomes were then banded using two different G-banding methods, the trypsin and BrdU G-banding techniques respectively. The BrdU G-banding technique produced a better resolution between G-positive and G-negative bands for chromosome identification and was used to form a karyotype and idiogram of the goat chromosome complement. The G-positive and G-negative bands were numerically identified and used to localize hybridization grains obtained from DNA-DNA hybridization of the 1.2 kb EcoR I/BamH I genomic DNA fragment of the epsilon IV globin gene to chromosomal DNA by <u>in situ</u> hybridization. This resulted in the assignment of the beta-globin gene cluster to goat chromosome 7 in region 33-35.

The contributions of this work to the field of cytogenetics are twofold:

(1) The numerical identification of the bands of the BrdU G-banded karyotype permits precise gene mapping in the goat in the future.

(2) The results permit provisional assignment of the beta globin gene cluster to a designated region (33-35) of goat chromosome 7.

CHAPTER SIX

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APPENDIX 1. PREPARATION OF SOLUTIONS REQUIRED FOR LYMPHOCITE CULTURE IN BrdU G-BANDING TECHNIQUE

BrdU (Sigma No. B5002):

This solution was prepared by dissolving 0.2 g of BrdU in 20 ml HBSS (pH-7.2). This was stirred for several hours resulting in a stock solution of 10,000 ug/ml. The solution was then sterilized by filtration and placed in a sterile lightproof bottle.

HOECHST SOLUTION.

This solution was prepared by dissolving 0.0208 g of Hoechst 33258 powder in 100 ml of 2X SSC which produces a 200 ug/ml stock solution. Then 250 ul of the stock solution was diluted to 300 ul. This working solution, which has a concentration of 1 ug/ml, was wrapped in tinfoil since it is light sensitive.