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A silver-staining study of the nucleolus organizer regions  
of the chromosomes of domestic sheep (Ovis aries)

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at Massey University

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ABSTRACT

A study was made of various aspects of the nucleolus organizer regions (NORs) on sheep mitotic chromosomes, using the Ag-AS and Ag-I techniques. The sheep used in this study were carriers of the  $t_1$ ,  $t_2$  and  $t_3$  Robertsonian translocations in various heterozygous and homozygous combinations. The locations of the NORs were determined to be at the telomeres on the metacentric chromosomes 1p, 2q and 3q and on the acrocentric chromosomes 4 and 25. The identity of the NO-chromosome was confirmed by a duplicate G-banding and Ag-I technique. The terminal location of the NORs indicates that they do not have a causative role in acrocentric association or Robertsonian translocation in domestic sheep. They also do not coincide with the regions in which secondary constrictions are sometimes seen.

Individual animals were found to have a characteristic silver-staining pattern. The chromosomal distribution of the Ag-staining NORs (Ag-NORs), the frequency and size of the Ag-NORs were fairly constant in different cells of an individual and in replicate cultures of an animal. Differences between cells and replicates in Ag-staining involved variation in the frequency of staining of Ag-NORs with small Ag-deposits. Overall, the metacentrics had the highest frequency of Ag-NORs, followed by chromosome 25 and then chromosome 4.

The association frequency of individual NO-chromosomes was found to be positively correlated with the frequency of Ag-NORs and the size of the Ag-deposit. The frequency of each pairwise combination of associating chromosomes was determined solely by the frequency of Ag-NORs of the component chromosomes. No evidence was found for a non-random fusion of NORs. These observations provide an explanation of the reports of non-random participation of acrocentric chromosomes in satellite association in man. The increased association of NO-chromosomes with large deposits could be due to an increased chance of fusion of larger nucleoli or to differences in the disintegration rate of different sized nucleoli.

The presence of a NOR on the chromosome 25 /  $t_3$  polymorphism was utilized in inheritance studies of Ag-stainability. Five pedigree groups in which this chromosome was segregating were studied and in all cases the size of the Ag-deposits and frequency of staining was consistent between consecutive generations. The value of Ag-staining in genetic mapping studies is discussed.

In 3 animals, the Ag-staining patterns of transformed lymphocytes and fibroblasts were compared and found to be similar.

A comparative study was made of 5 species of the Bovidae: domestic sheep (Ovis aries), domestic goat (Capra hircus), aoudad (Ammotragus lervia), bharal (Pseudois nayaur) and cattle (Bos taurus). Five NO-chromosome pairs were found in sheep, goat, aoudad and cattle and these chromosomes have homologous banding patterns. The bharal has at least four NO-chromosome pairs homologous to sheep. These results indicate a conservation of the NORs during evolution in this family.

Based on the results of the frequency of Ag-staining of NORs, the heritability of Ag-stainability and the constancy of Ag-staining patterns found in the tissue studies, the nature of the Ag-staining is discussed and a model on the basis of the Ag-stainability of individual chromosomes is presented.

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TABLE OF CONTENTS

	Page
Acknowledgements	iv
Table of Contents	v
List of Tables	xii
List of figures	xiv
Chapter 1: <u>Introduction</u>	1
Chapter 2: <u>Structure and Identification of Nucleolus Organizer Regions in sheep</u>	4
2.1. <u>Introduction</u>	4
2.1.1 The nucleolus	4
2.1.2 The formation of the nucleolus on specific chromosome regions	5
2.1.3 Secondary constrictions	6
2.1.4 Structure of the nucleolus organizer regions	6
2.1.4.1 Definition of the term 'nucleolus organizer region'	6
2.1.4.2 Location of 18S and 28S rRNA genes in the NOR	9
2.1.4.3 Structure of the NORs in man	10
2.1.4.4 Structure of the NOR in maize	10
2.1.4.5 Structure of the NOR in the sciarid fly	12
2.1.4.6 Structure of the NOR in salamander	12
2.1.4.7 Clarification of terminology	12
2.1.4.8 Other sites involved in nucleolus organ- ization	13
2.1.5 Nucleolus-associated heterochromatin	13
2.1.6 Staining Techniques in the identification of NORs	14
2.1.7 Duplicate banding	16
2.1.8 Robertsonian translocations and NORs in sheep	17

2.2.	<u>Materials and Methods</u>	18
2.2.1	Blood culture and harvesting techniques	18
2.2.2	Animals	18
2.2.3	Ag-AS technique	19
2.2.4	Ag-I technique	21
2.2.5	Duplicate G-banding and Ag-I staining	21
2.3.	<u>Results</u>	21
2.3.1	Location of nucleolus organizer regions	21
2.3.2	Ag-AS technique	30
2.3.3	Ag-I technique	31
2.3.4	Duplicate banding	31
2.3.5	An anomalous cell	34
2.3.6	Ag-staining in interphase, prophase and metaphase cells	34
2.4.	<u>Discussion</u>	34
2.4.1	Structure of sheep nucleolus organizer regions	34
2.4.2	Possible roles of the secondary constrictions on sheep chromosomes	35
2.4.3	Association of nucleolus organizer regions	38
2.4.4	Applications of silver-staining	39
2.4.5	Anomalous silver-staining region	39
Chapter 3:	<u>The Frequency of Ag-NORs and Regulation of NOR activity</u>	41
3.1.	<u>Introduction</u>	41
3.1.1	Evidence that silver stains active NORs	41
3.1.2	Regulation of NORs	41
3.1.2.1	Variation in the level of rDNA	41
3.1.2.2	NOR regulation in interspecies hybrids	44
3.1.2.3	rRNA gene activation during development	47
3.1.2.4	The effect of NOR dosage on NOR activity	48
3.1.2.5	The effect of NOR dosage on Ag-NOR numbers	50
3.1.2.6	Summary of evidence on the control of rRNA transcription	50
3.1.3	Variation in frequency of Ag-NORs	52

3.2.	<u>Materials and Methods</u>	52
3.2.1	Materials	52
3.2.2	Scoring of size polymorphisms	52
3.3.	<u>Results</u>	54
3.3.1	Average frequency and range of Ag-NORs per metaphase	54
3.3.2	Individual variation in number of Ag-NORs	55
3.3.3	Variation between replicates in the number of Ag-NORs	58
3.3.4	Chromosome distribution of Ag-NORs	58
3.3.4.1	Pooled data for all individuals	58
3.3.4.2	Frequency of Ag-NORs on C25 and $t_3$	59
3.3.4.3	Individual variation in distribution of Ag-NORs	60
3.3.5	Differences in deposit size between individuals	60
3.3.6	Relationship of deposit size to Ag-NOR frequency	65
3.3.7	Relationship of deposit size to between-culture differences	65
3.4.	<u>Discussion</u>	
3.4.1	Variation in the number of Ag-NORs between animals	67
3.4.2	Chromosomal distribution of Ag-NORs	68
3.4.3	Polymorphism in size of Ag-NORs	69
3.4.4	Relation of rDNA content to Ag-staining	69
3.4.5	Ag-stainability and NOR regulation	71
Chapter 4:	<u>Association of nucleolus organizer regions</u>	72
4.1.	<u>Introduction</u>	72
4.1.1	Satellite associations and nucleolar fusions	72
4.1.2	Studies on NOR-associations in species other than man	73
4.1.3	Participation of individual chromosomes in satellite association	74
4.1.4	Relationship of satellite association to NOR morphology	75

4.1.5	Relationship of satellite association to rDNA content	76
4.1.6	Relationship of satellite association to staining polymorphisms	77
4.1.7	Association of NORs in sheep	83
4.2.	<u>Materials and Methods</u>	83
4.2.1	Materials	83
4.2.2	Method of scoring associations	83
4.3.	<u>Results</u>	83
4.3.1	Observations on association complexes of NO- chromosomes	83
4.3.2	Frequency of association	84
4.3.3	Relationship of association frequency to the frequency of expression	84
4.3.4	Relationship of the association frequency to the deposit size	91
4.3.5	Combinations of associating chromosomes	91
4.4.	<u>Discussion</u>	95
4.4.1	Frequency of association	95
4.4.2	Factors affecting the association index of individual chromosomes	97
4.4.3	Combinations of chromosomes in association complexes	99
Chapter 5:	<u>Inheritance of Ag-stainability</u>	101
5.1.	<u>Introduction</u>	101
5.2.	<u>Materials and Methods</u>	101
5.2.1	Mating records	101
5.2.2	Establishment of mating groups	104
5.2.3	Determination of karyotypes of lambs	104
5.2.4	Culture staining and analysis techniques	105
5.2.5	Notation	105

5.3.	<u>Results</u>	105
5.3.1	Pedigrees taken from mating records	105
5.3.1.1	Pedigree 1	105
5.3.1.2	Pedigree 2	106
5.3.2	Results of mating experiments	106
5.3.2.1	Lambing results of Pedigree 3	106
5.3.2.2	Lambing results of Pedigree 4	109
5.3.2.3	Lambing results of Pedigree 5	115
5.3.3	Recombination of Ag-NORs	115
5.3.4	Inheritance of frequency of Ag-NORs and size polymorphism	117
5.4.	<u>Discussion</u>	118
5.4.1	Inheritance of Ag-stainability	118
5.4.2	Recombination of Ag-NORs	124
Chapter 6:	<u>Comparison of silver-staining patterns in fibroblasts and lymphocytes</u>	126
6.1.	<u>Introduction</u>	126
6.2.	<u>Materials and Methods</u>	127
6.2.1	Tissue Source	127
6.2.2	Fibroblast culture technique	127
6.2.3	Subculturing	128
6.2.4	Harvesting of fibroblast cultures	128
6.2.5	Ag-staining	128
6.3	<u>Results</u>	129
6.3.1	Frequency of Ag-NORs in different tissues	129
6.3.2	Size polymorphism of Ag-NORs in different tissues	132
6.4	<u>Discussion</u>	132
Chapter 7:	<u>Homology of NORs in the sheep, goat, aoudad, bharal and cattle</u>	137

7.1.	<u>Introduction</u>	137
7.1.1	Chromosomal evolution in the Bovidae	137
7.1.2	Studies on the evolution of NORs	139
7.1.3	NOR studies in the Bovidae	141
7.2.	<u>Materials and Methods</u>	144
7.2.1	Animals	144
7.2.2	Karyotyping and comparative studies	144
7.3.	<u>Results</u>	146
7.3.1	Location of NORs	146
7.3.2	Variation in number and size of Ag-NORs	152
7.4.	<u>Discussion</u>	152
7.4.1	Assignment of NORs to specific chromosomes	152
7.4.2	Nomenclature and presumptive homologies of the chromosomes of the Bovidae	155
7.4.3	The ring chromosome of the bharal	157
7.4.4	Frequency of Ag-NORs	157
7.4.5	Homology of NORs in sheep, goat, cattle, aoudad and bharal	158
7.4.6	Relationship of NORs to Robertsonian translocations in the Bovidae	160
Chapter 8:	<u>Conclusions</u>	163
8.1.	<u>Relation of Ag-staining to NOR activity</u>	163
8.2.	<u>The nature of the Ag-staining polymorphisms</u>	165
8.3.	<u>A model of NOR regulation and Ag-staining in diploid organisms</u>	166
8.4.	<u>The stability of NORs</u>	169
8.5.	<u>Summary of advances made in this study</u>	171

<u>Appendices</u>	173
I Personal communications	173
II Leucocyte culture technique	174
III Giemsa stain	175
IV Fibroblast culture materials	175
V G-banding technique	176
VI Washing of laboratory glassware	177
VII Photography techniques	177
VIII History and management of experimental animals	177
IX Distribution of counts of number of Ag-NORs per metaphase	179
X Two-way ANOVA for replicate cultures	180
XI Example of size polymorphism data score	181
XII Data on Ag-NOR frequency in inheritance study	182
XIII Copy of the publication "Association of nucleolus organizer chromosomes in domestic sheep ( <u>Ovis aries</u> ) shown by silver staining"	184
XIV Copy of the publication "Conservation of nucleolus organizer regions during evolution in sheep, goat, cattle and aoudad"	185
XV Abstract of paper presented to New Zealand Genetical Society Conference, 1978	186
<u>Bibliography</u>	following page 186

<u>List of Tables</u>	Page
2-1. Examples of varying usages of the term 'nucleolus organizer region' and its derivatives	8
3-1. Summary of evidence on the control of rRNA transcription	51
3-2. The distribution of average number of Ag-NORs per metaphase in 33 individuals	54
3-3. Average frequency and distribution of Ag-NORs in individual sheep	56
3-4. Analysis of variation due to replicate differences in between-animal differences	57
3-5. Data on the chromosomal distribution of Ag-NORs	58
3-6. Chromosome distribution of Ag-NORs in individuals other than those involved in pedigree study	59
3-7. Distribution of the number of cells in which a chromosome has a given size deposit	63
3-8. Relationship of deposit size to between-culture differences	66
4-1. Comparison of association frequency with expected values based on frequency of Ag-NORs	86
4-2. Relationship between deposit size and association frequency of C <sub>4</sub> , C <sub>25</sub> and t <sub>3</sub>	89
4-3. Relative participation in nucleolar organization of NO-chromosomes in 5 animals used in combinational analysis	92
4-4. Observed and expected values of pair-wise combinations of NO-chromosomes	94

4-5.	Reported frequencies of satellite association in man	96
5-1.	Lambing and Ag-staining results of pedigree 3	107
5-2.	Lambing and Ag-staining results of pedigree 4	109
5-3.	Lambing and Ag-staining results of pedigree 5.	116
5-4.	Inheritance of stainability in sheep heterozygous for Ag-stainability	117
5-5.	Size of Ag-NORs in 5 animals and their progeny	119
6-1.	The frequency of Ag-NORs in lymphocyte and fibroblast cultures from 3 animals	129
6-2.	Size polymorphism of Ag-NORs in each tissue from 3 animals	133
7-1.	Comparison of location and number of NORs in man, chimpanzee, orangutan and gorilla	140
7-2.	G-banding homology between sheep, aoudad, cattle, goat and bharal autosomes	145
7-3.	Modal values and range of number of Ag-NORs per metaphase in sheep, aoudad, cattle and goat	153
7-4.	Involvement of chromosomes in centric fusion during karyotypic evolution and within species	161

List of figures	Page
2-1. Drawing showing the morphology of the NOR in man (text figure)	7
2-2. Drawing showing the morphology of the NO-chromosome in maize (text figure)	11
2-3. Flow-diagram of Ag-AS and Ag-I staining techniques	20
2-4. Karyotype showing location of Ag-NORs on chromosomes 1, 2, 3, 4 and 25	22
2-5. Karyotype showing Ag-NOR on $t_3$ chromosome	23
2-6. Ag-I stained metaphase showing absence of Ag-staining on a secondary constriction	24
2-7. Absence of silver-staining on secondary constrictions in 3 metaphases stained by Giemsa, destained and Ag-stained	25
2-8. Metaphase stained by Ag-AS technique (colour plate)	26
2-9. Metaphase stained by Ag-I technique (colour plate)	27
2-10. Metaphase treated by Ag-I technique and then G-banded (colour plate)	28
2-11. Karyotype of NO-chromosomes identified by duplicate G-banding and Ag-I staining	29
2-12. Karyotype of a cell with Ag-deposits on both telomeres of a chromosome 1.	32
2-13. Ag-stained cells showing differences in amount of Ag-staining material at different stages of the cell cycle	33

3-1. Diagram of Nucleolar dominance in $F_1$ hybrids of <u>D.mulleri</u> and <u>D.arizonensis</u>	46
3-2. Two metaphase cells showing Ag-deposits of different sizes	53
3-3. Histograms illustrating the average number of Ag-NORs on the 3 chromosome types	61
3-4. Histograms of size of Ag-NORs in 8 animals	62
3-5. Graph showing correlation of deposit size and frequency of expression on chromosome 25 and $t_3$	64
4-1. Metaphase cell showing chromosomes in association	78
4-2. Metaphase cell showing chromosomes lying adjacent but not associated	78
4-3. Metaphase cell with chromosomes associated by one chromatid and by both chromatids	79
4-4. Two metaphases with associated chromosomes joined by a long connecting strand	80
4-5. Metaphase cell showing an associated chromosome with no detectable Ag-stain	81
4-6. Metaphase cell showing four chromosomes in one association complex	82
4-7. Metaphase cell with seven chromosomes involved in association complexes	82
4-8. Scattergram showing correlation of association index with frequency of Ag-NORs	88
4-9. Graph showing correlation between Ag-deposit size and association index	90

5-1. Pedigree chart of pedigree 1	102
5-2. Pedigree chart of pedigree 2	103
5-3. Ag-stained karyotype of one of the sires of pedigree 3 (121/5)	108
5-4. Karyotype of NO-chromosomes of two progeny of pedigree 3	108
5-5. Ag-stained karyotype of sire of pedigree 4 (60/5)	110
5-6. G-banded karyotype of lamb 123/8	111
5-7. Karyotype of NO-chromosomes of four progeny of pedigree 4	112
5-8. Karyotype of sire of pedigree 5 (176/6)	113
5-9. Karyotype of NO-chromosomes of six progeny of pedigree 5	114
5-10. Scattergram showing correlation between Ag-NOR frequency in parent and progeny	120
5-11. Scattergram showing relationship of Ag-NOR size polymorphism in parent and progeny	121
6-1. Histograms of average number of Ag-NORs per metaphase in fibroblasts and lymphocytes in three animals	130
6-2. Histograms of size polymorphism of Ag-NORs in lymphocyte cultures	131
6-3. Karyotype of NO-chromosomes from three tissues of 155/6	134

7-1. Taxonomy of bovid species used in this study	143
7-2. Karyotype of Ag-stained metaphase from cattle showing location of NORs	147
7-3. Metaphase cells from 1/29 bull showing Ag-NORs on 2 small acrocentric chromosomes	147
7-4. Karyotype of Ag-stained metaphase of a goat showing location of NORs	148
7-5. Karyotype of Ag-stained metaphase of an aoudad showing location of NORs	149
7-6. Partial metaphase from an aoudad showing an Ag-NOR on chromosome 1	149
7-7. Metaphase cell from bharal	150
7-8. Karyotype of an Ag-stained metaphase cell from a bharal showing the location of NORs	150
7-9. Two metaphases from a bharal showing 'ring' chromosomes	151
7-10. Idiogram of the NO-chromosomes in the five bovid species showing homologies	156

CHAPTER I

## INTRODUCTION TO THE STUDY

Nucleolus organizer regions are chromosomal regions which contain the DNA templates involved in the transcription of the precursors of the heavier ribosomal RNA subunits (18S and 28S rRNA). These regions may be identified by either the association of the nucleolus with the nucleolus organizer chromosome in mitosis or meiosis (Heitz, 1933; McClintock, 1934), by hybridization of rRNA or rDNA with fixed metaphase chromosomes (Pardue et al., 1970; Henderson et al., 1972; Henderson et al., 1974a, 1974b) or by cytological staining techniques e.g. N-banding (Matsui and Sasaki, 1973), 'satellite staining' (Eiberg, 1974) and silver staining (Goodpasture and Bloom, 1975; Howell et al., 1975; Bloom and Goodpasture 1976). In some species the nucleolus organizer regions (NORs) are manifest as secondary constrictions on metaphase chromosomes (Hsu et al., 1967).

Nucleolus organizer regions are a subject of interest to cytogeneticists due to their possible association with chromosome abnormalities in man. The phenomenon of satellite association, where the satellited human D and G group chromosomes are linked together by their short arms, was first described by Ferguson-Smith and Handmaker in 1961. They suggested the involvement of the acrocentric chromosomes in these associations is due to their role in nucleolus organization. Simultaneous breakage of two secondary constrictions in associating chromosomes, followed by rejoining, could provide a basis for Robertsonian translocations (Ohno et al., 1961; Ferguson-Smith, 1967). Satellite associations have also been suggested to have a causal role in promoting non-disjunction leading to the trisomy syndromes in man (Ferguson-Smith and Handmaker, 1961, 1963).

Robertsonian translocation (centric fusion) appears to be the main mechanism of chromosomal change in the Bovidae (Wurster and Benirschke, 1968). Three Robertsonian translocations have been identified in New Zealand sheep flocks (Bruère and Mills, 1971; Bruère et al., 1972; Bruère, 1973). At the start of this study the location of the NORs in sheep was unknown although a number of secondary constrictions occasionally can be seen on metaphase chromosomes (Bruère and McLaren, 1967). An accurate identification of the NORs was made possible by the use of silver-staining techniques. These were applied to sheep to identify the nucleolus organizer chromosomes and to determine their role in chromosome association and the formation of Robertsonian translocation chromosomes in this species. The results of this work are reported in Chapters 2 and 4.

Not all regions which contain the genes for 18S and 28S rRNA are stained by the Ag-I and Ag-AS techniques. These techniques appear to only stain those regions which have been active in synthesizing rRNA in the preceding interphase (O.J. Miller et al., 1976; D.A. Miller et al., 1976). Differences in frequency and distribution of silver-staining NORs (Ag-NORs) as revealed by these techniques, and reported in Chapters 2 and 3, presumably reflect differential activity of these regions. In this thesis, an attempt to study the regulation of the expression of the NORs in sheep is described. The distribution of Ag-NORs in various tissues from three animals was investigated to determine whether the ability of a NOR to be active in rRNA synthesis is inherited or is due to an inactivation phenomenon similar to that occurring in the inactivation of the mammalian X chromosomes. Also pedigree groups were mated to determine the inheritance of staining and non-staining chromosomes. The results of this work are reported in Chapters 5 and 6.

A further application of the silver-staining technique to an understanding of the cytogenetics of sheep was a comparative study

of the NORs in four species of Bovidae. These species show homology of G-banding patterns (Evans et al., 1973; Nadler et al., 1973, 1974; Schnedl and Czaker, 1974; Bunch et al., 1976). There are also similarities in satellite DNA composition and location in the subfamily Caprinae (Curtain et al., 1973; A.R. Mitchell, pers. comm.). The location of NORs in the sheep, goat, aoudad, bharal and cattle were determined to see whether the conservation of banding pattern and satellite DNA composition and location was accompanied also by a conservation in NOR location and number. This work is reported in Chapter 7.

In summary this work had three main aims; first to identify the nucleolus organizer chromosomes of sheep and report on their frequency of expression and role in chromosome association. Second, factors concerning the expression of these regions as shown by silver staining were investigated. And third, a comparative study of NOR location and number in sheep and four other bovids was made to add to our knowledge of the chromosomal evolution in this family.

## CHAPTER 2

### STRUCTURE AND IDENTIFICATION OF NUCLEOLUS ORGANIZER REGIONS

#### 2.1. Introduction

##### 2.1.1. The Nucleolus

The nucleolus is a non-membrane bounded body located in the nucleus which contains dense granular elements and fibrillar elements (Busch and Smetana, 1970). Although all aspects of its function are not yet understood, its main role is the synthesis of components of the ribosomes. The nucleolus is known to be involved in the synthesis of the precursors of the high molecular weight ribosomal RNA (rRNA) sub-units (18S and 28S rRNA). There are low molecular weight RNAs concentrated in the nucleolus but the role of the nucleolus in their synthesis is not clear. The nucleolus also contains histones and acidic proteins. The latter include biosynthetic enzymes (RNA polymerases and NAD pyrophosphorylases) and enzymes involved in the degradation of RNA (RNases). There may also be ribosomal proteins but again the role of the nucleolus in their synthesis is unknown. DNA occurs around the nucleolus ('para-nucleolar chromatin') and this chromatin probably has some role in nucleolar function. Thin strands of chromatin can also be seen to extend into the nucleolus.

The nucleolus is not a permanent structure. The nucleoli disintegrate during prophase and reform at telophase (Busch and Smetana, 1970). They increase in size as interphase proceeds due both to nucleolar growth and the fusion of smaller nucleoli (Gonzalez and Nardone, 1968). This fusion of nucleoli occurs mainly in late telophase and G1 and results in a decrease in nucleolar number from early to late interphase (Gonzalez and Nardone, 1968; Gani, 1978).

### 2.1.2. The formation of the nucleolus on specific chromosome regions

It has been known for a long time now that the nucleolus is closely associated with specific chromosome regions (e.g. Nawashin, 1912). The relationship was explained by Heitz (1931a,b) in his reports on the bean Vicia. He observed that satellite stalks and secondary constrictions were functionally synonymous and that the nucleolus originated in telophase at the position of the satellite stalk or secondary constriction. Heitz called these nucleolus-associated regions "SAT-chromosomes" (sine acido thymonucleinico) since he believed the secondary constriction lacked nucleic acid.

When precautions are taken to preserve nucleolar structure, the association of the nucleolus with a particular chromosome region may be seen. For example, these associations have been reported in mitotic prophase in man (Ohno et al., 1961) and rat kangaroo (Hsu et al., 1967), in mitotic telophase in Vicia (Heitz, 1931a,b) and in the pachytene stage of meiosis in man (Schultz and St Lawrence, 1949; Ferguson-Smith, 1964).

The early studies on the identification of the nucleolus-associated chromosomes in man involved the use of meiotic preparations. Schultz and St Lawrence (1949) reported the principal bivalent associated with the nucleolus was one of the larger bivalents and that the nucleolus was placed midway on this bivalent. However with the advent of reliable blood culturing techniques it was possible to show that each of the 5 pairs of acrocentric chromosomes of the D and G group in man may have satellites (Ferguson-Smith and Handmaker, 1961). The 5 pairs of acrocentric chromosomes were later confirmed to be associated with the nucleolus in pachytene (Ferguson-Smith, 1964). Nucleoli appeared to form at the terminals of 3 large bivalents and 2 small bivalents and were subtended by large deeply-staining chromomeres. It was suggested from this work that the bivalent described by Schultz and St Lawrence (1949) was in fact two acrocentric chromosomes associated with the nucleolus.

### 2.1.3 Secondary Constrictions

In some species, the region at which the nucleolus arises appears as a secondary constriction in metaphase cells (Heitz, 1931a,b). Transcription of the genes for rRNA, as shown by the uptake of tritiated uridine, occurs in prophase and prometaphase (Arrighi, 1967) when the rest of the genome is transcriptionally inactive. In most species and cell lines the nucleolus disintegrates at the end of the prophase, but its persistence to this stage of mitosis, while the chromosomes have been condensing, results in a delayed condensation of this region, manifest as a secondary constriction (Busch and Smetana, 1970). The time of disintegration of the nucleolus may vary between species. For example, in the rat, mouse and some other rodents, it is postulated that the nucleolus disintegrates early so that the NOR does not appear as a secondary constriction (Busch and Smetana, 1970).

The chromosome regions associated with the nucleolus may be extended in their functional state due either to the build-up of nucleolar gene products (McClintock, 1934) or to the requirement for the chromatin to be extended so that RNA transcription can occur (Hsu et al., 1967).

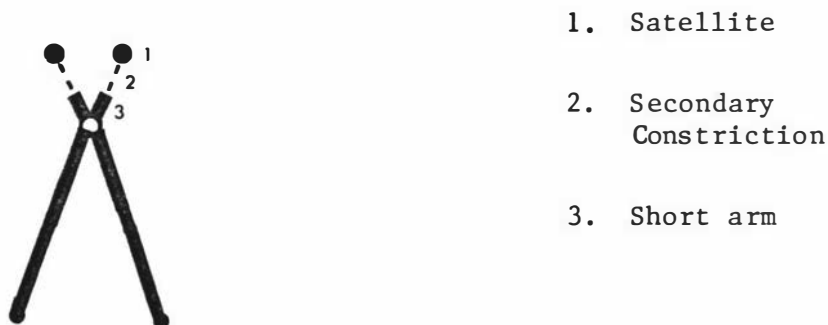
### 2.1.4. Structure of the nucleolus organizer regions

#### 2.1.4.1 Definition of the term 'nucleolus organizer region'

The term 'nucleolar organizer body' was coined by McClintock (1934) to describe the dark-staining body adjacent to the secondary constriction on chromosome 6 in maize. She envisaged the role of the nucleolar organizer body is to collect the 'nucleolar substances' produced by the chromosomes and to organize them into a definite body, the nucleolus. She did not see the role of this region as a synthesizer of the nucleolar products. The stalk was proposed to form as a result of chromosome stretching due to accumulation of nucleolar products. McClintock showed that the nucleolar organizer body can be split in two, and that each half

retains its capacity to 'organize' a nucleolus, indicating that this region is not a single gene.

While more modern work has indicated that the role of the secondary constriction and adjacent region is the synthesis of nucleolar products rather than 'organization', the term nucleolar organizer region (NOR) has been retained. However as pointed out by Doerschug (1976) there appears to be no adequate cytological or genetic definition of these regions. In addition, many authors use the term 'NOR' without defining which chromosomal regions they are referring to. Examples of varying usage of the term are shown in Table 2-1. While McClintock used it only to describe the heterochromatic body of maize, Phillips *et al.* (1971) and Givens and Phillips (1976) used the term NOR for the secondary constriction region and adjacent heterochromatic segment. In man, the short arms of the acrocentric chromosomes may have a 'satellite' connected to them by a secondary constriction (the satellite stalk) (Fig.2-1). The term NOR has been used to describe the satellite region (e.g. Matsui and Sasaki, 1973) or the short arm, satellite and stalk (Evans *et al.*, 1974)



**Fig. 2-1:** Diagram of a human acrocentric chromosome demonstrating the satellite, secondary constriction and short arm region.

Table 2-1: Examples of varying usages of the term  
'Nucleolus Organizer Region' and its derivatives

<u>AUTHOR</u>	<u>TERM</u>	<u>SPECIES</u>	<u>INTERPRETATION</u>
McClintock (1934)	NO-body	Maize	Heterochromatic body located adjacent to secondary constrict- ion
Hsu <u>et al.</u> (1967)	NOR	General definition	'Regularly occurring' secondary constrict- ions
Busch and Smetana (1970)	NOR	General definition	Ribosomal DNA and surrounding protein matrix
Phillips <u>et al.</u> (1971) Givens and Phillips (1976)	NOR	Maize	Heterochromatin and secondary constrict- ion
Matsui and Sasaki (1973)	NOR	Man  Rat Kangaroo, Muntjac	'Satellite bodies' of the acrocentric autosomes Secondary constrict- ion
Evans <u>et al.</u> (1974)	NO- segment	Man	Short arm, satellite and stalk of acrocentric autosomes

In many reports, the secondary constrictions have been assumed to be synonymous with the nucleolus organizers (e.g. Hsu et al., 1967) or the term NOR has been used without definition. Busch and Smetana (1970) modified the definition to include the ribosomal DNA and the surrounding matrix containing special proteins and other components essential for the synthesis of ribosomal RNA.

#### 2.1.4.2. Location of 18S and 28S rRNA genes in the NOR

Part of the confusion in terminology appears to be due to our continuing lack of knowledge of the structure of the chromosome regions associated with the nucleolus. By rRNA/rDNA hybridization techniques it has been possible to measure the amount of 18S and 28S rDNA. The technique involves the hybridization of isotopically labelled rRNA to denatured DNA. In the first experiment of this sort (Ritossa and Spiegelman, 1965) mutants of Drosophila melanogaster were constructed which possessed either duplications or deficiencies of the "NO" region such that 1-4 doses of the NOR were present. The amount of rRNA which hybridized to the DNA was proportional to the dosage of the NOR, confirming that the NOR is the site of 18S and 28S rRNA synthesis.

A similar result was obtained from mutants of the South African clawed frog Xenopus laevis, containing no nucleoli (anucleolate), one nucleolus and two nucleoli. It has been shown that the mutants which possess only one nucleolus lack the secondary constriction on one chromosome (Kahn, 1962). Again the degree of hybridization of rRNA was found to depend on the number of secondary constrictions (Wallace and Birnstiel, 1966).

More recently, confirmation of the localization of the 18S and 28S rRNA components to the nucleolus organizer region has been made by in situ DNA/RNA hybridization studies in a variety of species. In this technique, fixed metaphase cells are denatured, labelled rRNA is added under conditions which allow hybridization to occur and the slides allowed to develop in a photographic emulsion. It has been shown by this technique that the 28S and 18S rRNA

hybridizes to the NOR constrictions in 3 species of Diptera (Pardue et al., 1970), man (Henderson et al., 1972), Rhesus monkey (Henderson et al., 1974b), in 2 species of toad (Pardue, 1973), and mouse (Henderson et al., 1974b).

#### 2.1.4.3. Structure of the NORs in man

In man, the short arms of the D and G group chromosomes are polymorphic and variation is observed in satellite size, and secondary constriction length. In in situ experiments on an individual with an unusually long stalk on chromosome 15, and an individual with a very large satellite on chromosome 22, it was shown that the chromosome with the large stalk hybridized four times as much  $^3\text{H}$  - rRNA as its homologue whereas the large satellite does not show significantly more hybridization (Evans et al., 1974). From this, it was concluded that most, if not all, of the rDNA is localized in the stalk, and the satellites are not regions of condensed rDNA that can be unwound for transcription in the constricted regions. It has also been shown that four human satellite DNA components hybridize to the centromere and possibly the short arm and satellites of the acrocentric chromosomes (Gosden et al., 1975). Whether these heterochromatic regions have any functional role in nucleolus formation is not known at present (see Section 2.1.5). Thus the present state of our knowledge of the structure of the nucleolus organizer region of man is that it consists of a rDNA segment which is manifest as a secondary constriction or 'stalk' when the region is active in nucleolus organization and it is surrounded by two heterochromatic portions - the short arm and the 'satellite knob'.

#### 2.1.4.4. Structure of the NOR in maize

A quite different situation to that in man may exist in maize. As described by McClintock (1934) in the pachytene chromosomes of microsporocytes the short arm of chromosome 6 has a small satellite joined by an achromatic gap to a dark staining elongate knob (Fig. 2-2).

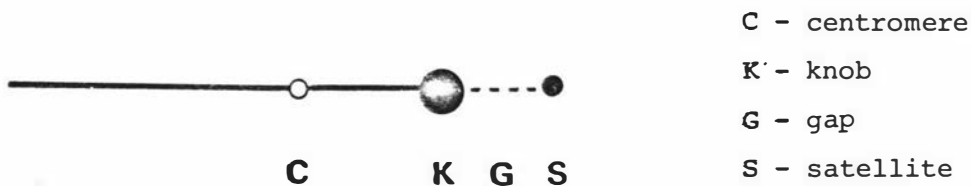


Fig. 2-2: Schematic diagram showing the morphology of the NO-chromosome in maize (after McClintock, 1934).

Several experiments show that the dark staining body contains rDNA. Doerschug (1976) used a reciprocal translocation between chromosome 6 and a B chromosome which involved a transection of the dark staining body (McClintock's NO body) at its mid-point, to form a series of plants with various dosages of the NO body fragments. Molecular hybridization experiments revealed there are genes coding for rRNA on either side of the breakpoint, and that an increase in dosage of chromosomes with the NO body fragment results in an increased rDNA content. A similar conclusion on the location of the rDNA in the dark-staining body of maize was reached by Givens and Phillips (1976). They produced plants partially triploid for the heterochromatic segment of the NOR or partially triploid or tetraploid for the site giving rise to the secondary constriction. By hybridization of  $^3\text{H}$  - rRNA with the DNA extracted from these plants it was shown that most of the rDNA is located in the heterochromatic segment. While DNA from plants trisomic for the secondary constriction do not have higher hybridization levels than normal diploids, it could not be excluded that the secondary constrictions do contain rDNA but that this could not be detected by this method. It would seem therefore that the NOR of maize consists of a condensed, probably inactive region of rDNA and an extended transcribed region.

#### 2.1.4.5. Structure of the NOR in the sciarid fly

In the sciarid fly, Rhynchosciara hollaenderi all regions observed to be producing nucleolar material appeared to extend chromatin strands from a 'rather circular piece of heterochromatin'. It is thought that the active strands retract back into these heterochromatin regions when they cease to be active (Stocker, 1978). This suggests a similar structure to the NOR in maize.

#### 2.1.4.6. Structure of the NOR in salamander

Another means of investigating the structure of the NOR is by microbeam irradiation (Berns et al., 1970; Berns and Cheng, 1971; Berns, 1973). In this series of experiments an argon laser microbeam was used to selectively irradiate the secondary constriction region or the adjacent region in the salamander, Taricha granulosa. Irradiation of the secondary constriction prevented nucleolus formation in 50% of the cases, whereas irradiation of the adjacent area consistently prevented nucleolus formation. The experimenters concluded that either the genes for rRNA are located in the adjacent region and the secondary constriction forms as a result of nucleolar product accumulation, or that the adjacent region is a 'control' region for the transcription of rRNA in the adjacent constriction.

#### 2.1.4.7. Clarification of terminology

It is apparent from this discussion that the structure of the NOR may not be uniform. In maize the adjacent heterochromatic segment contains rDNA and can produce a nucleolus. A similar situation probably exists in Rhynchosciara. Whether the adjacent heterochromatin in the NORs of man has a role in nucleolus organization is unknown although it probably does not contain rDNA. In the salamander the region adjacent to the secondary constriction is essential for nucleolus formation.

In the literature, the term 'secondary constriction' has been used synonymously with 'nucleolus organizer region'. This usage may be incorrect as the region adjacent to a secondary constriction may also have a functional role in nucleolus organization.

Therefore in this thesis the term NOR refers to the chromosomal location of the genes for 28S and 18S rRNA and the adjacent chromosomal regions which may function in the formation of the nucleolus or the regulation of these genes. The term Ag-NOR refers to a NOR which has a silver deposit after staining by the Ag-I or Ag-AS technique.

#### 2.1.4.8. Other sites involved in nucleolus organization.

It seems ironic that we retain the term 'nucleolus organizer' for the region transcribing the 18S and 28S rRNA genes when the true 'organizer' may be a different region. McClintock (1934) found that a microspore deficient for two-thirds of the long arm of chromosome 9 (but with the NOR region intact) had nucleolar material distributed into small bodies throughout the nucleus rather than into a nucleolus. Microspores without a NOR also have a diffuse distribution of nucleolar material. While nucleoli do not form during the early development of the homozygous anucleolate mutant of Xenopus, nucleolar-like bodies do form at later stages of development (Barr, 1966). Considering these two examples, it seems that sites other than those coding for 18S and 28S rRNA are necessary for nucleolus formation and also that rRNA is not essential for the formation of a nucleolus-like body. The genes for 5S rRNA have been located at sites other than the NOR in man (Johnson et al., 1974; Steffensen, et al., 1974) and other primates (Henderson et al., 1976a, Warburton et al., 1976b). Probably the genes for nucleolar proteins are also dispersed among the genome. These nucleolar components may form a nucleolus-like body in the absence of the 18S and 28S rRNA precursor.

#### 2.1.5. Nucleolus-associated heterochromatin

Nucleolus-associated chromatin can be detected in situ both at the periphery and within the nucleolar body. The ribosomal cistrons are localized within the nucleolus when they are being actively transcribed (Jordon and Luck, 1976). In some species the nucleolus has been reported to be surrounded by heterochromatin or associated with chromocentres, e.g. man (Lima-de Faria et al., 1965;

Stahl and Luciani, 1972; Stahl et al., 1975) and mouse (Natarajan and Cropp, 1972). Some of this nucleolus-associated chromatin may have a role in the structural arrangement of the nucleolus (Bachelier et al., 1977). To support this hypothesis, these authors point out that various factors such as antibiotics and thermic shock which affect nucleolar structure act primarily on the chromatin component.

While there may be a structural role for heterochromatin in the nucleolus, the high frequency of association of some specific chromosomal regions with the nucleolus suggests that they may have a more specific role. In man, several chromosome regions are frequently found associated with the nucleolus e.g. the heterochromatic portion of the Y chromosome (Bobrow et al., 1971; Gagné et al., 1972; Tishler et al., 1974) and the centromeric heterochromatin of chromosome 1 and chromosome 9 (Stahl et al., 1976). Each of these regions may sometimes contain a secondary constriction. Ferguson-Smith (1964) reported that a small nucleolus was sometimes seen to be located on the midpoint of a bivalent and that this was not due to the association of two bivalents. More recently, the elaboration of nucleolar constituents by chromosome 9 in pachytene and diplotene stages of human meiotic preparation has been shown (Page, 1973; Stahl et al., 1975). This site does not contain genes for 18S and 28S rRNA (Evans et al., 1974). Therefore some secondary constrictions other than those known to transcribe 28S and 18S rRNA may have a nucleolar function and this is manifest by occasional association with the nucleolus in pachytene and diplotene.

#### 2.1.6. Staining techniques in the identification of NORs

The first technique described which specifically stains the NORs was N-banding (Matsui and Sasaki, 1973; Funaki et al., 1975). This is a modified Giemsa technique involving serial extraction of DNA, RNA and acid soluble proteins. A second technique using  $\text{NaNO}_2$  was described by Eiberg (1974).

In 1974 an ammoniacal silver staining technique called AS-111 which selectively stains the secondary constriction region of chromosome 9 in man and occasionally the regions near the centromeres of the acrocentric D and G group chromosomes was reported. (Howell and Denton, 1974). This was followed by the development of the A-S technique which selectively stains the satellite regions of the human D and G group chromosomes (Howell et al., 1975). The N-banding technique, the satellite staining of Eiberg, and the A-S technique were thought by their authors to selectively stain the satellites of the acrocentric chromosomes but evidence is accumulating that it is the secondary constriction region or satellite stalk which is stained (Niikawa and Kajii, 1974; Goodpasture et al., 1976; Ferraro et al., 1977). The satellite body can sometimes be seen above the silver-stained or N-banded segment.

Two more silver-staining techniques have since been developed - the ammoniacal silver (Ag-AS) technique (Goodpasture and Bloom, 1975) and an improved silver technique (Ag-I) (Bloom and Goodpasture, 1976). These authors showed that the Ag-AS technique stains the chromosomal locations at which the genes for 18S and 28S rRNA are located as shown by in situ DNA/rRNA hybridization in several mammalian cell lines. In man, the 'stalks' of the D and G group satellites are stained, but not the other nucleolus-associated chromosome regions such as chromosome 9. Thus only secondary constrictions corresponding to 18S and 28S rDNA are equivalent to Ag-NORs in mammalian mitotic chromosomes.

Although the silver staining techniques are specific for the chromosomal location of the genes for 18S and 28S rRNA, the precise nature of the silver-staining material has not yet been determined. The techniques stain chromosomal proteins rather than nucleic acids as the reaction is not affected by either DNase or RNase but is prevented by brief pretreatments with trypsin or pronase (Goodpasture and Bloom, 1975). There is a direct correspondence between the areas positive for RNA and the silver-staining material, suggesting that the silver stains protein intimately associated with rRNA transcribed at these sites (Howell, 1977). The protein fraction

seems to be acidic as shown by its insolubility in acid but slow solubility in dilute alkali (Howell et al., 1975; Howell, 1977). The techniques show some non-specificity if the silver treatment is prolonged. The centromeres, whole chromosomes, Golgi apparatus, neurofibrils, reticular fibrils and centrioles may be silver stained (Howell et al., 1977; Schwarzacher et al., 1978).

The nature of the silver-staining reaction has been the subject of speculation. Since active chromatin is richer in non-histone proteins than heterochromatin, Ag-staining could indicate an acid protein present in high concentration at regions active in rRNA production (Stocker, 1978). An alternative hypothesis is that the Ag-positive material is a component of the ribonucleic protein of the nucleolus which accumulates around the NORs (Schwarzacher et al., 1978).

While the silver-staining techniques are apparently specific for active ribosomal cistrons in mammalian mitotic chromosomes, the technique is not as specific in other systems such as the lampbrush chromosomes of crested newts in which it stains additional sites (Varley and Morgan, 1978).

#### 2.1.7. Duplicate banding

The Ag-AS and Ag-I staining techniques described, identify the NORs but in karyotypes where more than one NO-chromosome has a similar morphology, the technique does not allow identification of individual chromosomes. Such is the case in the D and G group chromosomes in man. In these it is desirable to combine silver staining with a banding method. Zankl and Bernhardt (1977) used the Ag-I technique followed immediately by a G-banding technique using a trypsin pretreatment to obtain simultaneous Ag-staining and G-banding. A similar technique using an Ag-AS pretreatment has also been described (Howell and Black, 1978). Another approach to the problem is to use a banding technique followed by destaining and then silver-staining. Fluorescent banding followed by silver staining was used initially (Bloom and Goodpasture, 1976; Tantravahi et al., 1976). It has subsequently been shown that silver staining can be used after C-, R- and G-banding (Tantravahi et al., 1977).

### 2.1.8. Robertsonian translocations and NORs in sheep

The association of NORs in humans has been postulated to be a causative factor in Robertsonian translocations and non-disjunction in man (Ferguson-Smith and Handmaker, 1961, 1963; Ohno et al., 1961). Robertsonian translocation appears to be the major mechanism of chromosomal change during evolution in the genus Ovis (Nadler et al., 1971; 1973). Such changes also occur frequently during culture in cell lines derived from Ovis (Nelson-Rees et al., 1965).

In domestic sheep, Ovis aries three different Robertsonian translocations have been reported to be polymorphic in some New Zealand sheep flocks (Bruère and Mills, 1971; Bruère et al., 1972; Bruère, 1973). These have been called the  $t_1$ ,  $t_2$  and  $t_3$  translocation chromosomes and they are respectively -  $t(5,26)$ ,  $t(8,11)$  and  $t(7,25)$  (Bruère et al., 1974). In view of the possible role of NORs in Robertsonian translocations in man, it was important to determine whether NORs may be related to the chromosomes involved in Robertsonian translocations in sheep. Acrocentric associations occur frequently in sheep cells (Bruère, 1966) but the chromosomes involved have not been identified. The location of the NORs of sheep had not been previously determined although a detailed study of the location of secondary constrictions in sheep has been made (Bruère and McLaren, 1967). These authors observed secondary constrictions on both metacentric and acrocentric chromosomes. The higher frequency on the metacentric chromosomes led these authors to postulate that the metacentric chromosomes were the principal NO-chromosomes in sheep. More recently it has been shown that the areas which approximate constriction regions on the metacentric chromosomes are late-replicating (Zartman and Bruère, 1974). Since the NORs in man have also been shown to be late replicating (Schmid, 1963; Sofuni and Sandberg, 1967) , it was suggested that this was support for the hypothesis that these were the NORs of sheep. However, late replication may not be a property of NORs per se but due to their location within segments of heterochromatin in many species. Thus the NOR of the fruit bat, Carollia perspicillata is not bordered by detectable heterochromatin (Hsu, 1975) and this region is not late-replicating (Pathak et al., 1973). C-banding of sheep

metaphases shows that the heterochromatin is located at the centromeres only (Evans et al., 1973), although the frequent areas of secondary constrictions do correspond to zones of heavy G-bands (Zartman and Bruère, 1974).

No information on the association of nucleoli with specific chromosome regions in either mitotic or meiotic preparations of sheep has been reported. In a study on meiosis in the ram, no evidence of a nucleolus was seen in any meiotic preparations, probably due to the disruptive effects of the hypotonic used during preparation (Chapman, 1974). Basal 'knobs', thought to correspond to heterochromatin, were observed at the centromeres but were not reported at any other chromosome location.

When this study was begun the location of the NORs in sheep had not been determined, although the distribution of secondary constrictions was known. The Ag-AS and Ag-I techniques provided a simple method of identifying the NORs to determine their relationship to the Robertsonian translocations which have occurred in this species.

## 2.2 Materials and Methods

### 2.2.1 Blood culture and harvesting techniques

Whole blood was cultured by the standard procedure used in our laboratory (Appendix II). After 48-72 hours at 37° the cultures were harvested and flame-dried slides were prepared.

### 2.2.2 Animals

Robertsonian translocation homozygotes ( $52, t_1 t_1$ ,  $52, t_2 t_2$ ,  $52, t_3 t_3$ ), multiple heterozygotes with  $2n=51$  or  $52$  (e.g.  $51, t_1 t_2 t_3$ ,  $52, t_1 t_2$ ) and heterozygotes-homozygotes with a  $2n$  of 49, 50 and 51 (e.g.  $49, t_1 t_1 t_2 t_3 t_3$ ,  $51, t_1 t_2 t_2$ ) were used in the identification of the nucleolus organizer chromosomes. The sheep are mainly derived from the Romney, Drysdale and Perendale breeds (Appendix VIII).

### 2.2.3 Ag-AS technique

The technique used was essentially the method of Goodpasture and Bloom (1975). Three solutions were used, and each will keep for up to three days. Silver solutions were kept in foil-wrapped bottles in the refrigerator.

**Solution 1:** 50% aqueous solution of silver nitrate made up in deionized water.

**Solution 2:** Ammoniacal silver solution prepared by slowly stirring 4g of silver nitrate into a solution of 5cc deionized water and 5cc of concentrated ammonium hydroxide (pH 12).

**Solution 3:** Developing solution of 100cc of 3% formaldehyde neutralised with 3.2g of sodium acetate crystals and then adjusted to pH 5.5-5.7 with formic acid.

The aqueous silver solution was pipetted on to a slide, covered with an alcohol-washed coverglass, and the slide placed about 25cm below a 500 watt floodlight until white crystals appeared around the edge of the coverglass. This usually took 6-10 minutes. The coverglass was rinsed off in deionized water and the slide developed by adding four drops of the ammoniacal silver solution followed immediately by four drops of the developing solution. The slide was coverglassed and the staining process was monitored under the microscope. This usually took 10-60 seconds. When the chromosomes turned yellow, the slide was rinsed in deionized water, dehydrated through an ethanol series (70%, 90% and 100%) for about one minute in each, soaked for 10 minutes in xylol and mounted in DPX.

If the chromosomes did not stain with the ammoniacal silver and developing solutions the coverlips were rinsed off and the slide was treated again with the aqueous silver solution followed by the ammoniacal silver and developing solutions.

A flow diagram of the Ag-AS and Ag-I techniques is shown in Fig. 2-3.

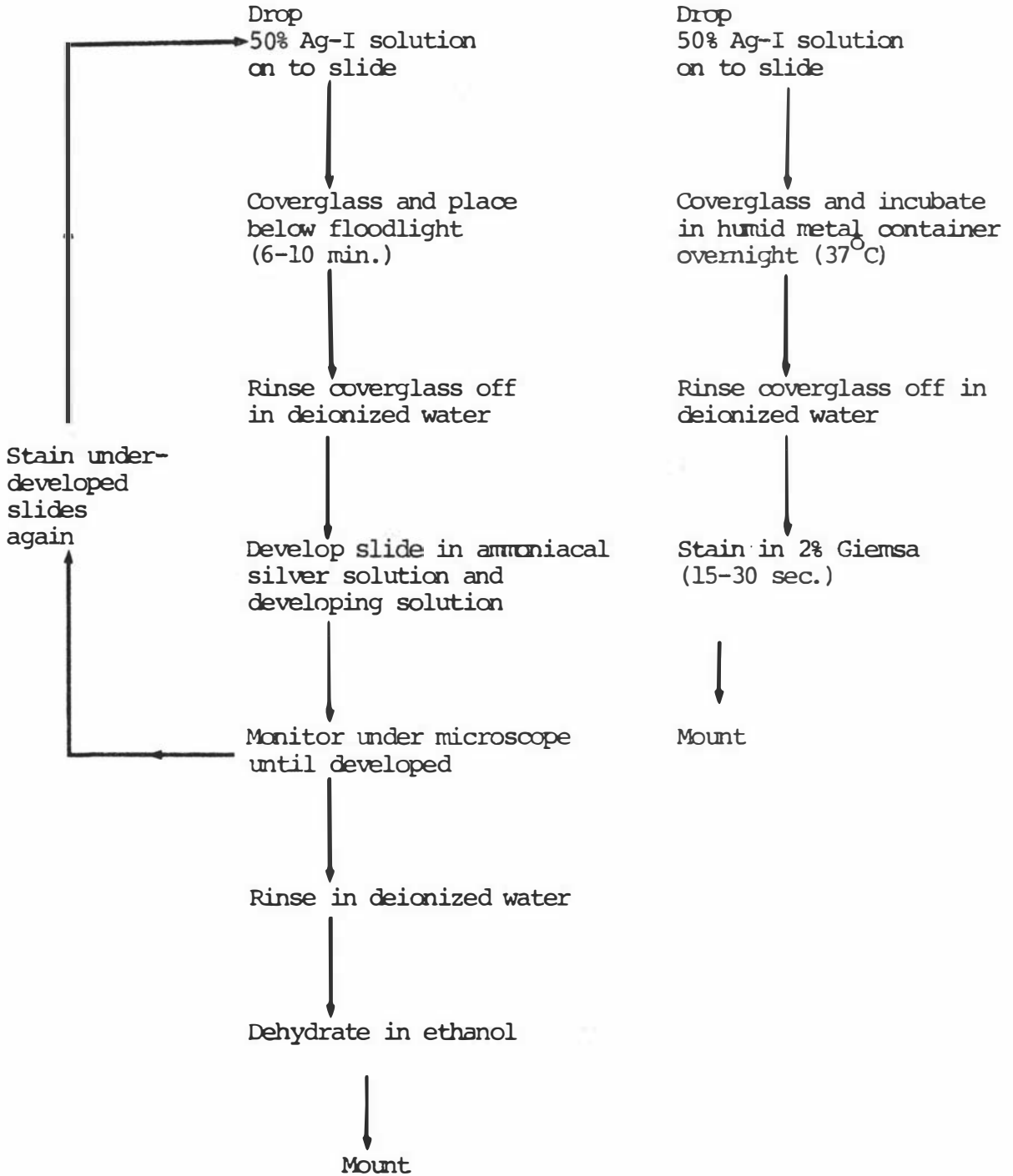
Ag-AS techniqueAg-I technique

Fig. 2-3: Flow diagram of Ag-AS and Ag-I staining techniques

#### 2.2.4 Ag-I technique

The technique used in this study was the technique described by Bloom and Goodpasture (1976). Three drops of a freshly prepared 50% solution of silver nitrate in deionized water were placed on each slide. The slides were then coverslipped and incubated in a sealed metal container in a 37°C incubator for 16 hours. The container was kept moist by covering the bottom with deionized water. The slides were rinsed twice in deionized water and stained in 2% Giemsa made up in buffered water (Gurrs buffer tablets pH 6.8) for 15-30 seconds. They were then mounted in DPX.

Ag-I staining was also used following normal Giemsa staining and destaining in a methanol-acetic acid (3:1) mixture. Giemsa-stained metaphases containing chromosomes with secondary constrictions were photographed, destained and then the location of the Ag-NORs was observed after Ag-staining.

#### 2.2.5 Duplicate G-banding and Ag-I staining

Slides were incubated overnight in a 50% silver solution as above. They were then rinsed in deionized water, dehydrated for two minutes in 95% alcohol and incubated in 2xSSC for 1½ hours. After rinsing in normal saline for one minute, the slides were flooded in 0.02% trypsin (1:250 Difco) in saline for about 60 seconds, washed in 1/15M Sorensen's buffer (pH 6.8) and then stained for 5-12 minutes in 2% Giemsa made up in Sorensen's buffer. Finally the slides were rinsed in Sorensen's buffer.

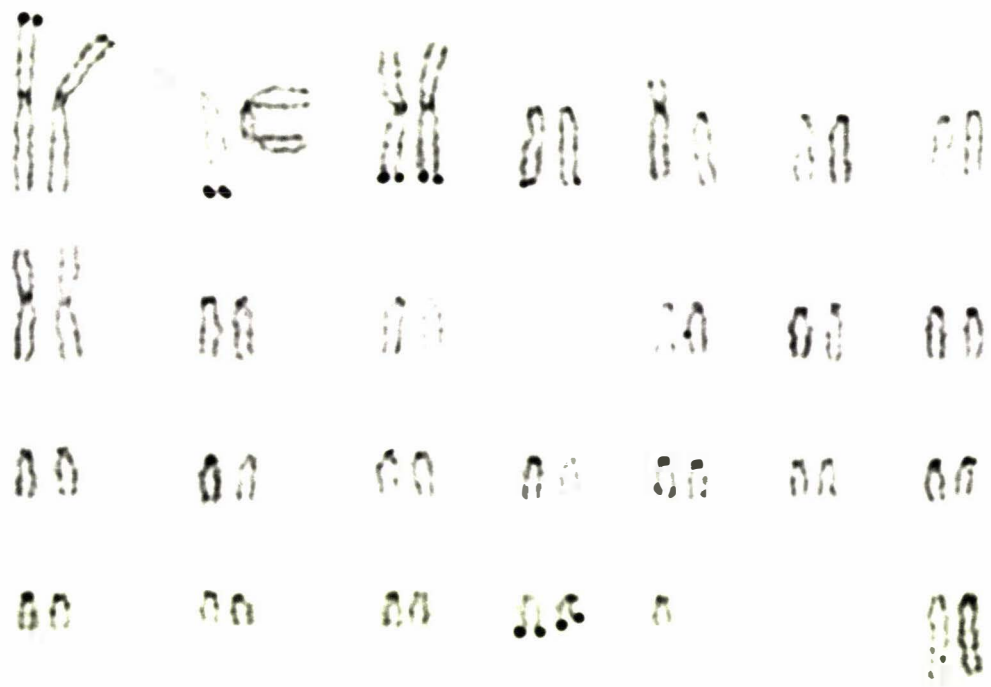
### 2.3 Results

#### 2.3.1 Location of nucleolus organizer regions

Silver was deposited terminally on one arm of each of the meta-centric chromosomes, a large acrocentric pair and a small acrocentric chromosome pair. Similar results were obtained for both the Ag-AS technique and the Ag-I technique. The maximum number of Ag-NORs per cell observed was 10, but not all cells showed this number (range 2-10).

Fig. 2-4: Karyotype of a  $51,t_1t_2t_2$  ewe  
showing Ag-NORs on both C1s, one C2,  
both C3s, C4s and C25s

(Ag-AS)



x x

Fig. 2-5: Karyotype of a  $50,t_1t_1t_2t_3$  ram showing the location of a NOR on the  $t_3$  chromosome. Ag-NORs are also present on 5 metacentrics, both C4s and C25 (Ag-I)

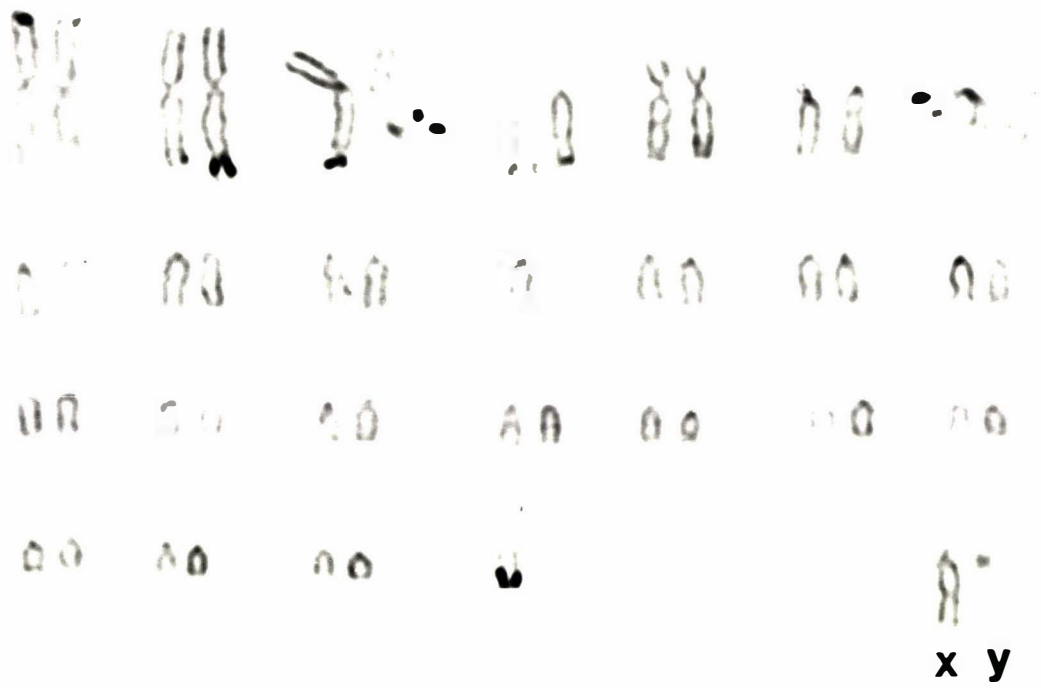


Fig. 2-6: Metaphase cell showing absence of  
Ag-staining on a secondary constriction  
(Ag-I, x 3,400)



Fig. 2-7: Absence of silver-staining on secondary constrictions in 3 metaphases stained by Giemsa, destained and Ag-stained. (Giemsa and Ag-I, x 2,500)

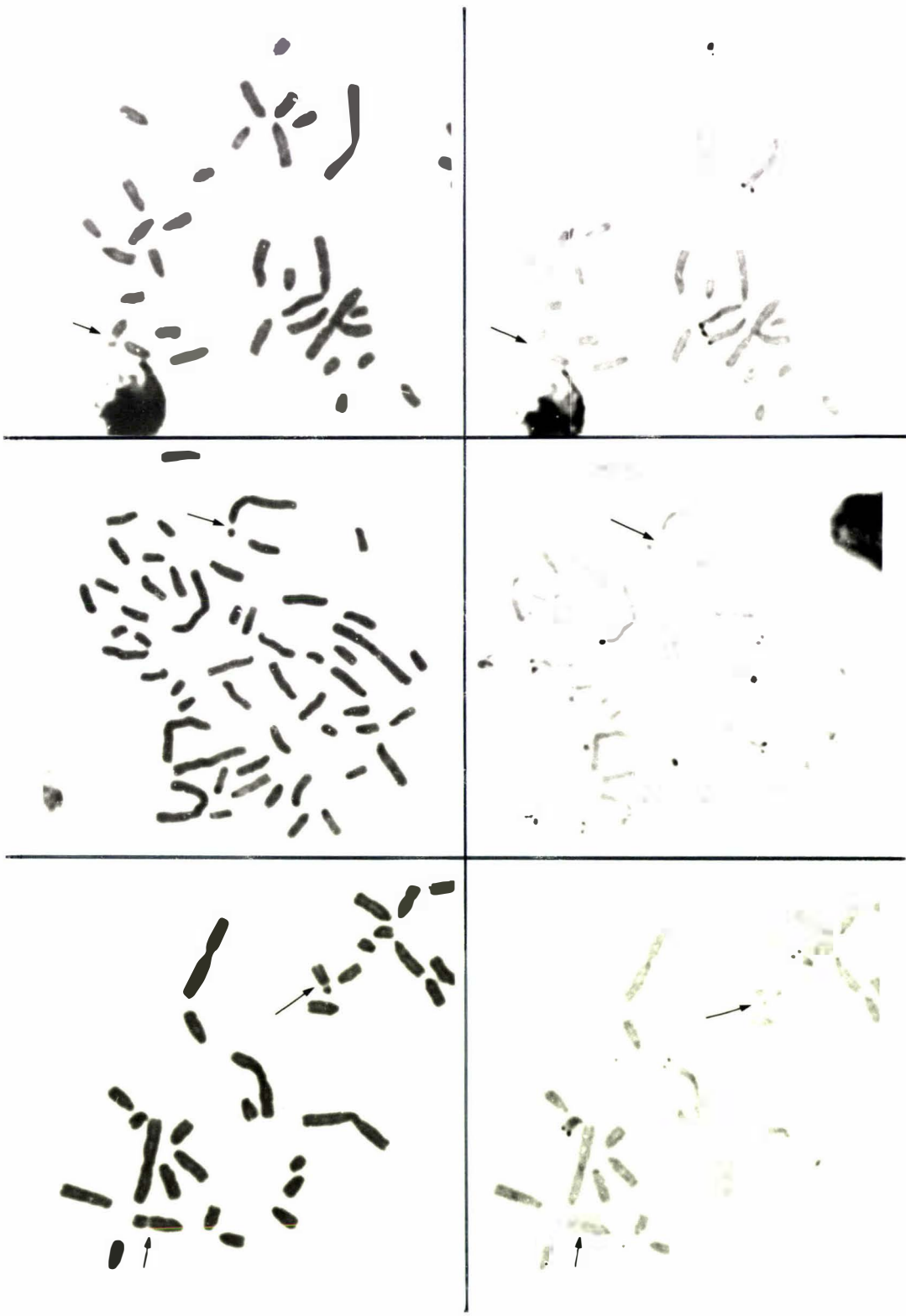


Fig. 2-8: Metaphase cell stained by the  
Ag-AS technique

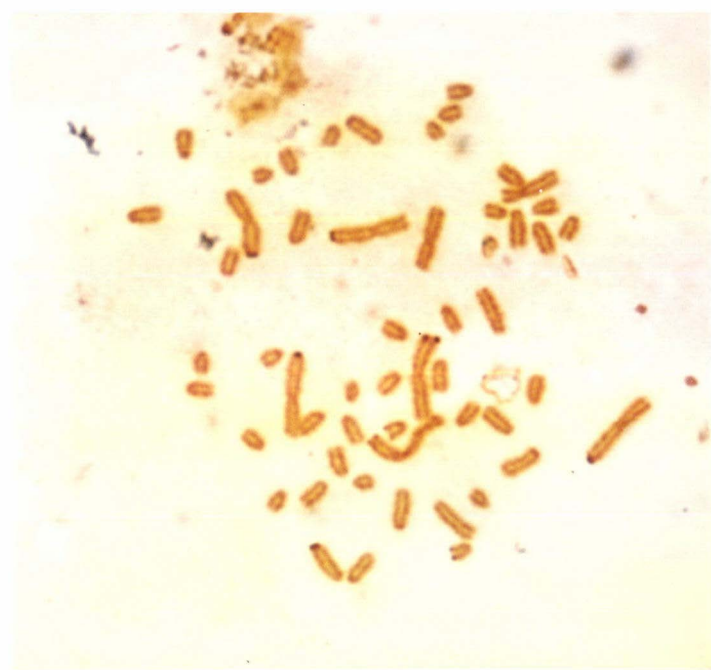


Fig. 2-9: Metaphase cell stained by the  
Ag-I technique

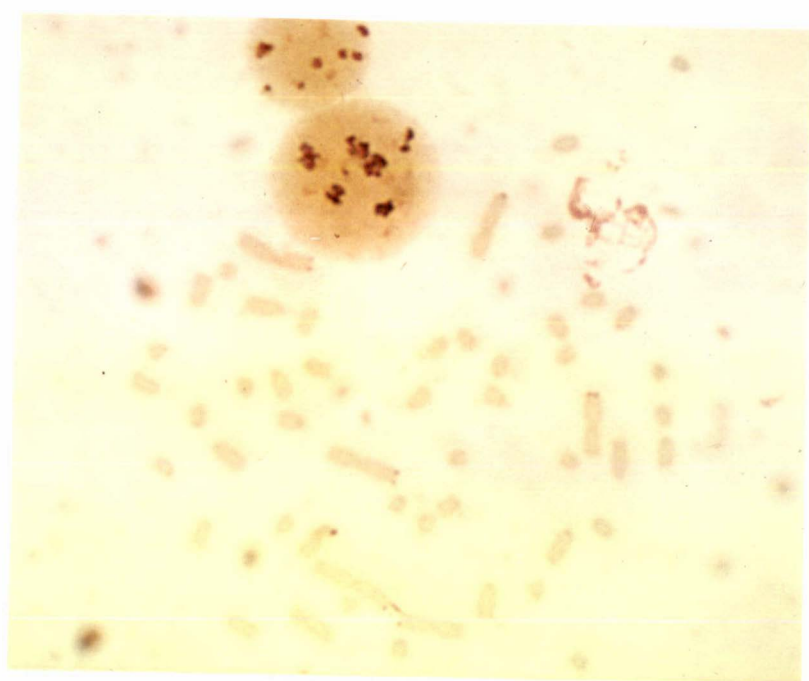


Fig. 2-10: Metaphase cell treated by the  
Ag-I technique and then G-banded.  
Ag-deposits are indicated by arrow heads

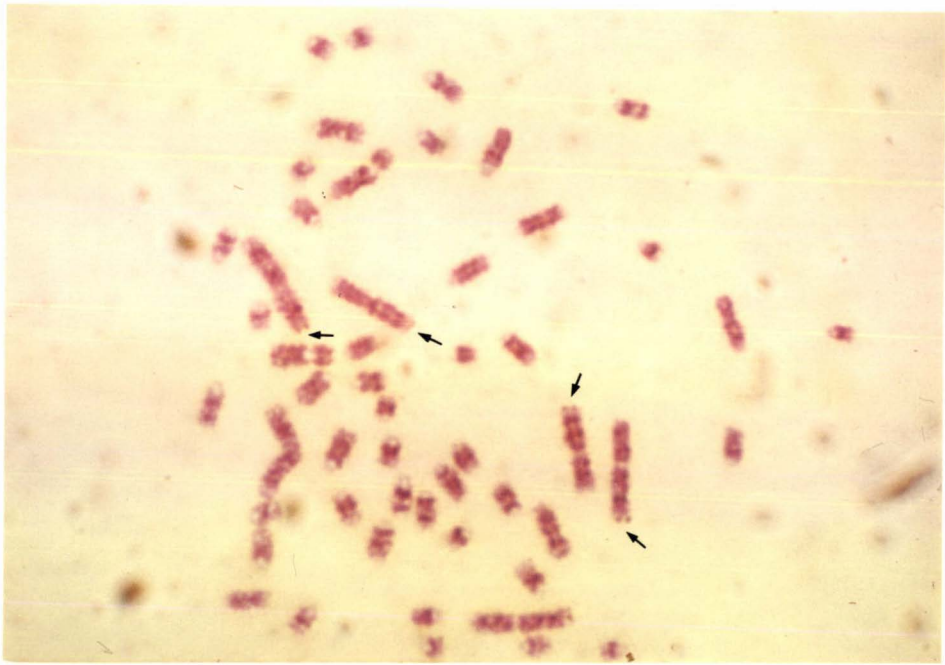
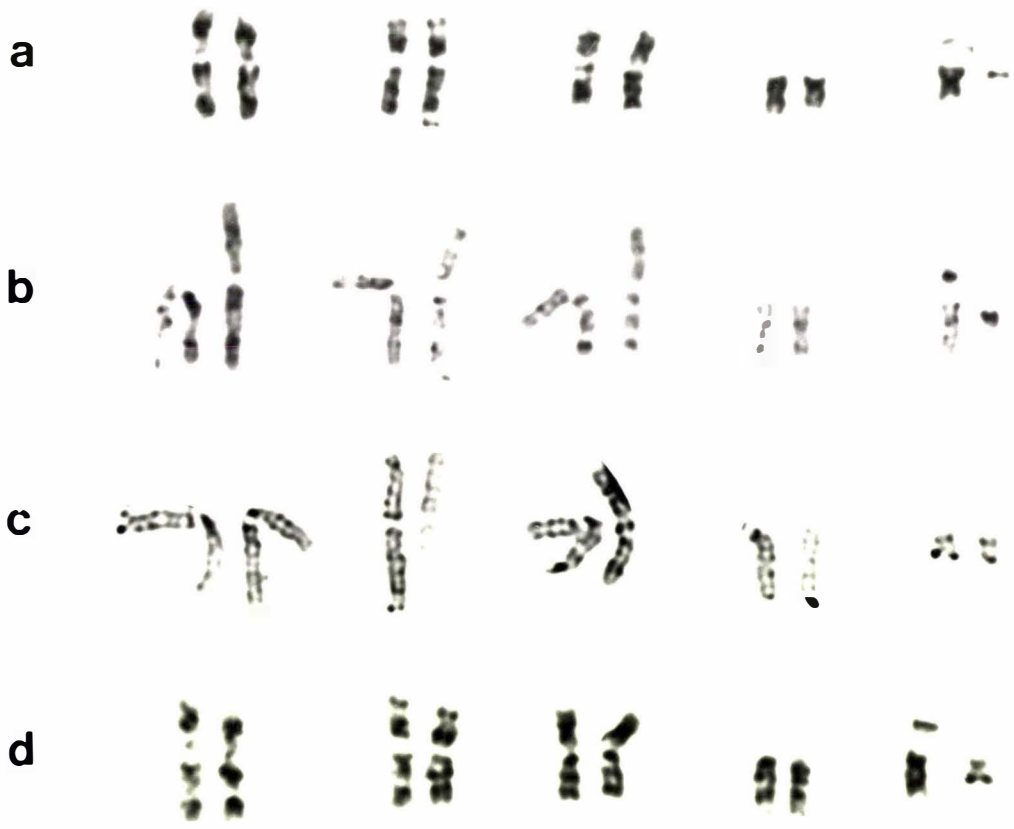


Fig. 2-11: Karyotype of NO-chromosomes  
identified by duplicate G-banding and  
Ag-I staining.

- a. NORs are located on both C1s and C2s
- b. NORs are located on C1, both C2s, C4 and t<sub>3</sub>
- c. NORs are located on C1, both C2s, C3, both  
C4s, and both C25s.
- d. NORs are located on C1, both C2s and C25



A study on the frequency of Ag-NORs is presented in the following chapter. Karyotypes were prepared of the metacentric chromosomes from 70 cells to determine which metacentric arms were involved in nucleolus organization. It is sometimes difficult to distinguish the metacentric chromosomes on the basis of arm length only in non-banded chromosomes. A tentative assignment was made to chromosomes 1p,2q and 3q. (In this work the p and q nomenclature is used to describe short and long arms respectively (Paris Conference, 1971).) A duplicate G-banding and Ag-I staining technique confirmed this (Section 2.3.4). The large acrocentric pair showing Ag-NORs appeared to be one of the three largest acrocentric pairs. The X chromosome was excluded by the presence of two Ag-NORs on a large acrocentric pair in rams. Since the  $t_1$  chromosome, t (5,26), did not show Ag-NORs, chromosome 5 was excluded and the Ag-NOR was assigned to chromosome 4. The smallest acrocentric pair was shown to be chromosome 25 since the short arm of the  $t_3$  chromosome t(7,25) had an Ag-NOR. Karyotypes showing the location of the ten Ag-NORs are shown in Figs. 2-4 and 2-5.

In the few metaphases in which secondary constrictions were visible, these did not have Ag-deposits (Fig.2-6). Also, in Giemsa stained metaphases with secondary constrictions, destaining and subsequent silver staining showed the secondary constrictions did not correspond to Ag-NORs (Fig. 2-7).

### 2.3.2 Ag-AS technique

In well stained cells treated by this technique the chromosomes appear yellow, with large black dots on the NORs (Fig 2-8). Frequently the centromeres also stain a brown-black colour. Variations in the size of the dots on individual chromosomes can be seen. The technique requires careful monitoring during the second Ag solution treatment for satisfactory results. The problems with the technique are that it is inconsistent, and often results in chromosome distortion to the extent that identification is impossible. Overstaining results in the loss of differential between the chromosomes arms and Ag-NORS.

### 2.3.3 Ag-I technique

This technique is simple and works consistently on good preparations. The Ag-NORs appear as black dots, while the chromosome arms stain blue or green depending on the extent to which the chromosomes have stained yellow with the Ag solution (Fig. 2-9).

In the early stages of using the Ag-I technique the slides were initially counterstained in Giemsa for 15 seconds, then observed under a microscope to check the intensity of stain. If this was too pale they were restained until a satisfactory intensity was observed. Subsequently it was found that staining for 30 seconds without monitoring gave consistent results.

With both the Ag-AS and Ag-I techniques the best preparations were obtained when the slides were less than one month old. Slides older than this often gave unsatisfactory results. Initially problems were experienced with the stain fading so that the chromosomes disappeared within a month of staining. This appeared to be due to the xylol used as subsequently slides which were mounted immediately after staining without immersion in xylol did not fade.

The Ag-AS technique was used only in the initial stages of this study as the Ag-I technique proved to be much simpler and more consistent.

### 2.3.4 Duplicate banding

A duplicate banding procedure was necessary to positively identify the metacentric chromosome arms with NORs and to confirm that chromosome 4 was the large acrocentric NO-chromosome pair. The technique used here produced faint black Ag-NORs against red-banded chromosomes (Fig. 2-10). The tentative assignments to 1p, 2q, 3q and chromosomes 4 and 25 were confirmed (Fig. 2-11).

The G-banding technique involved trypsin pretreatment, which resulted in a decrease in the size of the silver deposits so that

Fig. 2-12: Karyotype of a cell with  
Ag-deposits on both telomeres of a  
chromosome 1  
(Ag-I)



Fig. 2-13: Ag-stained cells showing differences  
in the amount of Ag-staining material at  
different stages of the cell cycle.

- a. Interphase cell
- b. Prophase cell
- c. Metaph<sup>s</sup><sub>h</sub>e cell surrounded by interphase cells
- d. Metaphase cell and interphase fibroblast cells  
(Ag-I, x 2,500)

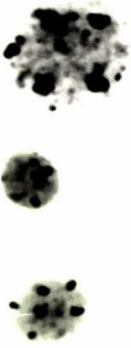
a



b



c



d



most metaphases showed only G-banding and not Ag-staining, or weak Ag-staining which made photography difficult, although identification of the chromosomes by microscope examination without karyotyping was possible.

#### 2.3.5 Anomalous cell

During the course of this study an estimated 2000 silver-stained metaphases were studied. In one cell stained with Ag-I an anomalous Ag-deposit was observed. In this cell chromosome 1 had Ag deposits occurring terminally on both p and q arms (Fig. 2-12). In 30 other cells from this animal only the lp had an Ag-NOR.

#### 2.3.6 Ag-staining in interphase,prophase and metaphase cells

The amount of Ag-staining in transformed lymphocytes at interphase exceeded the amount of Ag-staining material at metaphase. A decrease in Ag-staining material appeared to occur from interphase through prophase to metaphase. Fig. 2-13 shows the amount of staining on cells at different stages of the cell cycle.

### 2.4 Discussion

#### 2.4.1 Structure of sheep nucleolus organizer regions

The NORs of sheep chromosomes occur terminally and therefore they are not manifest as secondary constrictions in metaphase chromosomes. Since the telomeres of sheep chromosomes are not stained by the C-banding technique it appears that the NORs in this species are not located in heterochromatin. Although all the satellite DNA is found at the centromeres in sheep (A.R. Mitchell, pers.comm.) when satellite DNA is purified from sheep nuclei, the major satellite DNA fraction is found in the pure nucleolar fraction (Curtain et al., 1973). This suggests an association of heterochromatin and the NOR which is not the result of the chromosomal location

of the NOR in heterochromatin.

In many species the NORs are located adjacent to heterochromatin on the chromosomes e.g. in mice (Henderson et al., 1974a; Elsevier and Ruddle, 1975) and various primate species (Gosden et al., 1978). There is also evidence that heterochromatic regions which do not contain rDNA are associated with the nucleolus in interphase (see section 2.1.5). It has been suggested that the nucleolus-associated heterochromatin has some functional role (Stahl and Luciani, 1972; Gosden et al., 1978). The results from this study showing that the association of heterochromatin with nucleoli is not a result of the chromosomal location of the NOR, support this view that heterochromatin has a structural or functional role in the nucleolus.

#### 2.4.2 Possible roles of the secondary constrictions on sheep chromosomes

The Ag-I and Ag-AS techniques stain the regions at which 28S and 18S rRNA is transcribed (Goodpasture and Bloom, 1975). Therefore the secondary constrictions in sheep as studied by Bruère and McLaren (1967) are not the NORs as defined in this thesis.

Secondary constrictions which are not the nucleolus organizers may have several functions:

1. They produce nucleolar products other than 18S and 28S rRNA.

There is evidence from other species that chromosomal regions

other than the NOR, which have some nucleolar function may be manifest as secondary constrictions. The secondary constrictions of sheep may be similar.

In man the secondary constriction of chromosome 9 is capable of forming a nucleolar body (Page, 1973), although this region does not possess the DNA coding for 18S and 28S rRNA. This region is frequently associated with the nucleolus in interphase (Stahl et al., 1976). Chromosome 1 may have a similar role and it also is associated with the nucleolus in interphase (Stahl et al., 1976). In mutants of Xenopus which lack NORs, a nucleolar-like body forms (Barr, 1966). The nucleolus also contains the 5S rRNA precursor and proteins, and these may be transcribed elsewhere in the genome, outside the NOR.

Two explanations, not necessarily exclusive, have been presented for the lack of condensation of the secondary constrictions which function in nucleolus organization. In the first, lack of condensation is due to the physical restraint of the bulky nucleolus body preventing condensation until the nucleolus disintegrates (Busch and Smetana, 1970). In the second, since transcription of rRNA continues until prometaphase (Arrighi, 1967) and RNA synthetic activity takes place only when the chromatin is decondensed (Taylor, 1960; Prescott and Bender, 1962) the nucleolus organizer region remains decondensed to allow transcriptional activity to occur (Hsu et al., 1967). Considering these 2 factors, the secondary constrictions in sheep could be regions which produce nucleolar products other than 28S and 18S rRNA and which are

manifest as secondary constrictions either due to their transcription until late pro-metaphase or to their physical association with the nucleolus, resulting in a physical restraint to condensation.

2. They may be regions which have a structural role in the nucleolus

Heterochromatin is frequently seen associated with the nucleolus (e.g. Lima-de-Faria et al., 1965; Natarajan and Gropp, 1972) and it has been suggested some nucleolus-associated chromatin may have a role in the structural arrangement of the nucleolus (Bachelierie et al., 1977).

Intra-nucleolar chromatin would be expressed as a secondary constriction if its condensation was delayed by nucleolar persistence. The secondary constriction regions on the metacentric chromosomes of sheep are late-replicating and correspond to dark G-bands. This may mean they serve a similar role to the heterochromatic regions associated with the nucleoli in other species.

3. These regions may be completely unrelated to the nucleolus but are regions which are occasionally transcribed until either late prophase or prometaphase.

4. These regions may be extended or decondensed due to external factors.

Various drugs (e.g. bromodeoxyuridine and bromodeoxycytidine) may prevent chromosome condensation and produce extended chromosome segments (Zakharov and Egolina, 1972). Other external factors may also be capable of interrupting normal

condensation. Alternatively uncoiling of the chromatin might be induced during the harvesting process. The type of hypotonic solution has been shown to affect the frequency of secondary constrictions in sheep (Bruère and McLaren, 1967). Some chromosome regions could be more susceptible to stretching or uncoiling during hypotonic treatment.

It is probable that the secondary constrictions reported in sheep are not a homogeneous group but may incorporate more than one of these types. Now that the location of the NORs in sheep is known, it is possible to study whether the secondary constrictions, or chromosome regions known to contain them, associate with the NORs. This could distinguish whether the secondary constrictions in sheep do have some nucleolar function other than 28S and 18S rRNA production. Another possibility is to apply the G11 banding technique, which stains only the centromeric region in chromosome 9 in man, to sheep to determine if there is a similar staining region in this species.

#### 2.4.3 Association of NORs

The terminal location of the NORs and the non-involvement of most of the chromosomes involved in Robertsonian translocations in domestic sheep shows that the NORs are unrelated to the formation of Robertsonian translocations in this species (see also section 7.4.6). Since the terminal areas of sheep chromosomes are not C-banded, it appears the NORs in this species are not located in heterochromatin. This allows a separation of the effects of heterochromatin attraction and NOR association. In man, where the secondary constriction containing the rDNA occurs between the centromeric

heterochromatin and the satellites, these two effects can not be distinguished. NOR association in sheep is the subject of Chapter 4 of this thesis. The acrocentric association in sheep metaphases is presumably due to a heterochromatin attraction as has been reported in man (Schmid *et al.*, 1975). It has been suggested that homologies of satellite DNA at the centromere might facilitate interchromosomal exchange at these sites, resulting in Robertsonian translocation (Buckland & Evans, 1978b). This may be the mechanism of Robertsonian translocation formation in sheep and related species.

#### 2.4.4 Applications of silver-staining

The silver-staining technique has applications as a simple chromosome identification technique. In sheep, the  $t_1$  and  $t_3$  chromosomes can now be distinguished simply without the use of a banding technique.

The involvement of chromosome 25 in nucleolus organization allows the use of the  $t_3$  polymorphism in studies of the frequency of staining, association and inheritance of individual NO-chromosomes.

In the rest of this study, where the  $t_3$  polymorphism is involved, the unfused NO-chromosome will be referred to as chromosome 25 (C25) and the chromosome 25 component of the translocation chromosome will be called  $t_3$ . Chromosome 4 is abbreviated to C4, and the metacentrics are abbreviated to meta.

#### 2.4.5 Anomalous silver-staining regions

The anomalous silver-staining regions on the long arm of chromosome 1 in one animal found in this study could be a NOR which is normally inactive (Fig. 2-12). This seems unlikely in view of the fact that it was a unique occurrence.

It may be due to a somatic crossing-over event involving a NO-chromosome. Alternatively, the lq telomere might have some role related to the nucleolus which causes it to occasionally be

associated with NORs. The silver-staining protein associated with the NOR may sometimes become associated with this region.

### CHAPTER 3

#### THE FREQUENCY OF AG-NORS AND REGULATION OF NOR ACTIVITY

### 3.1 Introduction

#### 3.1.1 Evidence that silver stains active NORs.

Preliminary evidence that the silver staining techniques detect only those NORs which are active in rRNA production was found by silver-staining four mouse-human hybrid lines which selectively lost human chromosomes (D.A. Miller, et al., 1976). Although human acrocentric chromosomes were present, none of these had an Ag-NOR although the human parental cell line showed Ag-NORs when stained under the same conditions. Previously it had been shown that in a variety of mouse-human hybrid cell lines no human 28S rRNA is produced, even when a large number of human acrocentric chromosomes are present (Eliceiri and Green, 1969). In view of this, it was suggested that silver staining reflects the activity of rRNA genes rather than just the presence of these genes. In mouse-human hybrid cell lines which selectively lose mouse chromosomes and in which mouse 28S rRNA production is suppressed (Croce et al., 1977), only the human acrocentric chromosomes have Ag-NORs (O.J. Miller, et al., 1976). This suggests that there is some correlation between preferential chromosome elimination and preferential suppression of nucleolus organizer activity.

In contrast to the situation in mouse-human hybrid cells, where only one type of 28S rRNA is produced, in mouse-human heterokaryons, in which the mouse and human genomes are in separate nuclei, but within one cell, both mouse and human 28S rRNA is produced (Marshall et al., 1975). It was suggested that suppression of human 28S rRNA in hybrid cells was due to a product of the mouse genome which was unable to penetrate the nuclear membrane. In light of the findings that in mouse-human hybrid cells it is sometimes the mouse rRNA synthesis which is suppressed, it is apparent that one species is not always dominant in rRNA production.

The presence of Ag-NORs on both mouse and Syrian hamster chromosomes in individual somatic hybrid cells has recently shown that transcription of the rRNA genes of both parental species can take place within single hybrid cells (O.J. Miller et al., 1978b).

A second piece of evidence for the hypothesis that the silver techniques stain regions producing rRNA was obtained from work on cricket oocyte chromosomes (Howell, 1977). These chromosomes are active in rDNA amplification and rRNA transcription during pachytene. Howell reported that the quantity of silver-stainable protein increases as rRNA transcription proceeds (although no evidence for this is mentioned in the results section of this report). Cytochemical tests on the NORs of cricket oocytes supported the previous finding that silver stains acid proteins associated with rRNA transcribed at the rDNA sites. In this same material, Czaker (1978) studied the silver-staining of the amplified rDNA material at various stages of oogenesis and found that the amount and distribution of silver-staining material agreed with the amount and distribution of extrachromosomal rDNA at the various stages.

Additional evidence that silver stains genes active in 18S and 28S rRNA synthesis was presented by Engel et al. (1977). They showed that the silver staining patterns in the 2-cell, 4-cell and 8-cell stages of mouse embryos agreed with known patterns of ribosomal RNA synthesis in early mouse embryonic development. Ag-NORs were first observed at the 2-cell stage, their number increased at the 4-cell stage and at the 8-cell stage all the chromosomes with NORs showed silver staining. Similar results were reported by Hansmann et al. (1978).

### 3.1.2 Regulation of NORs

#### 3.1.2.1 Variation in the level of rDNA

While the dosages of most genes within an individual remain constant throughout its development, in some organisms the level of rDNA may not be constant. Changes in rDNA may be due to either

'amplification', 'magnification' or 'dosage compensation' of the genes for rRNA. A variety of vertebrate and invertebrate oocytes have been shown by RNA-DNA hybridization experiments to undergo selective amplification of their ribosomal DNA during oogenesis (Gall, 1969) so that a large number of rDNA genes exist extra-chromosomally. The amplification process provides sufficient rDNA genes to cope with the demands of the oocyte during the period of intensive synthesis of rRNA during oogenesis.

'Magnification' refers to a phenomenon observed in 'bobbed' mutants of Drosophila (Ritossa, 1968). These mutants apparently have a reduced rDNA level, but over several generations 'bobbed' stocks tend to revert to wild-type by a gain of rRNA cistrons. It has been suggested that this process may be due to disproportionate replication of the rDNA (Tartof, 1973).

In contrast to the heritable rDNA increase in the 'magnification' phenomena, a somatic regulation process, termed 'dosage compensation' has been reported to occur in the somatic cells in Drosophila. In flies with 1 NOR the number of rRNA cistrons appears to undergo a replication up to the wild-type level (Tartof, 1973). This may however be due to extra rDNA replication in the polytene chromosomes only, as analysis of polytene chromosomes showed that the level of rRNA in them is independent of the number of nucleolus organizers (Spear, 1974).

Some evidence for non-additivity in rRNA gene multiplicity has been presented in wheat (Triticum aestivum). The addition of extra dosages of NO-chromosomes in wheat does not always produce a proportional increase in rDNA level (Flavell and O'Dell, 1976; Liang et al., 1977). The presence of some factor controlling the total number of rDNA genes has been proposed. No evidence of disproportionate replication of rDNA as a function of different amounts of nucleolar organizing material has been found in maize (Phillips et al., 1974; Doerschug, 1976).

### 3.1.2.2 NOR regulation in interspecies hybrids

In interspecies hybrids of a variety of plants and animals the nucleolus organizer activity of one parental genome is suppressed. This phenomenon is termed 'nucleolar dominance'. The first observation of this was in some hybrids of the plant genus Crepis where the secondary constriction of one of the parental genomes disappears (Navashin, 1934). This work was extended by Wallace and Langridge (1971) who showed various Crepis species could be ranked in a dominance series such that one species could prevent secondary constriction formation in any other species lower in the series. In certain hybrids the repressed chromosome can resume its NOR activity to form nucleoli during pollen formation when it is isolated from the dominant chromosomes only by a nuclear membrane and the intervening cytoplasm. Wallace and Langridge suggested that the nucleolus organizers of the various species were isoalleles which differ in their repressibility and the dominant organizer is the source of repression of its recessive iso allele. This is 'allelic repression', where one allele is dominant over another and represses its synthesis. 'Allelic repression' in Crepis is apparently not related to rDNA content as C. capillaris is dominant over C. dioscorides although these both have the same number of rRNA genes (Doerschug et al., 1976).

The dominance of one species' NOR over another has also been reported in Ribes, where crosses between gooseberry (R. grossularia) and blackcurrant (R. nigrum) result in the disappearance of the blackcurrant satellite (Keep, 1962). The satellite reappears when the parental genomes are separated by segregation.

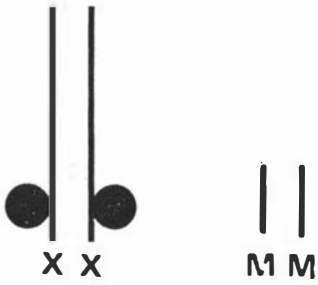
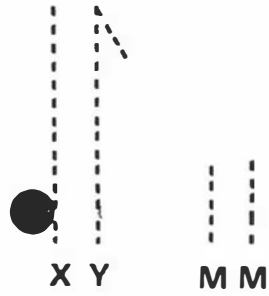
Evidence for a cytoplasmic repressor and a repressor product of the rDNA region of one parent have been obtained from crosses of the toads, Xenopus laevis and Xenopus mulleri. Cytological observations on the number of nucleoli (Cassidy and Blacker, 1974) have been coupled with the biochemical analysis of the rRNA products of the hybrids (Honjo and Reeder, 1973). In these hybrids the synthesis of X. mulleri rRNA is suppressed. However if the X. laevis rDNA is not present (as in crosses involving a X. laevis parent heterozygous for

the anucleolate mutant (see section 2.1.4) the X. mulleri rRNA synthesis is transiently suppressed if X. laevis is the maternal parent but no suppression occurs if X. laevis is the paternal parent. This suggests two factors may be involved in the suppression of X. mulleri rRNA synthesis. A product of the X. laevis rDNA may function as a repressor of X. mulleri rDNA transcription or the X. laevis rDNA may have a higher affinity than X. mulleri for some essential molecule in short supply. The maternal effect of X. laevis is suggestive of a repressor substance.

In Drosophila, evidence for regulation of NOR activity of one chromosome by another within a diploid organism, as well as interspecific regulation has been obtained. In D. mulleri and D. arizonensis, the NOR occurs only on the X chromosomes. Bicuodo and Richardson (1977) have studied the dominance relations of the NOR in hybrids of these species (see Fig. 3-1).

In female  $F_1$  hybrids of matings of D. mulleri females and D. arizonensis males only the D. arizonensis X chromosome is attached to the nucleolus. This again suggests an 'allelic repression' of the D. mulleri rDNA. However, in  $F_1$  males, the NOR on the X (derived from D. mulleri) is not expressed but instead a previously 'latent' NOR on the microchromosome derived from D. arizonensis is attached to the nucleolus. In this case suppression of the D. mulleri NOR cannot solely be due to the dominance of the D. arizonensis X chromosome. Bicuodo and Richardson suggested on the basis of this work that the D. arizonensis microchromosome and X chromosome contain ribosomal cistrons and a regulatory site which represses the D. mulleri NOR, and that in addition the D. arizonensis microchromosome is regulated by the D. arizonensis X chromosome so that it is only expressed in its absence.

Further evidence of nucleolar dominance in interspecific Drosophila hybrids have been found in crosses of D. simulans and D. melanogaster (Durica and Krider, 1977, 1978). The presence of the D. melanogaster NO-chromosomes prevented secondary constriction

D.mulleri femaleD.arizonensis male

M : Microchromosome


 : Chromosomal region associated with nucleolus

Fig. 3-1: Diagram of nucleolar dominance in F<sub>1</sub> hybrids of D.mulleri and D. arizonensis

formation in D. simulans and this was independent of any maternal effect. This dominance effect was not simply a function of the presence of D. melanogaster rDNA. Using an array of heterochromatic deficiencies surrounding the NO, it was shown that the establishment of nucleolar dominance was correlated with the presence of at least one heterochromatic region separate from the NO.

### 3.1.2.3 rRNA gene activation during development

Evidence that a cytoplasmic factor can control rRNA transcription was produced by the classic transplantation experiments of Gurdon and Brown (1965) in X. laevis. During development in this species, rRNA synthesis starts at the beginning of gastrulation. Gurdon and Brown showed that when a nucleus is transplanted from a tissue which is actively synthesizing rRNA into an unfertilised egg, rRNA synthesis stops. However rRNA synthesis recommences during gastrulation in nuclear-transplant embryos. These results suggest that the synthesis of rRNA is regulated by a cytoplasmic factor.

The hypothesis of a repressor substance regulating rRNA synthesis during development in Xenopus was supported by experiments in which extracts from blastula cells (in which no rRNA synthesis occurs) were added to isolated cells obtained from the gastrula stage (Yamana and Shiokawa, 1966). The extract inhibited rRNA synthesis while not affecting the synthesis of other RNAs, in the gastrula cells. This effect was reversible: removal of the inhibitor resulted in resumption of rRNA synthesis.

Additional information on the nature of the control of rRNA transcription has been provided by observations on cytological preparations of the oocyte chromosomes of the alpine newt, Triturus alpestris (Scheer et al., 1976 a and b). In this species marked differences in rRNA synthesis occur during the prolonged diplotene stage of oogenesis. The transcriptional activity of the oocyte chromosomes can be viewed directly by electron microscopic examination. Transcriptionally active units, characterised by the attachment of ribonucleo-protein fibrils that contain the growing precursor molecules for the rRNAs can be distinguished from the transcriptionally inactive, or 'naked' rDNA units. While in lampbrush chromosomes of the vitellogenic oocytes, all units are

transcriptionally active, mature oocyte nucleoli contain active and inactive regions, suggesting control of rRNA synthesis is mediated by the number of genes active in synthesis rather than alteration of the rate of transcription. The occurrence of transcribed and non-transcribed units on the same nucleolar axis suggested that each pre-rRNA gene is individually controlled, rather than the whole NOR being regulated by a single promoter unit. These investigators proposed that the regulation of transcription of rRNA genes is controlled by regulatory factors, probably protein, which interact either with RNA polymerases or directly with the template.

This work is in close agreement with the electron microscopical observations, also employing a microspreading technique, on spermatocyte nucleoli of D. hydei (Meyer and Hennig, 1974). They reported the synthesis of rRNA appeared to be primarily regulated by the activation or inactivation of varying numbers of ribosomal cistrons. Meyer and Hennig suggest that the regulation of adjacent units may be correlated to the gradual unfolding of the DNA of the nucleolus organizer.

A similar suggestion that chromatin structure has a role in the regulation of the transcriptional activity of the rRNA genes has been made on the basis of an electron microscopic study of different embryonic stages of the milkweed bug, Oncopeltus fasciatus (Foe, 1978). Transcriptional activation of ribosomal genes appears to be preceded by a change in chromatin structure from beaded to non-beaded chromatin. In this report data was also presented to show that changes in rRNA synthesis result from changes in the mean number of active ribosomal genes per nucleus, as well as from changes in the level of transcriptional activity of each activated gene.

#### 3.1.2.4 The effect of NOR dosage on NOR activity

In the absence of control mechanisms, changes in the dosage of NORs in an organism might be expected to lead to a change in the rRNA

content and number of nucleoli in a cell. In some organisms a linear relationship between the number of NORs and the number of nucleoli is found. Normally X. laevis has two nucleoli formed per cell but animals heterozygous for the anucleolate mutant produce only one nucleolus (see section 2.1.4). The number of nucleoli in human meningioma cell cultures is dependent on the number of acrocentric chromosomes (Zankl and Zang, 1972). And in rat kangaroo cells, one nucleolus is found in diploid cells, two nucleoli in tetraploid cells and three nucleoli in hexaploid cells (Branch and Berns, 1976).

Several studies have looked at the effect of changes in NOR dosage on nucleolar mass or rates of rRNA synthesis. In wheat, an increase in the number of nucleolar chromosomes was reported to result in an increased number of nucleoli per cell, and also an increase in the volume and dry mass of the nucleoli (Longwell and Svihla, 1960), indicating a lack of regulation of rRNA production. In other reports a regulation of rRNA production appears to occur when different dosages of the NOR are present. In X. laevis although the heterozygous anucleolate mutant produces only one nucleolus, it is equal in size to that formed by the fusion of the two nucleoli in homozygous normal individuals (Barr and Esper, 1963), indicating some compensation in rRNA production when only one NOR is present. In human meningioma cell cultures, the nucleolar area is independent of the number of acrocentric chromosomes (Zankl et al., 1973) although the number of nucleoli is dependent on the number of acrocentric chromosomes. Again, this indicates that the overall production of rRNA in a cell is independent of the number of NORs. A similar situation occurs in D. melanogaster, where the X and Y chromosome each has a NOR. Thus XO, XX, XY and XXY flies have 1, 2, 2 and 3 NORs respectively. However, the rRNA content of these flies is similar (Kiefer, 1968) and the rate of synthesis of nucleolar RNA appears to be independent of the number of nucleolus organizers (Krider and Plaut, 1972). Krider and Plaut also found that the equality of the rates of nucleolar RNA synthesis in stocks with 2, 3, or 4 dosages of the NOR is accounted for by their having only two functional organizers.

Thus there is regulation in the number of active NORs. However, since XO males with only one organizer synthesize rRNA at the same rate as flies with two NORs, another control form which modulates the activity of the cistrons of the organizer must also be present.

### 3.1.2.5 The effect of NOR dosage on Ag-NOR numbers

Conflicting studies have been reported on the effect of dosage of NOR on the number of active NORs as shown by silver-staining. In the first of these, human tumours with an increased number of acrocentrics were found to have no increase in the number of Ag-NORs above control diploid cell lines (Hubbell and Hsu, 1977). This was thought to indicate a regulation of NOR activity. In contrast, cells with an increased number of NORs from a human fibrosarcoma line were found to have an increase in the number of Ag-NORs compared with the normal cells from this cell line (D.A. Miller *et al.*, 1978a). Similarly in cell lines derived by hybridization of this cell line and mice cells and rat cells, an increase in the number of Ag-NORs was found. Thus there was no evidence for inactivation.

In another study, the numbers of Ag-NORs in a HeLa derivative cell line and a daughter cell line which is a carrier of measles virus were compared (Heneen, 1978). The measles carrier cell line had a lower number of Ag-NORs although these appeared to have larger deposits than the Ag-NORs of the control cell line. Although both cell lines are near triploid, the measles carrier line has a lower number of acrocentrics present so that there is a correlation between the average number of acrocentrics and the number of Ag-NORs. In all except one case chromosomes which were active in the control cell line were active, if present, in the carrier cell line. The increased size of the Ag-NORs in the carrier cell line was suggested to indicate compensation for the lower number of active sites.

### 3.1.2.6 Summary of evidence on the control of rRNA transcription

The evidence presented above for mechanisms controlling the

TABLE 3-1: Summary of findings on the control of rRNA transcription

Type of control	Organism	Author	Nature of Evidence
1. Alteration of rRNA synthesis rate	<u>Drosophila</u>	Krider and Plaut (1972)	Flies with 1 active NOR and 2 active NORs have same rates of synthesis of nucleolar RNA
2. Dominance and epistatic effects	<u>Triticum</u>	Longwell and Svihla (1960)	Normally latent chromosomes become active in the absence of the 'strong' pair of nucleolar chromosomes
	<u>Crepis</u>	Wallace and Langridge (1971)	'Allelic repression' by dominant iso-allele in hybrids
	<u>Xenopus</u>	Honjo and Reeder (1973) Cassidy and Blacker (1974)	'Allelic repression' in hybrids
	<u>Drosophila</u>	Bicuodo and Richardson (1977)	'Allelic repression' in <u>Drosophila</u> hybrids; suppression of latent NOR by dominant NOR
3. Inactivation of complete NOR	<u>Drosophila</u>	Krider and Plaut (1972)	All NORs in excess of two are non-functional
4. Inactivation of some cistrons within the NOR	<u>Triturus</u>	Scheer <i>et al.</i> (1976b)	Active and inactive cistrons can be observed within a NOR in mature oocytes where rRNA transcription is below maximum level
5. Presence of regulators of rRNA synthesis	<u>Xenopus</u>	Gurdon and Brown (1965)	Nuclei transplanted into cytoplasm of eggs which are inactive in rRNA transcription, stop synthesizing rRNA
	<u>Xenopus</u>	Yamana and Shiokawa (1966)	An extract from the blastula stage inhibits rRNA synthesis in gastrula cells
	<u>Xenopus</u>	Honjo and Reeder (1973)	<u>X. laevis</u> cytoplasm prevents rRNA transcription in <u>X. mulleri</u> nuclei
	<u>Crepis</u>	Wallace and Langridge (1971)	When a repressed NOR is isolated from its dominant iso-allele by a nuclear membrane, it is expressed again
	Mouse-man cell hybrids	Marshall <i>et al.</i> (1975)	Human NORs are expressed in heterokaryons but not synkaryons

transcription of rRNA is summarized in Table 3-1. The types of control categorized in this table are not necessarily exclusive. For example, the 'alteration' of the rRNA synthesis rate in Drosophila with one NOR could be due to differences in the number of cistrons within the NOR being transcribed in one and two NOR flies. Similarly the dominance and epistatic effects may be mediated by regulator molecules.

### 3.1.3 Variation in frequency of Ag-NORs

The variation in frequency of Ag-NORs within individuals is now a well-established phenomenon (Bloom and Goodpasture, 1976; Miller et al., 1977; Varley, 1977). There is a characteristic number and pattern of distribution of Ag-NORs within a cell line as well as within an individual (Goodpasture and Bloom, 1975). This is thought to indicate a differential activity of NORs within an individual. In addition individual NORs tend to have a characteristic size of Ag deposit (Miller et al., 1977) which may also reflect differential activity. In this chapter results on the distribution and size of Ag-NORs in sheep are reported, and discussed in terms of the regulation of these regions.

## 3.2 Materials and Methods

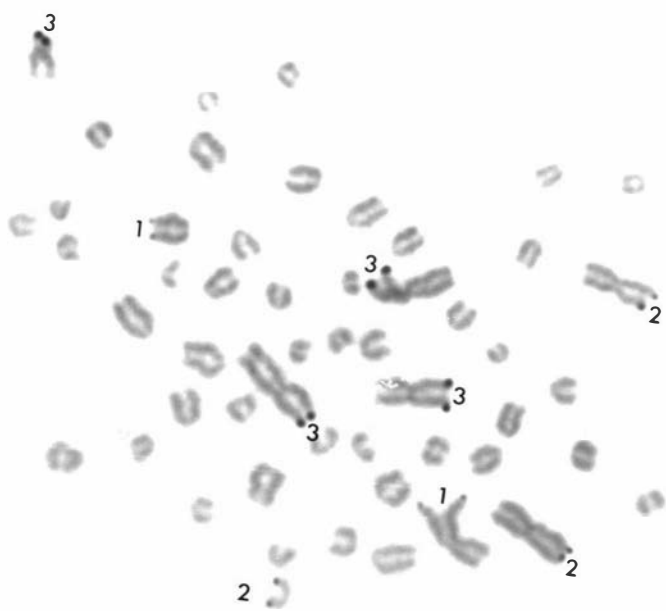
### 3.2.1 Materials

Ag-AS and Ag-I preparations from blood leucocyte cultures from translocation and normal animals were used, as described previously (see section 2.2).

### 3.2.2 Scoring of size polymorphisms

The method of Miller et al., (1977) was used. The size of the Ag-deposit was scored as 0 (absent), 1 (small), 2 (medium) and 3 (large). Examples of different size deposits are shown in Fig. 3-2. If homologues could not be distinguished they were scored on the basis of the difference in the amount of Ag-stain. The member of the pair with the less Ag-stain was always put in one column and the member of the pair with more Ag-stain was put in the other column. No attempt was made to distinguish the metacentrics by microscopic

Fig. 3-2: Two metaphase cells showing  
Ag-deposits of different sizes. Dark  
deposits are scored as 3, medium deposits  
as 2 and faint deposits as 1.  
(Ag-I, x 2,650)



analysis. The metacentrics were ranked on the basis of the difference in the amount of Ag-stain. Thus the largest deposit score for a metacentric chromosome was placed under the metacentric A column, the next largest under metacentric B and continuing in decreasing order to the smallest deposit score under the metacentric F column. Similarly the largest deposit on C4 was put under the C4<sub>a</sub> column and the smaller deposit under the C4<sub>b</sub> column. The mean Ag-size of a chromosome was calculated by totalling the score and dividing by the number of cells scored. An example of the data collected is shown for one animal in Appendix XI.

### 3.3 Results

#### 3.3.1 Average frequency and range of Ag-NORs per metaphase

The number of NO-chromosomes with silver deposits in a metaphase is often less than the maximum of 10. The number of Ag-NORs per metaphase ranged from 2 to 10 with an average frequency of 7.82 in 1026 cells from 33 animals. The range of the average number of Ag-NORs in an individual is much narrower, with most animals having an average frequency of 7-9 as shown in Table 3-2.

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Table 3-2: The distribution of average number of Ag-NORs per metaphase in 33 individuals

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	<u>Average No. of Ag-NORs</u>	<u>No. of animals with this average</u>
	6 - 6.9	6
	7 - 7.9	11
	8 - 8.9	10
	9 - 10	6
Overall	7.82	33

---

### 3.3.2 Individual variation in number of Ag-NORs

The frequency of Ag-NORs in 32 different individuals is shown in Table 3-3. Marked differences were found between animals in the frequency of Ag-NORs. ( $F_{31,980} = 17.76$   $P < 0.01$ ). The cell counts used to calculate this statistic are given in Appendix IX. Variation could be due either to variation between animals or culture variation.

Table 3 - 3 : Average frequency and distribution of Ag-NORs in individual sheep

Animal No.	Karyotype	No. of cells	Mean Ag-NORs per cell	Mean Ag-NORs Meta	per group C4	group C25	t <sub>3</sub>
61/6	52,XX,t <sub>1</sub> t <sub>2</sub>	15	7.5	5.3	1.1	1.1	-
174/6	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>2</sub>	49	8.0	5.1	1.0	1.9	-
124/3	52,XX,t <sub>3</sub> t <sub>3</sub>	31	9.1	5.7	1.7	-	1.7
169/1	52,XX,t <sub>1</sub> t <sub>1</sub>	36	7.5	5.9	0.4	1.2	-
166/1	52,XX,t <sub>3</sub> t <sub>3</sub>	23	7.0	4.9	0.7	-	1.4
176/6	50,XY,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub> t <sub>3</sub>	124	7.4	5.9	1.0	-	0.5
45/6	50,XX,t <sub>1</sub> t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	49	9.1	5.7	1.5	0.9	1.0
67/5	52,XX,t <sub>1</sub> t <sub>1</sub>	21	6.0	4.8	0.2	1.0	-
18/6	49,XX,t <sub>1</sub> t <sub>1</sub> t <sub>2</sub> t <sub>3</sub> t <sub>3</sub>	18	9.0	5.7	1.4	-	1.9
166/7	54,XX	16	8.1	5.0	2.0	1.1	-
156/7	54,XX	9	9.1	5.7	1.3	1.9	-
60/5	51,XY,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	30	6.8	5.6	0.1	0.1	1.0
73/4	51,XY,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	30	6.8	5.0	0.1	0.8	0.9
106/3	52, XY, t <sub>3</sub> t <sub>3</sub>	30	6.5	4.1	0.4	-	1.9
34/3	53,XX,t <sub>3</sub>	30	7.5	5.3	1.1	0.1	1.0
74/2	53,XX,t <sub>3</sub>	30	8.7	5.8	1.1	0.9	0.8
157/5	53,XX,t <sub>3</sub>	30	8.0	5.6	0.7	0.8	1.0
67/3	53,XX,t <sub>3</sub>	30	7.3	5.4	0.3	0.8	0.8
80/5	52,XY,t <sub>2</sub> t <sub>3</sub>	10	8.2	4.3	1.9	1.0	1.0
114/5	52,XY,t <sub>1</sub> t <sub>3</sub>	10	7.1	4.2	0.9	1.0	1.0
121/5	51,XY,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	30	6.9	4.2	0.8	1.0	0.9
91/5	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	30	7.6	4.9	0.8	1.0	1.0
101/6	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	30	6.5	4.5	1.0	0.0	1.0
128/7	50,XY,t <sub>1</sub> t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	30	8.9	6.0	1.1	0.8	1.0
68/5	52,XX,t <sub>1</sub> t <sub>3</sub>	30	8.7	5.8	1.0	0.8	1.0
116/2	52,XX,t <sub>1</sub> t <sub>3</sub>	30	7.9	5.9	0.6	0.4	1.0
98/5	53,XX,t <sub>3</sub>	30	8.3	5.7	1.3	0.3	1.0
199/5	53,XX,t <sub>3</sub>	30	9.1	5.6	1.6	0.9	1.0
198/5	53,XX,t <sub>3</sub>	30	8.8	5.3	1.6	1.0	1.0
155/6	52,XY,t <sub>1</sub> t <sub>2</sub>	25	9.0	5.4	2.0	1.6	-
90/6	53,XY,t <sub>2</sub>	25	7.0	3.9	1.2	1.9	-
165/6	51,XY,t <sub>2</sub> t <sub>2</sub> t <sub>3</sub>	25	8.1	4.8	1.7	0.6	1.0

Table 3-4: Analysis of Variation due to replicate differences  
in between-animal differences

Animal No.	Repli- cate No.	No. of cells	Mean No. of Ag-NORs	No. of cells having this no. of Ag-NORs					
				5	6	7	8	9	10
174/6	1	31	8	0	4	6	10	8	3
		28	8	0	0	10	13	1	4
	Overall	59	8.0	0	4	10	23	9	7
176/6	1	25	7.4	0	1	14	9	1	0
	2	28	7.4	0	1	18	7	2	0
	3	29	7.9	0	1	9	13	4	2
	4	50	7.6	0	1	20	29	0	0
	5	24	7.0	2	3	12	7	0	0
	Overall	156	7.5	2	7	73	65	7	2
101/6	1	12	6.2	3	4	5	0	0	0
	2	18	6.7	1	5	10	2	0	0
	Overall	30	6.5	4	9	15	2	0	0
37/5	1	15	8.0	0	3	2	5	4	1
	2	17	8.3	0	0	5	4	6	2
	3	30	7.5	2	4	9	7	7	1
	Overall	62	7.8	2	7	16	16	17	4
60/5	1	13	6.7	1	3	8	1	0	0
	2	14	6.8	1	1	12	0	0	0
	3	21	7.4	0	2	13	3	2	1
	4	8	6.8	1	1	5	1	0	0
	Overall	56	7.0	3	7	38	5	2	1
121/5	1	23	6.7	4	4	10	5	0	0
	2	14	7.9	0	0	3	9	2	0
	3	22	7.9	0	0	6	13	3	0
	Overall	59	7.4	4	4	19	27	5	0

### 3.3.3 Variations between replicates in the number of Ag-NORs

In order to distinguish whether the differences between animals in the number of Ag-NORs was due to technical factors or was an individual characteristic, an analysis was made of between-animal and between-replicate variation. Six animals were tested and the number of replicates ranged from two to five. Table 3-4 shows the cell counts, and average frequencies for the replicate  $t$  test. In 176/6 the first four replicates were set up and harvested simultaneously. In the other cases the cultures were set up on separate occasions. The analysis was done by a two-way nested ANOVA with uneven sample sizes (Sokal and Rohlf, 1969). Details are presented in Appendix X. Both the between-animals and between-replicates test statistics are significant. For the between-replicates  $F_{(13;403)} = 4.29, > F_{0.1(12;400)} = 2.23$ . The results of the analysis of variance between animals was  $F_{(5,13)} = 3.69 > F_{.05(5,12)} = 3.11$  so that again the result is significant.

### 3.3.4 Chromosome Distribution of Ag-NORs

#### 3.3.4.1 Pooled data for all individuals

The number of metacentrics, chromosomes 4, chromosomes 25 and  $t_3$  chromosomes with Ag-NORs was counted in 30 cells each from 22 animals. The number of each chromosome type, and the expected number based on the hypothesis that each chromosome type has an equal frequency of Ag-NORs is given in Table 3-5.

Table 3-5: Data from 22 animals on the number of Ag-NORs on each chromosome type

	Meta	C 4	C 25	$t_3$	Overall
Observed	3503	608	436	612	4159
Expected	2495	832	397	435	4159

There is a significant variation in the frequency of involvement of each chromosome type ( $\chi^2 = 548$ , 3 df,  $p \ll 0.01$ ). The meta-centric chromosomes most frequently have Ag-NORs, chromosome 25 and  $t_3$  less often, and chromosome 4 least often. Several of the animals that were used in this analysis were from a pedigree group which had inherited a  $t_3$  chromosome with a high Ag-stainability (Chapter 5) which could have contributed to a bias in the number of chromosomes 25/ $t_3$  chromosomes having Ag-NORs. An analysis for the frequency of involvement of each chromosome type using only unrelated animals not involved in this pedigree is set out in Table 3-6.

Table 3-6: Pooled data on chromosome distribution of Ag-NORs from 8 animals not involved in pedigree groups

	Meta	C 4	C 25	$t_3$	Overall
Observed	1349	237	192	173	1951
Expected	1171	390	195	195	1951

The counts from 30 cells from each of 8 animals were pooled to give this data. The results agree with those obtained above ( $\chi^2 = 89$ ; 3 d.f.  $P \ll 0.01$ ) i.e. the frequency of Ag-NORs is given by the following series - metacentrics > chromosome 25/ $t_3$  chromosome > chromosome 4.

#### 3.3.4.2 Frequency of Ag-NORs on C25 and $t_3$

In the early stages of this study, preliminary results suggested a higher frequency of Ag-NORs on the  $t_3$  chromosome than chromosome 25. To determine whether there was a difference in the staining frequency between these, an analysis was made of animals not involved in the high  $t_3$  staining pedigree. In Table 3-6 the number of chromosomes 25 with Ag-NORs was 192, and the number of staining  $t_3$  chromosomes was 173. This data was collected from two animals homozygous for chromosome 25, two

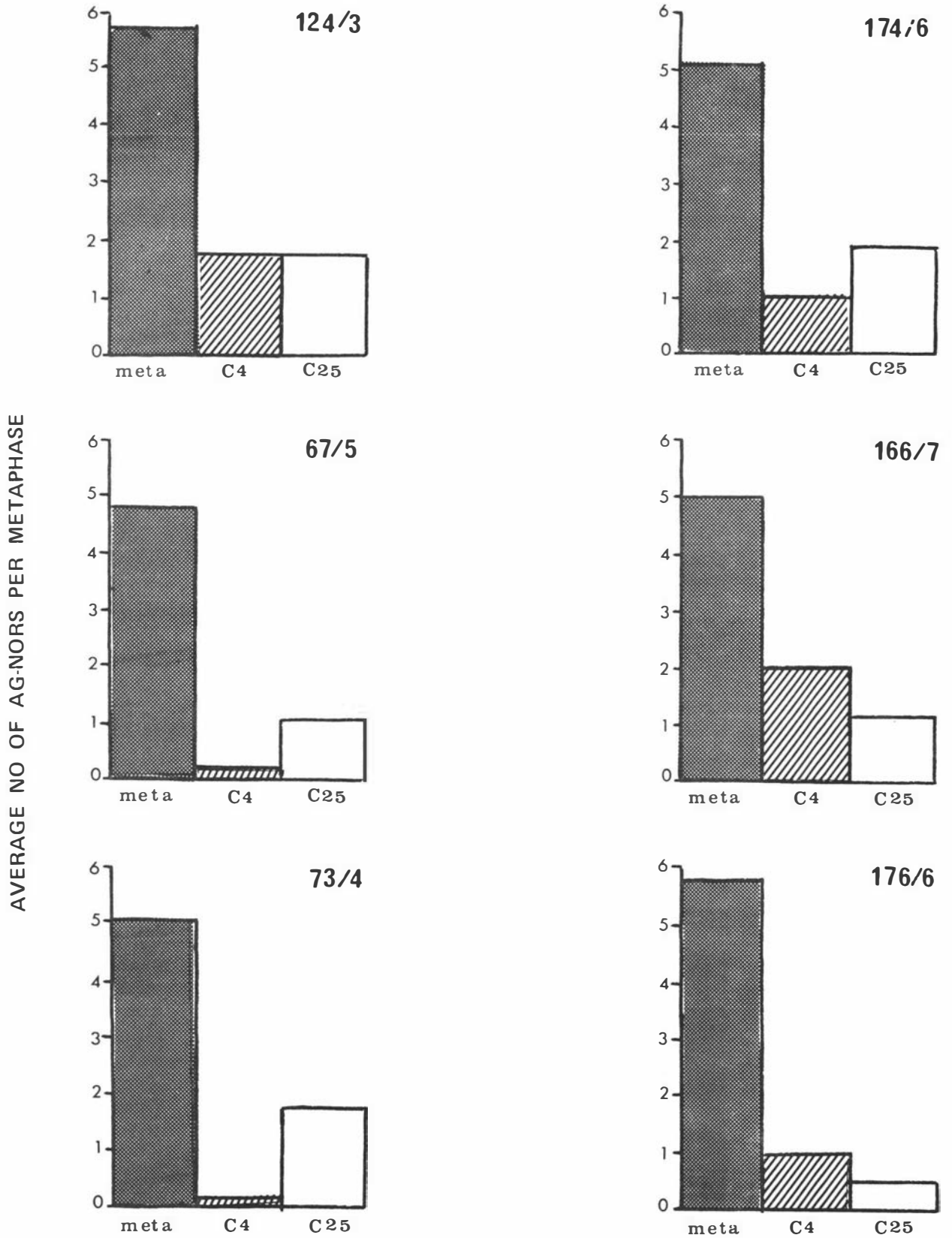
animals homozygous for the  $t_3$  chromosome and four animals heterozygous for these chromosome types. Thus the number of  $t_3$  chromosomes equalled the number of chromosomes 25. There was found to be no significant difference in staining frequency between the  $t_3$  chromosome and chromosome 25 ( $\chi^2 = 0.96$ , 1 d.f.  $P > 0.1$ ).

### 3.3.4.3 Individual Variation in distribution of Ag-NORs

Table 3-3 shows the variation between animals in the mean number of Ag-NORs per chromosome type. The maximum and minimum frequency for each chromosome type are 3.9 and 6.0 for the three metacentric pairs; 0.1 and 2.0 for the chromosome 4 pairs, 0.3 and 1.0 for the  $t_3$  chromosome and 0 and 1.0 for chromosome 25. Thus each individual may have a different distribution of Ag-NORs over the NO-chromosomes as illustrated in Fig. 3-3. This is a fairly constant feature within the cells of an individual.

### 3.3.5 Differences in deposit size between individuals

The size of the silver deposit on a NO-chromosome can vary from small to large (Fig. 3-2). Each animal has a different distribution of the amount of Ag-stain on the various chromosome types. This is illustrated in the histograms of the amount of stain on each NO-chromosome in eight animals (Fig. 3-4). The amount of stain is fairly constant on a given chromosome in all cells of an individual. This is shown by the distribution of the number of cells having a chromosome with a given size deposit (Table 3-7). This data was collected from animals heterozygous for chromosome 25 and the  $t_3$  chromosome, which enabled individual chromosomes to be scored.



**Fig. 3 - 3 :** Histograms illustrating the average number of Ag-NORs per metaphase on the 3 chromosome types in 6 animals.

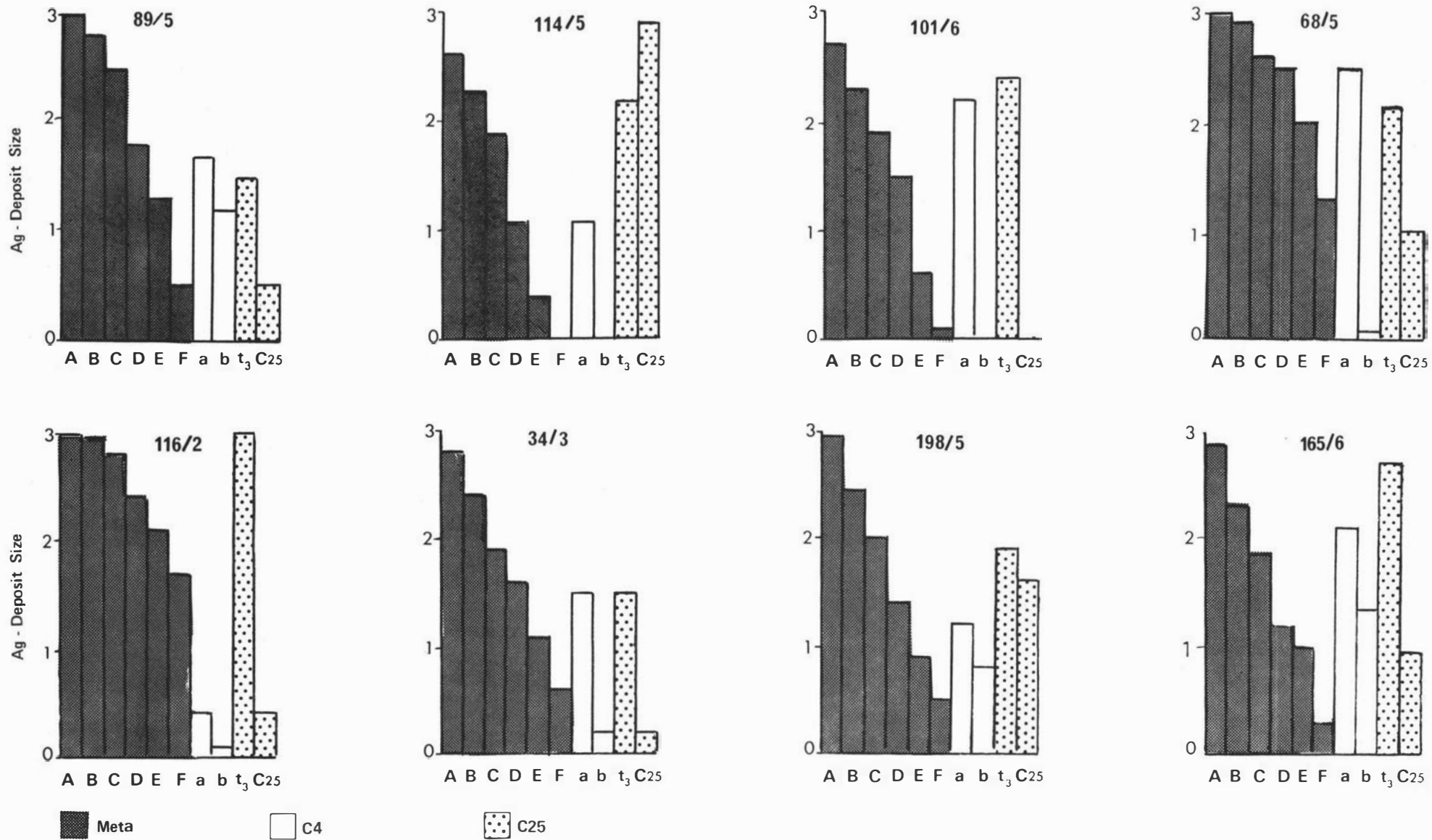


Fig. 3 - 4 : Histograms of size of Ag-NORs in 8 animals. (for explanation of chromosome numbering system see section 3.2.2)

Table 3-7: Distribution of the number of cells in which a chromosome has a given size deposit

Animal No.	No. of Cells	Chromosome involved	No. of cells having this size deposit*			
			3	2	1	0
34/3	30	C25	0	1	4	25
		t <sub>3</sub>	2	14	13	1
67/3	29	C25	4	14	6	5
		t <sub>3</sub>	7	13	2	7
68/5	25	C25	0	4	17	4
		t <sub>3</sub>	8	13	4	0
74/2	30	C25	1	16	11	2
		t <sub>3</sub>	1	11	13	5
91/5	26	C25	7	15	4	0
		t <sub>3</sub>	1	18	6	1
98/5	30	C25	0	0	9	21
		t <sub>3</sub>	3	21	6	0
101/6	18	C25	0	0	0	18
		t <sub>3</sub>	9	7	2	0
116/2	29	C25	1	1	9	18
		t <sub>3</sub>	27	2	0	0
157/5	30	C25	0	10	13	7
		t <sub>3</sub>	7	14	8	1
198/5	30	C25	2	14	14	0
		t <sub>3</sub>	3	21	6	0
199/5	29	C25	1	15	11	2
		t <sub>3</sub>	22	6	1	0
165/6	23	C25	3	3	7	10
		t <sub>3</sub>	17	6	0	0

\* For explanation of scoring see section 3.2.2

0 = absent; 1 = small; 2 = medium; and 3 = large)

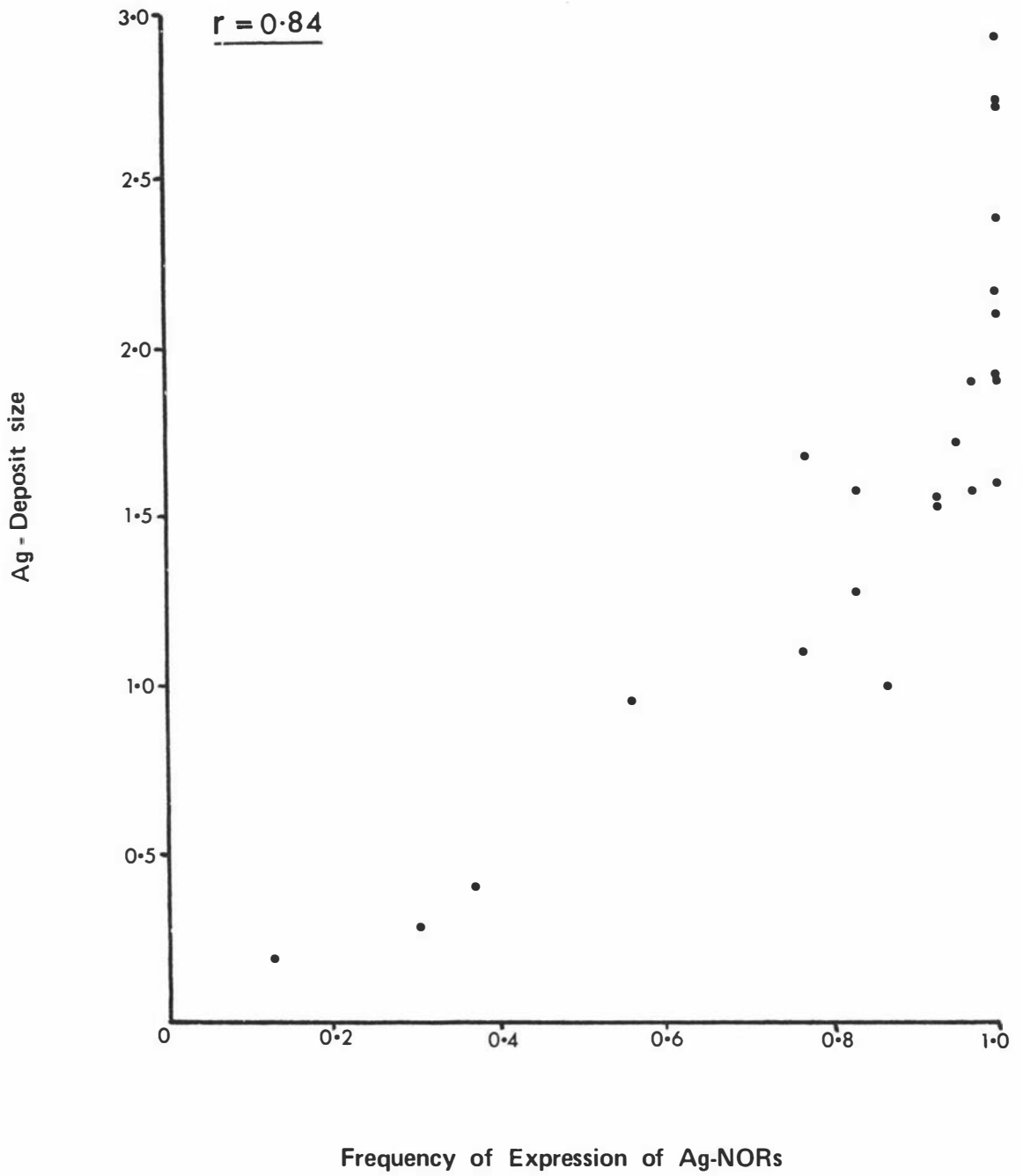


Fig. 3 - 5 : Graph showing Correlation of deposit size and frequency of expression of Ag-NORs on chromosome 25 and  $t_3$

### 3.3.6 Relationship of deposit size to Ag-NOR frequency

In 12 animals heterozygous for the  $t_3$  chromosome, the frequency of Ag-NORs was plotted against the deposit size for each chromosome. The frequency of Ag-NORs was found to be highly correlated ( $r = 0.84$ ) with the size of the Ag deposit (Fig.3-5).

### 3.3.7 Relationship of deposit size to between-culture differences

In five of the animals in which replicate cultures were set up, and differences between cultures determined, the deposit size of the chromosomes were also scored (Table 3-8). For comparative purposes, the frequency of staining has been set out as for the deposit size scoring. That is, chromosomes are ranked on the frequency of staining so that where one metacentric chromosome is not stained in a metaphase, it is assumed to always be metacentric "F" and where 2 chromosomes are unstained they are assumed to be chromosome "E" and "F" etc. It can be seen that chromosomes with a large deposit score show a smaller fluctuation between replicates than those with a small score. For example the variation between replicates of animal 101/6 is due to differences in the metacentric frequency and the two lowest deposit scores for the metacentrics in this animal were 0.6 and 0.1. The deposit scores for C4 and  $t_3$  in this animal are above 2 and the frequency of expression of these chromosomes shows little variation between cultures. In animal 176/6, replicate 3 which had the highest average frequency had an increase in staining of the second C4 and the  $t_3$  chromosomes. These chromosomes had low deposit scores. Similarly in animal 121/5, two metacentrics and chromosome 4 (scores 0.8, 0.2 and 1.5) show variation between replicates whereas the  $t_3$  (deposit score of 2.2) and C25 (deposit score of 2.8) show a smaller variation.

Table 3-8: Relationship of Ag-deposit size to between culture differences

	No. of Cells	Meta +						Total of Meta	C4 a	Total of C4 b	t <sub>3</sub>	C25	Over- all	
		A	B	C	D	E	F							
<u>Animal 101/6</u>														
Frequency of R1*	12	1.0	1.0	1.0	0.8	0.4	0	4.2	0.9	0	0.9	1.0	0	6.1
Frequency of R2	18	1.0	1.0	1.0	0.9	0.7	0.1	4.7	1.0	0	1.0	1.0	0	6.7
Deposit Size (R1 and R2)	30	2.7	2.3	1.9	1.5	0.6	0.1	-	2.2	0	-	2.4	0	-
<u>Animal 176/6</u>														
Frequency of R1	25	1.0	1.0	1.0	1.0	1.0	1.0	6.0	1.0	0	1.0	0.4	0.1**	7.5
Frequency of R2	28	1.0	1.0	1.0	1.0	1.0	0.9	5.9	1.0	0	1.0	0.3	0.1	7.3
Frequency of R3	25	1.0	1.0	1.0	1.0	1.0	0.9	5.9	1.0	0.1	1.1	0.6	0.2	7.8
Frequency of R4	50	1.0	1.0	1.0	1.0	1.0	0.9	5.9	1.0	0	1.0	0.7	0	7.6
Frequency of R5	30	1.0	1.0	1.0	1.0	0.9	0.7	5.6	0.9	0	0.9	0.4	0	6.9
Deposit Size (R5)	30	2.8	2.4	2.1	1.9	1.5	0.9	-	2.2	0	-	0.4	0	-
<u>Animal 37/5</u>														
Frequency of R1	15	1.0	1.0	1.0	0.8	0.7	0.3	4.8	1.0	0.2	1.2	1.0	1.0	8.0
Frequency of R2	17	1.0	1.0	1.0	1.0	0.5	0.2	4.7	1.0	0.7	1.7	0.9	1.0	8.3
Frequency of R3	30	1.0	1.0	1.0	0.8	0.4	0.1	4.3	1.0	0.4	1.4	1.0	0.9	7.6
Deposit Size (R1 and R2)	32	2.3	1.9	1.6	1.3	0.7	0.2	-	2.2	0.4	-	2.5	2.5	-
<u>Animal 60/5</u>														
Frequency of R1	13	1.0	1.0	1.0	1.0	0.9	0.7	5.6	0	0	0	0.9	0.2	6.7
Frequency of R2	14	1.0	1.0	1.0	1.0	0.9	0.8	5.7	0.1	0	0.1	1.0	0	6.8
Frequency of R3	21	1.0	1.0	1.0	1.0	1.0	0.9	5.9	0.4	0.1	0.5	1.0	0.1	7.5
Frequency of R4	8	1.0	1.0	1.0	1.0	0.9	0.6	5.5	0.1	0	0.1	1.0	0.1	6.7
Deposit Size (R3 and R4)	29	2.7	2.4	2.2	1.8	1.6	1.0	-	0.3	0.1	-	2.0	0.2	-
<u>Animal 121/5</u>														
Frequency of R1	23	1.0	1.0	1.0	0.8	0.3	0	4.1	0.7	0	0.7	0.9	1.0	6.7
Frequency of R2	14	1.0	1.0	1.0	1.0	0.8	0.1	4.9	0.9	0.1	1.0	1.0	1.0	7.9
Frequency of R3	22	1.0	1.0	1.0	1.0	0.7	0.2	4.9	1.0	0	1.0	1.0	1.0	7.9
Deposit Size (R2 and R3)	36	2.4	2.1	1.9	1.5	0.8	0.2	-	1.5	0	-	2.2	2.8	-

\* R1 refers to replicate 1, R2 to replicate 2 etc.

\*\* This column refers to the second t<sub>3</sub> chromosome in 176/6 only

† For explanation of scoring see Section 3.3.7

### 3.4 Discussion

#### 3.4.1 Variation in the number of Ag-NORs between animals

Each animal was found to have a characteristic number of Ag-NORs. In this study some of the differences between animals in the number of Ag-NORs was found to be due to differences between replicates. Five of the 6 animals used in the replicate study had an average of 7-8 Ag-NORs per metaphase. This contrasts with the data on 33 animals (Table 3.2) where only 11 animals were in this range. Consequently the variance in this analysis due to between-animal differences was probably lower than would be found in a larger, or more divergent, sample.

It was also shown that differences in the staining 'patterns' of Ag-NORs exist between animals. Each individual has a characteristic chromosomal distribution of Ag-NORs, and the size of each Ag-deposit is also an individual characteristic. Differences between replicates is due to variation in staining of NO-chromosomes with small Ag-deposits.

The differences between replicates may be due to technical variation. Some of the Ag-staining protein may be removed during hypotonic treatment or fixation and the smaller deposits would be expected to show a greater variation in this respect. Alternatively, during culture the number of nucleoli and the number of NORs activated may show variation so that the number of Ag-NORs may be affected by factors such as variation in the number of cell cycles which have occurred. NORs which have a small deposit may be more variable in activity in nucleolar organization.

In the only published study in which replicate cultures were compared, 'closely similar' results on Ag-NOR frequency were found in replicates from four people taken three months apart (Varley, 1977). Neither the results nor statistical analysis were presented.

The modal numbers of Ag-NORs reported in different studies

show a large variation. For example, in Ag-AS stained preparations from humans, modal numbers of 8-10 (Mikelsaar et al., 1977) 5-9 (Miller et al., 1977) and 4-7 (Varley, 1977) have been recorded. Similarly in cattle, Diamond et al. (1975) recorded a modal number of 4-6 whereas in this study a modal number of 6-7 was found (Chapter 7). These differences can not be due entirely to differences between the individuals in the study groups. They suggest that technical factors such as intensity of staining may be involved. Observer differences may also be significant in these comparisons whereas they would not be involved in between-replicate comparisons. A high correlation ( $r=0.84$ ) was found between the deposit size and Ag-staining frequency of individual chromosomes. The system of scoring Ag-deposit sizes uses an arbitrary scale so that the Ag-deposit scores of 0, 1, 2 and 3 marked in Fig. 3-5 do not necessarily indicate equal intervals. Nevertheless, these results suggest that within individuals, the frequency of staining of an Ag-NOR is related to the deposit size. That is, some of the variation in number of Ag-NORs between cells of an individual is due to variation in the presence of stain on NO-chromosomes which have small Ag-deposits. This again could be due to technical variation or to a selective regulation of small Ag-NORs.

Most of the differences in the frequency of Ag-NORs between animals appear to be due to intrinsic differences in the frequency and chromosome distribution of Ag-NORs. Each chromosome has a characteristic ability to stain with silver and this is related to the size of its Ag deposit.

#### 3.4.2 Chromosomal distribution of Ag-NORs

While each of the NO-chromosomes may either have an Ag-NOR or not in an individual, the pooled data on chromosome distribution indicates that the frequency of silver-staining of the various chromosome types is not equal. Metacentrics are most frequently stained, followed by chromosome 25 and then chromosome 4. Few reports have appeared on the distribution of Ag-NORs in other species. In a sample of 20 humans, the observed number of Ag-NORs

on the D group chromosomes is close to the figure expected on the basis of random distribution (Varley, 1977). This study however did not differentiate individual chromosome pairs. In a study of 51 people the frequency of Ag-positive NORs was found to be similar in all 5 acrocentric pairs although chromosome 22 had a non-significant lower frequency (Mikelsaar et al., 1977). In mice, the distribution of chromosomes with Ag-NORs varies among inbred strains. The relevance of the difference in Ag-staining frequencies of the different chromosome types to hypotheses on the nature of Ag-staining and variation in the level of rDNA, is discussed further in Chapter 8.

#### 3.4.3 Polymorphism in size of Ag-NORs

The size of the Ag-deposit on a given NO-chromosome is fairly constant within an individual, with little variation between metaphases. The identity of the protein material staining with silver is still not known so that interpretation of size differences as an indicator of the degree of rRNA gene activity is only speculative at this stage. It has been observed in the lampbrush chromosomes of the newt, T. alpestrus that some cistrons are inactive while others in the same NOR are active in rRNA transcription (Scheer et al., 1976 a and b). Possibly the difference in size of the Ag-deposit may be a reflection of differential activity within a NOR.

#### 3.4.4 Relation of rDNA content to Ag-staining

The reason for the differential Ag-stainability of the NORs, has not been found yet. There has been a great deal of speculation that Ag-staining reflects rDNA content, or in other words, that the ability of a NOR to be active in rRNA synthesis is dependent on its rDNA content (e.g. Dev et al., 1977; Ward, 1977).

In several species, the amount of rDNA varies between individuals and between chromosomes. In the toad, Bufo marinus a two-fold range in the amount of 18S and 28S rRNA was found between animals (Miller and Brown, 1969). A similar range of variation was found in a cultivar of onion, Allium cepa which was derived by mixed random breeding and selfing, whereas A. sativum which

propagates vegetatively had a constant value for the rDNA content (Maggini et al., 1978). The extent of the variation in rDNA levels which may exist in a population was shown in two small interbreeding populations of the salamander, Plethodon cinereus. In one of these populations a 7.5 times range in the ribosomal cistron number was found and a 2.5 fold range was found in the other population (MacGregor et al., 1977). Variation between animals in the total rDNA content has also been demonstrated in mice (Henderson et al., 1974 a,1976b) and in humans (Evans et al., 1974; Warburton et al., 1976a). In these studies differences between NO-chromosomes in the amount of rRNA hybridized was found to occur in all cases.

Various mechanisms have been suggested to be involved in the production of this heterozygosity in ribosomal cistron number. Differences in rRNA gene number could result from various types of unequal exchange within rDNA segments i.e. sister chromatid, between homologues, or between regions associating in nucleolus organization (Henderson et al., 1976b; MacGregor et al., 1977).

Some of the studies on rRNA cistron number have attempted to correlate cistron number with secondary constriction length. In mice where homologues differ in the presence of a secondary constriction, the amount of rDNA was found to be larger in the chromosome with the secondary constriction than in the homologue lacking the secondary constriction. However that this was not an all-or-none occurrence was shown by the presence of substantial rDNA in a region with no secondary constriction on one NO-chromosome (Henderson et al., 1976b). Similarly, in situ hybridization of rDNA to lampbrush nucleolus organizers of P. cinereus showed that long organizers have more ribosomal cistrons than shorter ones (MacGregor et al., 1977). In man, in situ, hybridization experiments on an individual with a very long nucleolar constriction resulted in a four times greater level of <sup>3</sup>H-rRNA hybridization on the large stalk than on the homologous chromosome (Evans et al., 1974). However, conflicting results were reported

by Warburton *et al.* (1976) who observed no relation between the degree of  $^{125}\text{I}$ -rRNA labelling and stalk size of the NOR.

As previously discussed, the relationship of Ag-staining to 18S and 28S rRNA cistron number of a nucleolus organizer region is still unclear. However, circumstantial evidence indicates Ag-staining may reflect rDNA content. When the observation of Dev *et al.* (1977) that the size of the secondary constriction in mice NORs is related to the size of the Ag-stained region is viewed in the context of the positive correlation between rDNA content and secondary constriction length (preceding paragraph), it is possible that the Ag-staining reflects rDNA content. In ten cases studied sequentially with hybridization *in situ* and silver-staining, an overall correlation between rDNA content and silver stainability was found in man (D. Warburton, pers. comm.). However, exceptions to this rule were found, involving chromosomes with very large amounts of rDNA but normal amounts of silver staining material. It was suggested that this probably represents regulation of the NOR.

Further evidence on the relationship of rDNA to silver staining is available from studies on the relationship of rDNA content to association of the NORs, and the relationship of Ag-stainability to NOR association as will be discussed in the following chapter. Irrefutable evidence on the basis of Ag-stainability will require further studies on the rDNA content of individual NORs and their Ag-stainability. This may also elucidate the basis of the size polymorphism of the Ag-NORs observed in this and other studies.

#### 3.4.5 Ag-stainability and NOR regulation

In this chapter it has been shown that individual sheep have a characteristic number and chromosomal distribution of Ag-NORs. The size of the Ag-deposit on each NO-chromosome was also found to be an individual characteristic. As discussed in this chapter, this probably represents differential activity of the NORs. Results on the differences in frequency of Ag-NORs between the cells of an individual and between replicate cultures have also been presented. On the basis of these results, and those on the heritability and tissue distribution of Ag-NORs (Chapters 5 and 6) the nature of the Ag-stainability and regulation of NORs is discussed in Chapter 8.

## CHAPTER 4

### ASSOCIATION OF NUCLEOLUS ORGANIZER REGIONS

#### 4.1. Introduction

##### 4.1.1 Satellite associations and nucleolar fusions

The term 'satellite associations' describes the linking of the short arms of the satellited D and G group chromosomes in man (Ferguson-Smith and Handmaker, 1961). On the basis of cytological evidence in other species that the nucleolus arises from a secondary constriction region (see section 2.1.2), it was suggested that the phenomenon of satellite association was related to the formation of nucleolar material in the region of the satellite. These authors proposed the hypothesis that satellite association is one of the determining factors in the production of autosomal non-disjunction during meiosis.

This idea was extended by Ohno et al. (1961) who suggested satellite association was caused by the fusion of nucleoli. They suggested the extended chromosomal regions involved in nucleolus organization might be prone to breakage, which could lead to a Robertsonian translocation event if the long arm of one chromosome rejoins with the short arm of the other. It was later suggested that both breaks could occur in the secondary constriction resulting in a dicentric chromosome (Ferguson-Smith, 1967).

Proof that the phenomenon of satellite association resulted from participation in nucleolus organization was given in a report on the association of the terminal regions of 3 long and 2 short bivalents with the nucleoli in human pachytene chromosomes (Ferguson-Smith, 1964). It was suggested that the 5 bivalents were the 5 pairs of satellited chromosomes in the human mitotic idiogram.

The association of nucleolus organizer regions in metaphase is believed to reflect nucleolar fusion in the preceding interphase. In mouse L cells (Gonzalez and Nardone, 1968) human embryonal kidney and ovary cells (Anastassova-Kristeva, 1977) and human lymphocytes (Gani, 1978) cyclic changes in nucleolar behaviour occur. In mouse L cells, a large number of small nucleoli are observed in G<sub>1</sub>, which are incorporated into larger nucleoli by nucleolar fusions in S and G<sub>2</sub>. The nucleoli become progressively more diffuse in G<sub>2</sub>

prior to disintegration (Gonzalez and Nardone, 1968). In human fibroblasts the occurrence of fusion events is similar to those reported in mouse L cells. However a dissociation of nucleoli occurs in early prophase, producing a large number of nucleoli (Anstassova-Kristeva, 1977). In human lymphocytes the fusion events have been observed to occur only in late telophase, with no fusion occurring during the later stages as in mouse L cells (Gani, 1978). In all cases NORs which have been brought together by the fusion of the nucleoli attached to them during the interphase stages may remain in proximity in the following metaphase.

#### 4.1.2 Studies on association of NORs in species other than man

There have been only 3 reports published on NOR association in species other than man. The contribution of association of NORs and heterochromatin attraction to overall chromosome association patterns may differ between species. In the mouse, Mus musculus no evidence for a non-random association of nucleolus organizer regions was found (Spence and Luthardt, 1975). This study involved a statistical analysis of the distances between pairs of chromosomes. The 3 chromosome pairs, 15, 18 and 19, found by Henderson et al. (1974a) to contain rDNA were not found to be more closely positioned than other combinations. On the basis of these findings, it was suggested that the mere presence of rDNA does not obligate these chromosomes to be involved in the formation of a common nucleolus. The localisation of the NORs to chromosomes 15, 18 and 19 (Henderson et al., 1974a) was based on an analysis of a mouse stock of mixed origin. It has since been found that the location of rDNA differs between strains of mice (Elsevier and Ruddle, 1976; Atwood et al.,<sup>1976</sup> Henderson et al., 1976b). Animals from 5 different strains were used in the mitotic association analysis of Spence and Luthardt (1975) and in at least 3 of these the location of the NORs has been shown by Ag-staining to differ from that found by Henderson et al. (1974a) (Dev et al., 1977) thus invalidating the conclusion of Spence and Luthardt that there was a lack of association of NORs in the mouse.

The short arms of the sex chromosomes of the brush-tailed possum Trichosurus vulpecula have been observed to show non-random association (Murray, 1977). NORs are found on the long arm of the acrocentric X chromosome and on the short arm of the acrocentric Y chromosome. Both short arms are heterochromatic.

It was therefore suggested that sex-chromosome association was due to heterochromatin attraction rather than NOR association. However more recently it has been found that the short arms of the X chromosome show silver-staining (J.D. Murray, pers. comm.) thus indicating a role of the NOR in the non-random association in this species.

In the Syrian hamster, Mesocricetus auratus and the Djungarian hamster, Phodopus syngorus terminal associations occur frequently (Bigger and Savage, 1976). The chromosomes involved in these associations were shown to contain the NORs, suggesting non-random association of chromosomes due to NOR association in these species.

#### 4.1.3 Participation of Individual Chromosomes in Satellite Association

While few studies of association of NORs in species other than man have been reported, a large number of papers have been published on the phenomenon of satellite association in man. Some of these were published before reliable chromosome identification techniques became available (e.g. Cohen and Shaw, 1967; Galperin, 1968; Zang and Back, 1968) but more recently the use of banding techniques has allowed the determination of the frequency of participation of individual chromosomes in satellite association (e.g. Mattei et al., 1976; Galperin-Lemaitre et al., 1977; Ardito et al., 1978). Silver staining techniques without chromosome identification have also been used (Denton et al., 1976; Liem et al., 1977). Some of the studies have analysed the chromosomes involved in association complexes (e.g. Jacobs et al., 1976; Ardito et al., 1978) while others have studied the association of acrocentric chromosomes by less direct means such as intercentromeric distances (e.g. Galperin, 1968; Warburton et al., 1973b; Kirsch-Volders et al., 1977).

Attempts to quantify differences in the frequency of association complexes per metaphase, have been made. Differences in association frequency have been reported between macrocultures and microcultures (Zang and Back, 1968), between different age groups (Cooke, 1972; Mattevi and Salzano, 1975), between 3 day and 2 day cultures (Mattevi and Salzano, 1975), between males and females (Galperin, 1968;

Liem et al., 1977) and between normal children and children with Down's syndrome (Rosenkranz and Holzer, 1972). Many of these differences have been disputed by other investigators. Estimates of the frequency of satellite association have varied from 13.3% (Zellweger et al., 1966) to 96% (Cohen and Shaw, 1967).

Some studies have reported a random frequency of involvement of chromosomes in satellite association in normal subjects (e.g. Nakogome, 1969; Curtis, 1974; Denton et al., 1976 and Liem et al., 1977). That is, each acrocentric chromosome shows an equal frequency of participation in satellite association. In some of these studies the association frequencies of the D and G group chromosomes have had to be determined rather than the association frequency of individual chromosomes because chromosome identification techniques were not used. Several more recent studies using banding techniques have shown differences in the association frequency of the acrocentric pairs but there is a lack of agreement over which chromosomes are most frequently involved. The order of participation in satellite association has been variously reported as 14>21>13>22>15 (Patil and Lubs, 1971), 21>13>15>14>22 (Ing et al., 1973); 21>22>15>14>13 (Jacobs et al., 1976), 21,13>22,15>14 (Mattei et al., 1976) and 13>21>22>14>15 (Ardito et al., 1978). These estimates are based on pooled samples and several authors have noted the wide individual variation in association frequencies. It has been suggested that the frequency of association is a characteristic property of a given acrocentric chromosome (Jacobs et al., 1976; Mattei et al., 1976) and that this is a heritable property (Phillips, 1975). While Jacobs et al. (1976) and Ardito et al. (1978) reported no preferential combinations of the chromosomes in association complexes, a non-random pattern of association was reported by Mattei et al. (1976).

#### 4.1.4 Relationship of Satellite Association to NOR Morphology

In man, structural polymorphisms of the short arm regions of the acrocentric chromosomes are fairly frequent. These involve variations in the size of the short arm, the secondary constriction and the satellite. Several studies have been made on the relationship of these

polymorphisms of the short arm to the association frequency. As with the reports on the randomness and non-randomness of acrocentric association, several conflicting reports have appeared. An increase in the size of the satellites has been associated with an increased frequency of satellite association (de Capoa et al., 1973; Zankl and Zang, 1974). Mattei et al. (1976) also noted an increase in association frequency of acrocentrics with an elongated short arm but reported that this was not consistently found. Other studies have looked at the relationship between constriction length and satellite association. The satellite association frequency is reported to be positively correlated with the size of the satellite stalk (Schmid et al., 1974; Phillips, 1975). A more complex relationship was reported by Orye (1974). He found that intermediate values for the secondary constriction length showed higher association frequencies than either shorter or more elongated secondary constrictions. The third region, the short arm, has been reported to be negatively correlated with the association frequency (Zankl and Zang, 1974). In contrast to all the preceding reports, Jacobs et al., (1976) found no obvious correlation between any heteromorphism of the satellite stalk and the degree of satellite association.

#### 4.1.5 Relationship of satellite association to rDNA content

In 1973 an in situ hybridization technique was used to demonstrate rDNA connectives between acrocentrics (Henderson et al., 1973). It was proposed that the frequency with which connectives involve the rDNA of a given nucleolar organizer is a function of its rDNA content. Under this hypothesis subjects which have a random-involvement of chromosomes in satellite association would be expected to have an equal amount of rDNA in each of the nucleolus organizer regions. Non-random association would be accounted for by differences in the amount of rDNA on different acrocentrics. The earlier observation of Henderson et al. (1972) that there is no significant difference in the rDNA content of associated chromosomes compared to unassociated chromosomes could be accounted for by a random association of NO-chromosomes in their sample. Evans et al. (1974) have also shown by in situ hybridization that chromosomes in association do not hybridize more tritiated rRNA than those not in association.

Other reports have suggested a correlation between rDNA content and satellite association. Schmid et al. (1974) found the frequency with which acrocentric chromosomes are found in association is proportional to the length of the satellite stalk. They later found a correlation between the amount of rDNA and the lengths of the nucleolar constriction (Dittes et al., 1975), thus suggesting that the frequency of participation in satellite association of a chromosome is related to its rDNA content. A correlation between the amount of rDNA and the frequency of participation in satellite associations was observed in a double satellited human acrocentric chromosome (Henderson and Atwood, 1976).

A significant level of correlation between the amount of rDNA and the proportion of satellite association has been reported in a study of 10 individuals (Warburton et al., 1976a). But in this study a few chromosomes with a high rDNA content were not found especially often in satellite association. It was suggested that while the rDNA content was a major factor in determining the satellite association frequency, other factors could also be involved.

#### 4.1.6 Relationship of satellite association to staining polymorphisms

Using N-banding, it has been shown that chromosomes with longer satellite stalks have a larger N-band and participate in satellite association at a higher frequency than those with shorter stalks (Hayata et al., 1977). This study used differences in the frequency of appearance of distinctive N-bands as an indirect measure of the size of the N-bands.

Similarly a correlation has been found between the size of Ag-stained NORs and their frequency in satellite association (D.A. Miller et al., 1977).

Fig. 4-1: Metaphase cell showing  
3 pairs of chromosomes in association  
(Ag-I, x 3,050)

Fig. 4-2: Metaphase cell showing chromosomes  
lying adjacent but not associated  
(Ag-I, x 3,100)

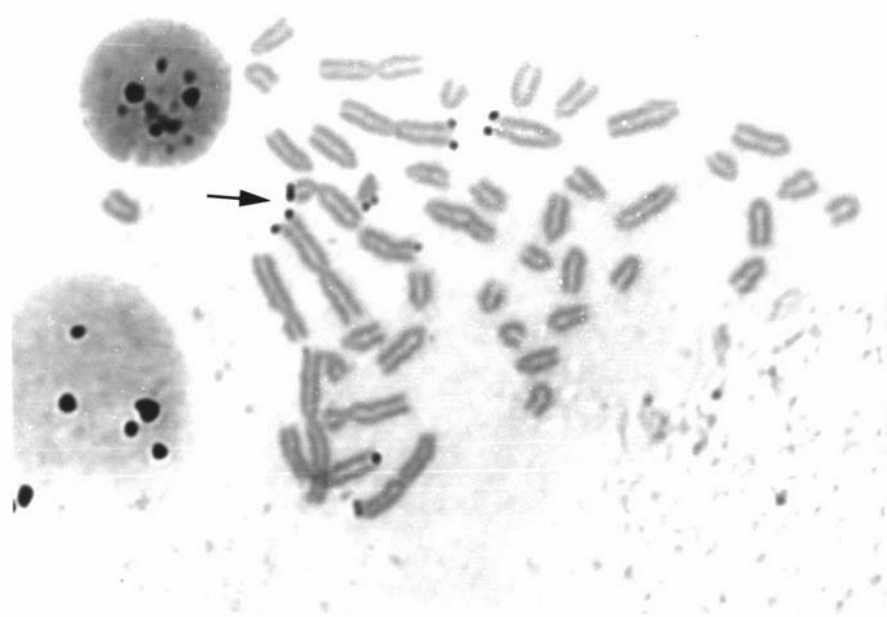


Fig. 4-3: Metaphase cell with 2 pairs of chromosomes associated by one chromatid and one pair of chromosomes associated by both chromatids (Ag-AS, x 3,100)



Fig. 4-4: Two metaphases with associated  
chromosomes joined by a long connecting  
strand  
(Ag-I, x 3,100)

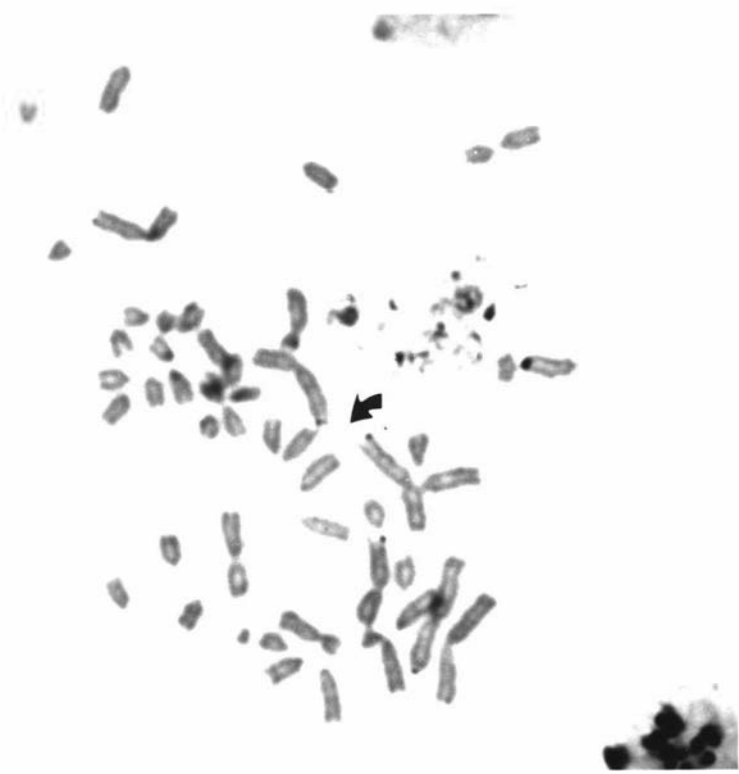
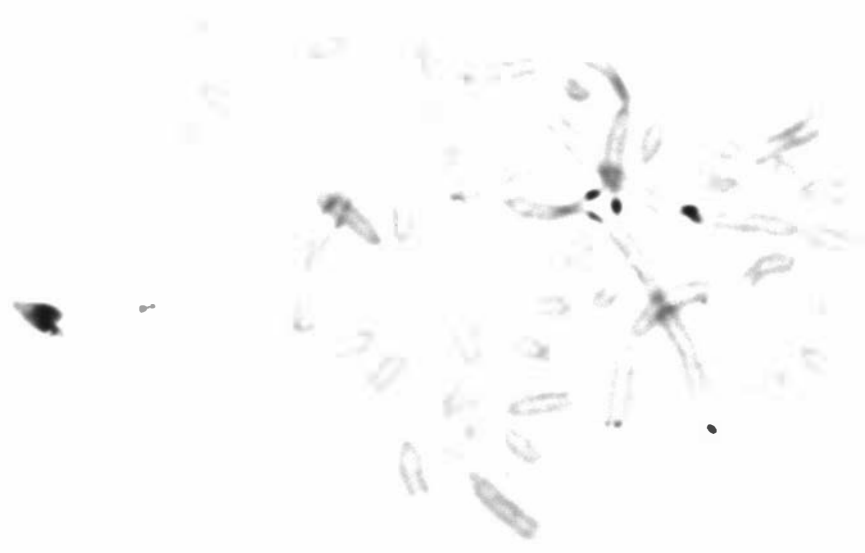
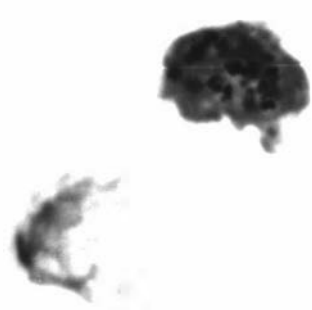


Fig. 4-5: Metaphase cell showing an  
associated chromosome with no  
detectable Ag-stain  
(Ag-I, x 3,000)



Fig. 4-6: Metaphase cell showing four  
chromosomes in one association complex  
(Ag-I, x 3,000)

Fig. 4-7: Metaphase cell with seven  
chromosomes involved in association complexes  
(Ag-I, x 3,500)



#### 4.1.7 Association of NORs in sheep

This study of the association of nucleolus organizer regions aimed to determine whether there was random or non-random participation of NO-chromosomes in association in sheep and whether the combinations of chromosomes in association complexes were random. A study was also made of the effect of Ag-deposit size on association frequency.

### 4.2 Materials and Methods

#### 4.2.1 Materials

Ag-AS and Ag-I preparations from blood leucocyte cultures of normal and translocation animals were used, as described previously (section 2.2).

#### 4.2.2 Scoring of associations

The use of silver-staining techniques allows the scoring of chromosomes in association without arbitrary criteria on which constitutes an association complex. Only chromosomes with NORs which were joined by silver-staining material were scored as associated (Fig. 4-1). Thus silver-staining NORs which lay adjacent but were not connected at metaphase were not scored as associated (Fig. 4-2). These chromosomes were probably associated at the previous interphase but this stringent criteria was adhered to to prevent bias in omitting scoring weakly stained NORs which were only obvious when connected by a silver-stained strand.

### 4.3 Results

#### 4.3.1 Observations on association complexes of NO-chromosomes

NO-chromosomes in association were either joined together by one chromatid or by both chromatids (Fig. 4-3). Occasionally associating NORs were observed to be some distance apart but still joined by a silver-staining strand. This indicates the disruptive forces involved during slide preparation (Fig. 4-4). Occasionally one of the chromosomes in an associating pair or group of chromosomes appeared to have almost no stain (Fig. 4-5). When more than 2 chromosomes were involved in an associating complex, they appeared either as a chain, a circle, or tightly clumped. The maximum number

of chromosomes in an association complex was 4 (Fig. 4-6). Most association complexes had only 2 chromosomes. The maximum number of chromosomes associating in a single metaphase was 8, in a metaphase containing 4 pairs of associating chromosomes. A metaphase containing 7 associating chromosomes is shown in Fig. 4-7. All possible pair-wise combinations of NO-chromosomes were observed during the course of this study.

#### 4.3.2 Frequency of association

The frequency of metaphases containing associating chromosomes varied widely between individuals from 0% to 90%. Out of 1320 metaphases from 65 animals, 464 contained association complexes, giving an overall association frequency of 35%. The total number of chromosomes involved in these association complexes was 1345, giving an average number of associating chromosomes per metaphase of 1.02. The frequency of association appeared to be related to the spread of the metaphases rather than being a property of the individual.

#### 4.3.3 Relationship of association frequency to the frequency of expression

The association index (A.I.) is a measure of the relative participation of a chromosome in association complexes. The term is defined as the number of acrocentrics participating in satellite association divided by the total number of acrocentrics examined (Nankin, 1970). For the purpose of this study on the NORs in sheep, the association index of a particular chromosome or chromosome type ( $C_x$ ) is calculated as:

$$\text{A.I. } (C_x) = \frac{\text{number of } C_x \text{ associating in a sample}}{\text{total number of associating chromosomes in that sample}}$$

This allows a within-sample comparison and is independent of the frequency of association. If there is a random involvement of NO-chromosomes in association, then the expected association indexes are:

$$\text{A.I. (meta)} = 0.6$$

$$\text{A.I. (C4)} = 0.2$$

$$\text{A.I. (C25)} = 0.2$$

In a preliminary study (Henderson and Bruère, 1977), on 438 associating chromosomes in 161 cells from 15 individuals, the number of associating chromosomes was 311 metacentrics, 48 chromosomes 4 and 79 chromosomes 25, corresponding to association indexes of 0.71, 0.11 and 0.18 respectively. These values differ significantly from the expected values based on random association shown above ( $\chi^2 = 28.26$ , 2 d.f.;  $P < 0.001$ ). However they are in close agreement with the values of the relative frequency of expression (R.E.F.) of the NO-chromosomes in this sample. This is computed similarly to the association index as follows:

$$\text{R.E.F. } (C_x) = \frac{\text{number of } C_x \text{ with Ag-NORs in a sample}}{\text{total number of Ag-NORs in that sample}}$$

The relative expression frequency values for this sample of 0.70 for the metacentrics, 0.14 for chromosome 4 and 0.16 for chromosome 25 do not differ from the observed association frequencies ( $\chi^2 = 3.33$ , 2 d.f.,  $P > 0.10$ ).

To determine whether there are deviations from random participation of NO-chromosomes in association complexes in individual sheep, the observed number of each of the NO-chromosome types in association complexes involving 1037 chromosomes from 26 animals was compared with the expected frequency calculated from the frequency of Ag-NORs. Thus the expected values were calculated as:

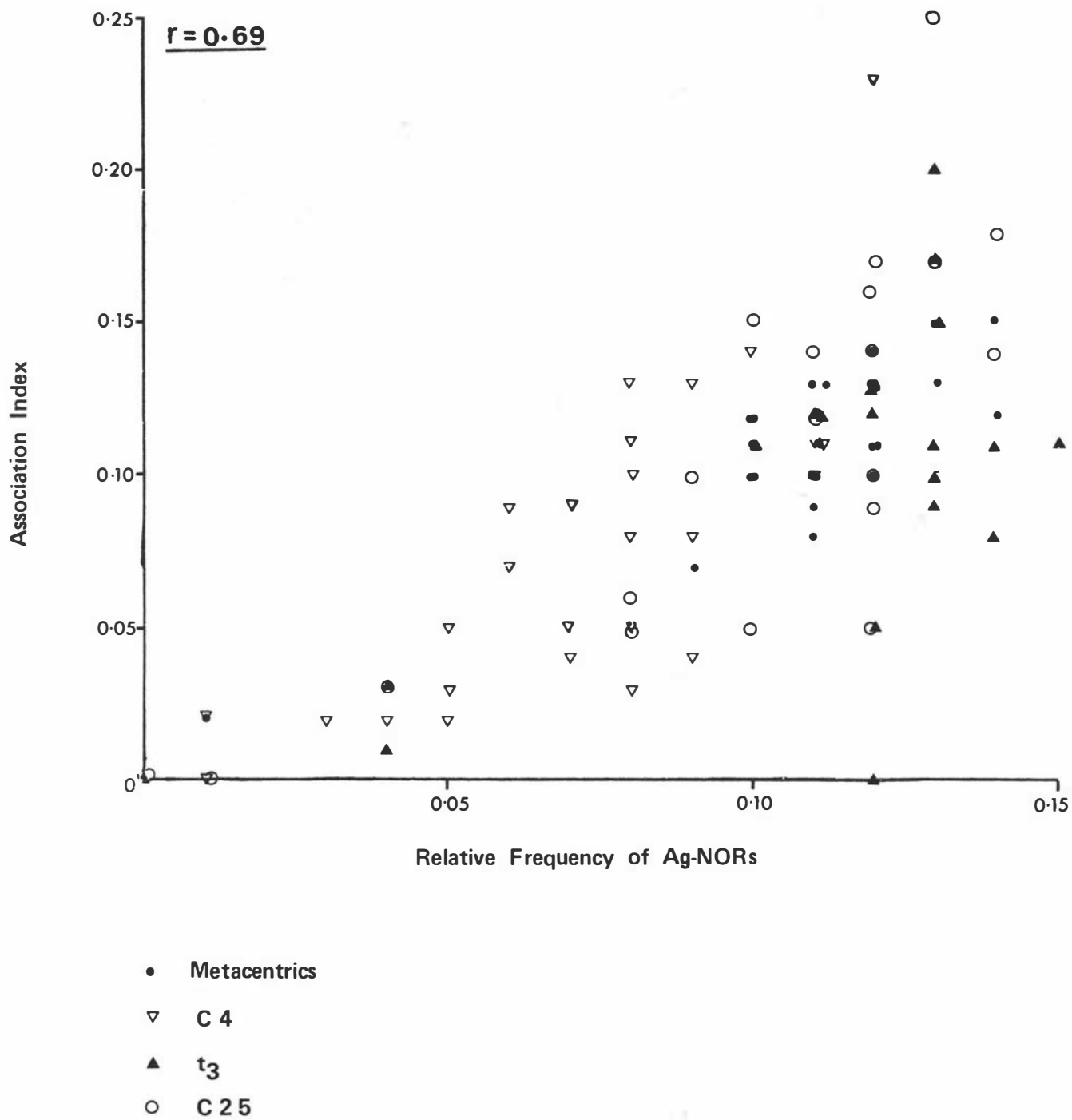
No. of associating chromosomes x R.E.F.

The Ag-NOR frequencies used for computing the R.E.F. values are taken from the data in Table 3-3 and Appendix XII. In each animal, at least 20 associating chromosomes were scored. These values, and the results of the  $\chi^2$  test are shown in Table 4-1. In all animals, except one, the  $\chi^2$  values are non-significant. In 176/6 the  $\chi^2$  is significant ( $0.025 < p < 0.01$ ). Therefore it seems that, with the possible exception of 176/6, the association of chromosomes within an animal is largely accounted for by the frequency of involvement in nucleolus organization as shown by silver-staining. The significant result in 176/6 is due to a low observed value for the involvement of the  $t_3$  chromosomes in association.

Table 4-1: Comparison of association frequency with expected values based on frequency of Ag-NORs

Animal No.	No. in sample	Meta		C4		t <sub>3</sub>		C25		X <sup>2</sup>
		Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
37/5	41	16	23	7	7	8	5	10	6	6.60
68/5	22	17	15	4	3	0	2	1	2	3.09
73/4	24	18	18	1	1	2	2	3	3	0
91/5	35	23	22	3	4	3	4	6	5	0.75
98/5	40	32	28	2	6	5	5	1	1	3.24
101/6	44	30	31	9	6	5	7	0	0	2.10
121/5	93	57	57	13	11	10	12	13	13	0.70
67/8	29	18	19	6	6	1	1	4	3	0.39
198/5	51	35	30	4	9	6	6	6	6	3.60
165/6	24	16	14	5	5	3	3	0	2	2.28
123/8	22	18	16	2	3	2	3	0	0	0.92
130/8	24	19	19	1	2	4	3	0	0	0.83
92/8	38	27	24	3	6	3	5	7	5	3.48
148/8	27	20	23	7	4	0	0	0	0	2.64
155/8	35	22	24	10	7	0	0	3	4	1.70
156/8	22	11	14	10	5	0	0	1	3	6.98
176/6	155	124	122	28	21	3	11	-	-	8.20
90/6	28	11	15	7	5	-	-	10	8	3.87
155/6	49	28	29	11	11	-	-	10	9	0.15
45/6	34	20	21	5	6	4	4	5	3	1.55
174/6	41	23	26	4	5	-	-	14	10	2.15
61/6	32	21	22	7	5	-	-	4	5	1.05
169/1	35	31	28	1	2	-	-	3	5	1.62
166/1	37	28	26	1	4	8	7	-	-	2.55
112/8	20	14	13	1	2	3	3	2	2	0.58
60/5	35	31	29	0	0	4	5	0	1	1.34

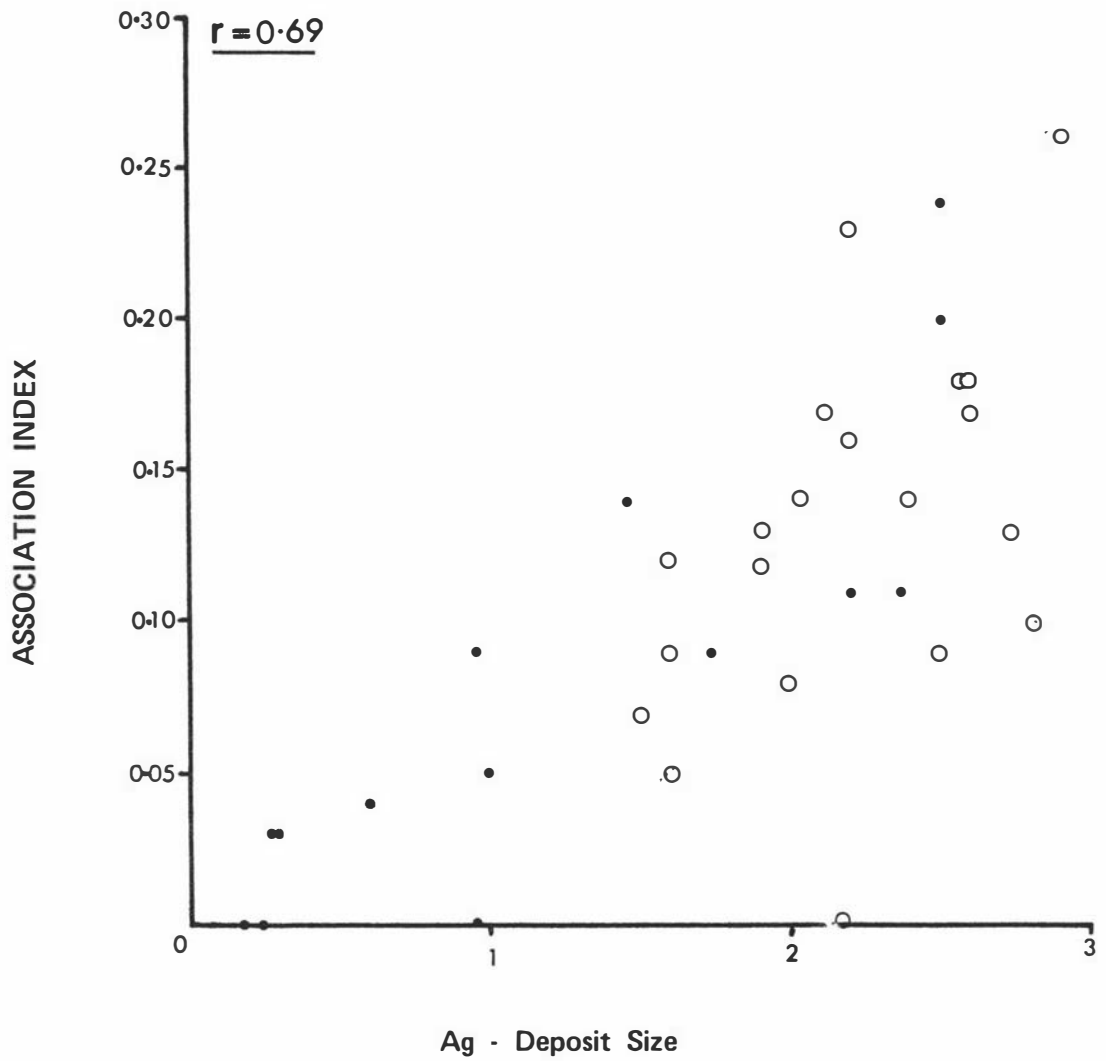
This data was analyzed further to determine the extent of the relationship between association index and Ag-staining frequency of the NORs. The association frequency was plotted against Ag-NOR frequency for each of the NO-chromosome types (Fig. 4-8). In order to standardize the values, the metacentric scores were divided by 6 and the C4 values by 2 to give values of association index and expression frequency per chromosome. If the chromosomes with zero staining frequency are excluded, the correlation is 0.69, and if the zero values are included, the correlation is 0.76 (50 d.f.  $p < 0.01$ ). Therefore the frequency of association is highly correlated with the frequency of participation in nucleolus organization.



**Fig. 4 - 8 :** Scattergram showing correlation of association index with frequency of Ag-NORs

Table 4-2: Relationship between deposit size and association frequency of C4, C25 and  $t_3$

Animal No.	No. of associating chromosomes	C25		$t_3$		C4	
		Size	A.I.	Size	A.I.	Size	A.I.
91/5	35	2.12	0.17	1.73	0.09	0.96	0.09
98/5	40	0.30	0.03	1.90	0.13	-	-
101/6	44	0	0	2.39	0.11	2.22	0.23
198/5	51	1.60	0.12	1.90	0.12	-	-
165/6	24	0.96	0	2.74	0.13	-	-
68/5	24	1.00	0.05	2.16	0	2.56	0.18
37/5	41	2.50	0.24	2.50	0.20	-	-
123/8	22	0	0	2.50	0.90	-	-
130/8	24	0	0	2.60	0.17	0.6	0.04
148/8	27	0	0	0	0	2.9	0.26
155/8	35	1.60	0.09	0	0	-	-
156/8	22	1.60	0.05	0	0	-	-
67/8	29	2.40	0.14	0.30	0.03	-	-
92/8	40	2.60	0.18	2.00	0.08	1.5	0.07
121/5	93	2.80	0.14	2.20	0.11	1.47	0.14
60/5	33	0.2	0	2.04	0.14	0.25	0
176/6	138	-	-	-	-	2.17	0.16



**Fig. 4 - 9 :** Graph showing correlation between Ag-deposit size and association index

#### 4.3.4 Relationship of the association frequency to the deposit size

In 17 animals the deposit size of the Ag-NORs on individual chromosomes was compared to the association frequency (Table 4-2). These chromosomes were either  $t_3$  and C25 in  $t_3$  heterozygotes or chromosomes 4 from animals which never had both C4 staining. (In animals with only one C4 staining it is assumed it is always the same homologue.) As shown in Fig. 4-9 a high correlation exists between the deposit size and the association index ( $r = 0.69$ , 32 d.f.  $p < 0.01$ ). As discussed previously, the deposit size is subjectively determined and therefore the axis in Fig. 4-9 does not correspond to a linear scale. Chromosomes which were expressed in every metaphase were analysed to see whether there was any effect of deposit size when the frequency of expression is maximum. The points corresponding to these chromosomes are indicated in Fig. 4-9. A lower correlation is found between the association frequency and the deposit size in these cases ( $r = 0.54$ ).

#### 4.3.5 Combinations of associating chromosomes

In sheep, with three types of NO-chromosomes, there are six possible combinations of two chromosomes (M-M; M-C4; M-C25; C4-C4; C4-C25; C25-C25) and eight possible combinations of three chromosomes (M-M-M; M-M-C4; M-M-C25; M-C4-C4; M-C4-C25; M-C25-C25; C4-C4-C25; and C4-C25-C25). During the course of this study all of these possible combinations were seen except for C4-C4-C25.

It is of interest to know not only whether there is a non-random participation of individual chromosomes in association as shown in section 4.3.3 but also whether there are preferential combinations of chromosomes in association complexes. The hypothesis that the combinations of chromosomes in association complexes is random, implies that the frequency of the types of association complexes will be directly proportional to the frequency of participation in nucleolus organization of the individual chromosomes involved in the complexes. Thus if each of the NO-chromosomes participated with an equal frequency in nucleolus organization and there was no

preferential association, then the ratios of the pairs of associating chromosomes would be expected to be:

15 M-M: 12 M-C4: 12M-C25: 1 C4-C4: 4 C4-C25: 1 C25-C25.  
(These are the numbers of different combinations possible in choosing 2 chromosomes from the 6 metacentric, 2 C4 and 2 C25 NO-chromosomes).

To determine whether the combinations of chromosomes in pair-wise associations was random, the number of associating pairs of each of the two-chromosome combinations was compared with expected values based on participation in nucleolus organization. Five animals were used, each having more than 20 pairs of associating chromosomes. The numbers of three-chromosome complexes were insufficient to be analysed separately so only two-chromosome complexes were scored. The relative frequencies of participation in nucleolus organization of each of the NO-chromosomes in these five animals is shown in Table 4-3.

Table 4-3: Relative participation in nucleolus organization of NO-chromosomes in five animals used in combinational analysis

Animal No.	Meta	C4	C25
37/5	0.57	0.18	0.25
121/5	0.61	0.12	0.27
155/6	0.60	0.22	0.18
176/6	0.79	0.13	0.08
174/6	0.64	0.13	0.23

From this data the expected frequency of two-chromosome combinations based on the hypothesis of random association was calculated. Thus, the frequency of each of the pairwise combinations is:

$$\begin{aligned}
 \text{M-M} &= (\text{R.E.F. (M)})^2 \\
 \text{M-C4} &= 2(\text{R.E.F. (M)}) (\text{R.E.F. (C4)}) \\
 \text{M-C25} &= 2 (\text{R.E.F. (M)}) (\text{R.E.F. (C25)}) \\
 \text{C4-C4} &= (\text{R.E.F. (C4)})^2 \\
 \text{C4-C25} &= 2 (\text{R.E.F. (C4)}) (\text{R.E.F. (C25)}) \\
 \text{C25-C25} &= (\text{R.E.F. (C25)})^2
 \end{aligned}$$

For example, in animal 37/5 the expected frequencies for each of the pair-wise combinations was 0.32 M-M: 0.21 M-C4: 0.29 M-C25: 0.03 C4-C4: 0.09 C4-C25: 0.06 C25-C25.

The expected frequencies are an approximation only as homologues are not distinguished in this analysis and it is therefore based on an equal association frequency for both homologues of a pair. For example, 176/6 rarely has both C4 chromosome<sup>s</sup> staining so that in fact there is a very small chance of a C4-C4 pair associating.

The expected and observed values for the five animals are shown in Table 4-4. The C4-C4, C4-C25 and C25-C25 classes were pooled to give sufficient numbers. The results on this small group indicate that there are no preferential combinations of chromosomes in pair-wise combinations in these animals ( $P > 0.05$  in all cases, 5 d.f.).

Table 4-4: Observed and Expected values of pair-wise combinations of NO-chromosomes

Animal No.	No. of associating pairs	<u>M-M</u>		<u>M-C4</u>		<u>M-C25</u>		<u>C4-C4</u>		<u>C4-C25</u>		<u>C25-C25</u>		$\chi^2$
		Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	
37/5	26	5	8	5	6	7	8	0	1	4	2	5	1	7.67
121/5	45	18	17	6	6	13	15	0	1	6	3	2	3	0.51
155/6	31	15	11	8	8	4	7	2	2	2	2	0	1	2.94
176/6	64	40	40	21	13	3	8	0	1	0	1	0	1	11.04
174/6	28	11	11	2	5	9	8	0	1	4	2	2	1	2.93

#### 4.4 Discussion

##### 4.4.1 Frequency of association

The frequency of metaphases containing associating chromosomes was found to vary widely (0% - 90%) between cultures and therefore could not be used to measure interindividual variability in association. In man also a range of association frequencies has been reported (Table 4-5) and it has been pointed out that the total number of associating chromosomes is an unreliable measure of biological variation (Jacobs et al., 1976). This makes suspect several reports on the variation of the frequency of satellite association between different age groups and sexes. The variation in the frequency of satellite association in different reports is believed to be due to differences in the criteria of association, differences in culture technique, slide preparation and quality and observer differences.

The average incidence of 33% of metaphases containing associations and the average number of associating chromosomes per metaphase of 1.02 found in this study is lower than most studies of satellite association in man. For comparison, these values have been either taken directly or calculated where possible from eight reports on satellite association in man (Table 4-5). As noted above, the wide variation in frequency of association in different studies is obvious. However, with the exception of one study (Ardito et al., 1978) the frequency of satellite association and number of associating chromosomes is smaller in sheep than in man.

Table 4-5: Reported frequencies of satellite association in man

No. of associating chromosomes per metaphase	Percentage of metaphases with associations	Author
1.93	60%	Ferguson-Smith and Handmaker, 1961
4.46	96%	Cohen and Shaw, 1968
-	88%	Zang and Back, 1968
-	89%	Nankin, 1970
2.59	-	Jacobs <u>et al.</u> , 1976
1.91	-	Mattei <u>et al.</u> , 1976
2.65	-	Liem <u>et al.</u> , 1977
0.49	21%	Ardito <u>et al.</u> , 1978

There are several possible explanations for the lower association values found in sheep. (1) This may simply be due to technical variation. (2) The stringent criteria adopted for scoring associations will underestimate the true association frequency. (3) The size of the metacentric chromosomes could lead to more association complexes being disrupted during harvesting and spreading. Such a hypothesis was originally invoked to explain the higher association index of the G than D group chromosomes in man (Zang and Back, 1968). However, in this present case this explanation appears to be invalid because the association index of the metacentrics does not differ significantly from their frequency of expression, and chromosome 25 does not have a significantly higher association frequency. (4) There may be differences in the frequency of nucleolar fusion between sheep and man. In a survey of the number of nucleoli in lymphocytes from various species of domestic animals, rodents and man, only rats and man were found to have a prevalence of uninucleolar lymphocytes. In goats and cows a much higher proportion of multinucleolar lymphocytes was found (Beran and Pospisil, 1967). It is possible that the proportion of multinucleolar lymphocytes is also higher in sheep, thus accounting for a lower association index.

#### 4.4.2 Factors affecting the association index of individual chromosomes

The results of this investigation of the association frequency of individual chromosomes and groups of chromosomes in sheep show that the ability to participate in nucleolus organization is a characteristic property of each chromosome. When the data on the involvement of the three types of NO-chromosomes from several animals was pooled, it was found that the metacentrics associated more often than chromosome 25, which in turn showed a higher association frequency than chromosome 4. The frequency of association was highly correlated with the frequency of expression. Thus chromosomes which were expressed in most metaphases had a higher association index than those that were expressed less often. The order of association indexes agrees with the relative expression frequencies of the metacentrics, chromosomes 4 and chromosomes 25.

Further, there was found to be a high correlation of association index with deposit size ( $r = 0.69$ ). Since the frequency of expression has been shown to be correlated with deposit size (see section 3.3.6) it was necessary to determine whether this high correlation of deposit size and association was simply a result of large deposits being more frequently expressed, or whether the large deposit size per se resulted in a higher tendency for association. When chromosomes were analysed which were expressed in every metaphase but which differed in deposit size, a correlation value of 0.54 was found between deposit size and association frequency. This means chromosomes with large deposits may be more likely to engage in associations than chromosomes with lower deposit sizes but equal expression frequencies. This is lower than the correlation between deposit size and association frequency for all the chromosomes analysed ( $r = 0.69$ ). This suggests that two factors may affect the frequency with which a chromosome participates in association, the frequency of participation in nucleolus organization and the size of the Ag-NOR.

In two instances, the  $t_3$  chromosome in animal 68/5 and chromosome 25 in 156/8 had fairly high R.E.F. values and deposit sizes but low association frequencies. There may be other factors than frequency of expression affecting the ability to be involved in NOR association in these cases. Alternatively these may be chance occurrences.

This finding of the relationship of expression frequency and deposit size to association frequency allows an interpretation of the different association indexes reported for individual chromosomes in different subjects. Each chromosome has a characteristic frequency of association which is dependent on its Ag-staining frequency and deposit size. In studies in man the association index of each of the five acrocentric pairs in pooled samples has been found to differ (see section 4.1.3) although there is a lack of uniformity in the relative order of participation in association complexes in different reports. In man, as in sheep, the NO-chromosomes may have a different capacity to be expressed. It has been reported that each individual has a unique pattern of silver staining (Mikelsaar et al., 1977; Varley, 1977). The relative order of participation in association complexes that has been reported in man may therefore reflect both this overall capacity of a chromosome pair to be expressed, and the composition of the individuals in the study.

The association frequency is highly correlated with the deposit size of the Ag-NOR. This is partly due to the relationship of deposit size to frequency of participation in nucleolus organization. In addition, the size of the Ag-deposit may have some other effect on association frequency. Larger Ag-NORs are more likely to undergo fusion than smaller Ag-NORs with the same expression frequency. A positive correlation of deposit size and association frequency was also reported by Miller et al. (1977). No cytological interpretation of this observation has been offered. One possible interpretation is that NORs with a large amount of silver-staining material may tend to be more resistant to the disruptive forces that lead to association complex breakdown during the harvesting and slide preparation procedures. It has already been noted that association frequencies show a large amount of variability, presumably due to technical variation. NOR association is postulated to be due to the entanglement of

rDNA fibres during the contraction of chromosomes in prophase and metaphase (Henderson et al., 1973). Regions with a large number of rRNA cistrons may be more likely to remain entangled either during the chromosome movements prior to metaphase or during technical manipulations in chromosome preparations and this could account for the effect of Ag-deposit size on NOR-association frequency.

The other possibility is that large deposits are more likely to lead to nucleolar fusion. A probability model for the fusion of nucleoli in metabolic cells has been published (Hasofer, 1974). In this model, the probability of fusion is partly determined by the radius of the nucleolus and the assumption is that if, during the process of growth, two nucleoli touch each other, fusion will occur. Both this model and Gani (1978) assume fusion is a random event, resulting from contact between aggregations of ribonuclear protein of two different nucleoli. Under this hypothesis, larger deposits may be more likely to come into contact and fuse. However nucleolar fusion may be determined by some factor other than growth and random fusion i.e. there may be a directed movement of nucleoli within the nucleolus. In this case, the increased association frequency of NORs with larger deposits could be due to a stronger attraction force of larger deposits.

#### 4.4.3 Combinations of chromosomes in association complexes

The results of the analysis on the frequency of the types of two-chromosome association complexes indicated that there are no preferential combinations of NO-chromosomes. The frequency of the various pairs is determined by the association frequency of the component chromosomes. Jacobs et al. (1976) also failed to find any significant evidence for preferential association between any of the acrocentric chromosomes in man. Their mathematical model developed to analyse pair-wise combinations used an estimate

for the association probability of each homologous pair of acrocentrics. While they do not give a value for the association probability for each of the homologous pairs in their subjects they state that their model does not fit the null hypothesis of equal association frequencies. Their analysis using different association probabilities gave non-significant results. These values presumably reflect the unequal association indexes of the chromosomes and are therefore comparable to the results of this study.

In the only similar study to the one presented here, the association indexes and frequency of associating pairs of chromosomes in people with Robertsonian translocations were scored (Hansson, 1975). A formula was derived for the expected frequency of pairs of associating chromosomes based on the association indexes of the individual chromosomes. A large difference was found between expected and observed numbers, which was interpreted as a specific tendency of the intact homologues of translocated chromosomes to associate with each other. The frequency of pair-wise associations in Robertsonian translocation carriers in man was therefore concluded to be not simply dependent on the association frequencies of the individual chromosomes. This contrasts with the results reported here of random combinations of NO-chromosomes in sheep when the relative expression frequencies are taken into consideration. No studies on normal people which take account of the association indexes of individual chromosomes have been reported.

## CHAPTER 5

### INHERITANCE OF AG-STAINABILITY

#### 5.1 Introduction

It has been shown that each chromosome has a characteristic ability to stain with silver. Since the factors determining this property are not yet fully understood, it was important to determine whether this is a heritable property. The presence of a NOR on the  $t_3$ /chromosome 25 polymorphism allowed studies to be made of the transmission of the Ag-stainability from parent to offspring. By mating sheep of appropriate karyotypes and Ag-stainability, the inheritance of the NO-chromosomes could be determined.

Recently two studies have been reported on the inheritance of Ag-stainability in humans. Inheritance studies in humans rely on the use of short arm polymorphisms to determine parentage. Mikelsaar et al. (1977b) reported a study on seven children with trisomy 21 and their parents. They concluded that the Ag-stainability of the NORs in general is a heritable characteristic. A similar study, also using quinacrine fluorescent polymorphisms in families with Down's syndrome children, also found that the degree of silver-staining of a NOR is an inherited property (Markovic et al., 1978).

#### 5.2 Materials and Methods

##### 5.2.1 Mating records

Since 1969 a flock of sheep carrying the  $t_1$ ,  $t_2$  and  $t_3$  polymorphisms has been established at Massey University. These sheep have been used to obtain fertility and segregation data and for this purpose accurate mating records have been kept (Bruère, 1974, 1975a). An analysis was therefore made of all the breeding data of the years 1972-1977 to determine mating groups which would be of use in a study of the inheritance of the Ag-stainability of NO-chromosomes. Useful matings were deemed to be those in which either the  $t_3$  and C25 chromosomes were segregating or the maternal or paternal origin of the NO-chromosomes could be determined.

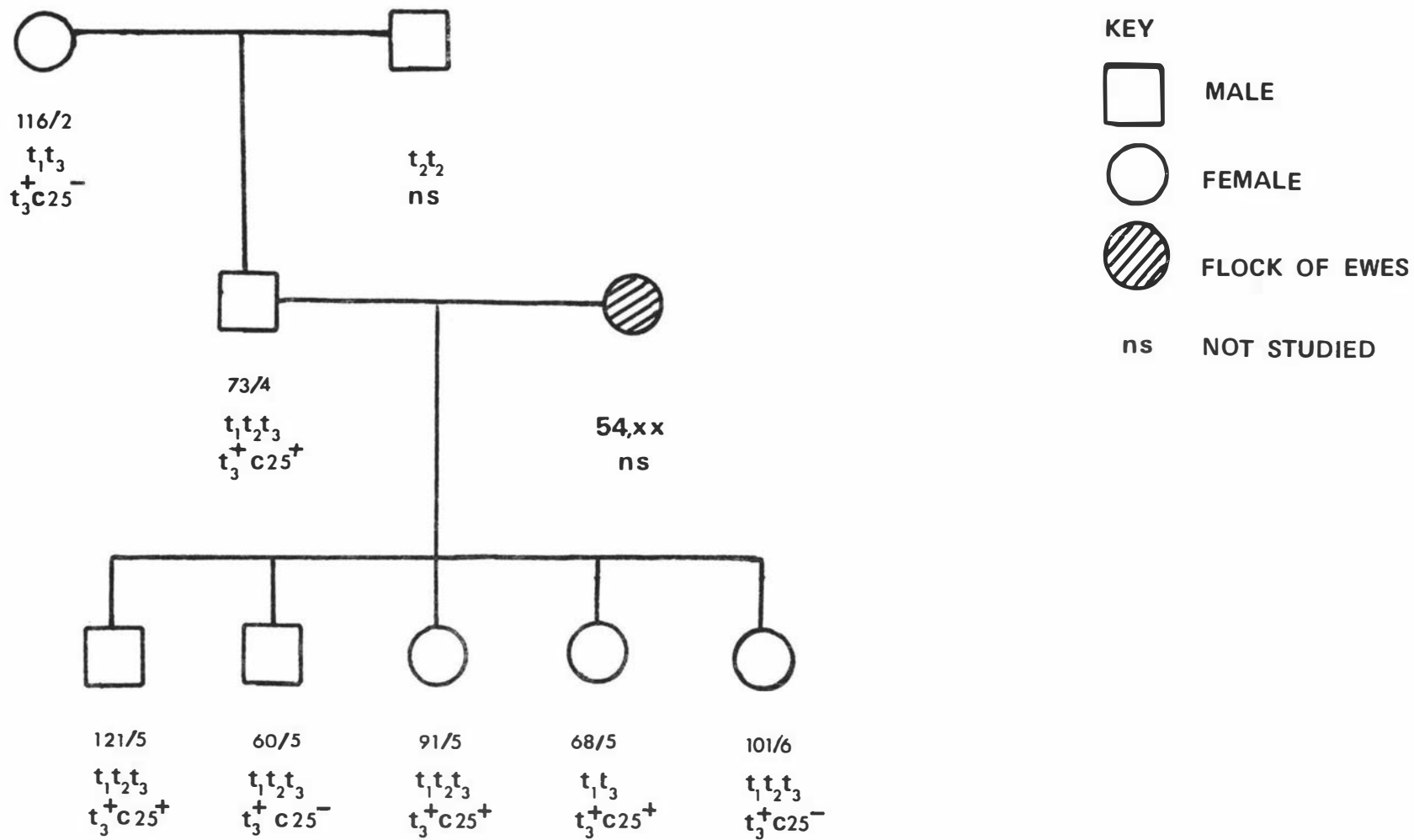
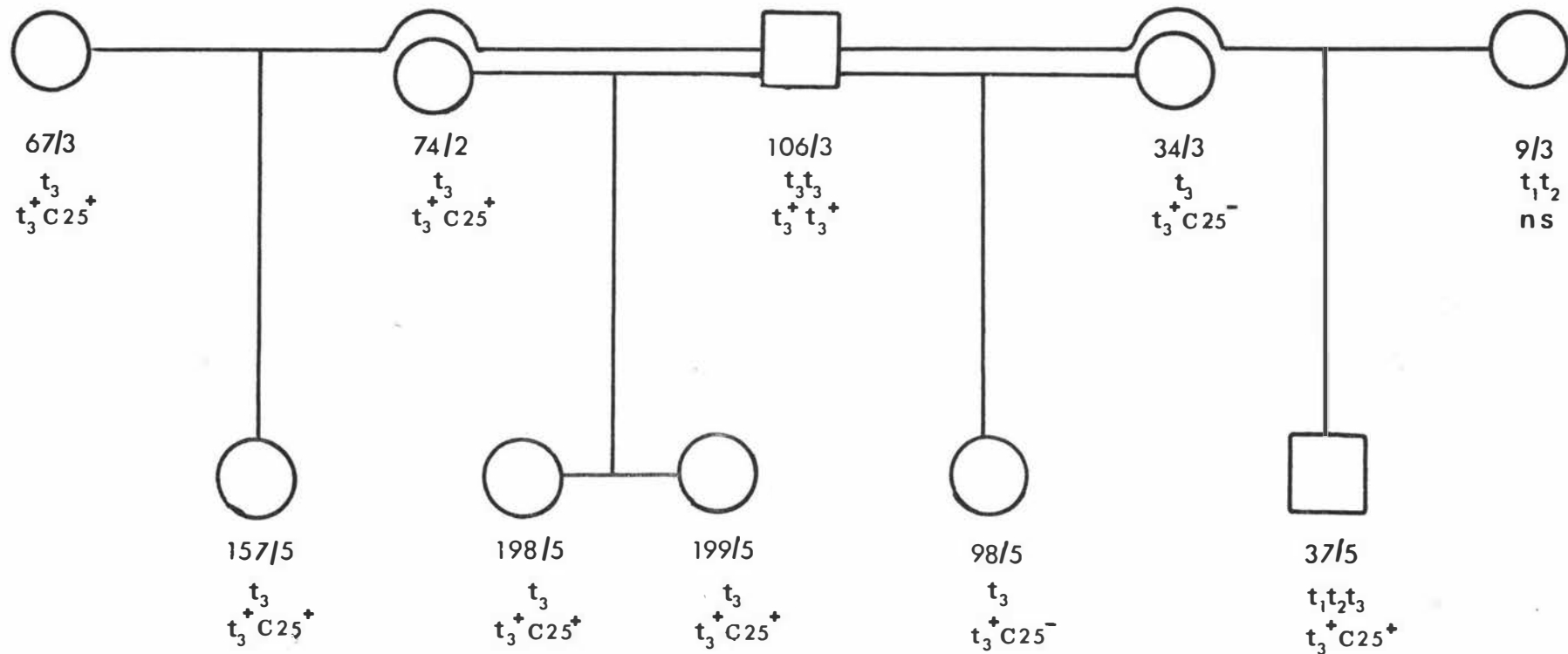


Fig. 5 - 1 : Pedigree chart of pedigree 1.



Key



Male



Female

ns

Not Studied

Fig. 5 - 2 : Pedigree chart of pedigree 2.

Two mating groups were found which satisfied these conditions. Pedigree 1 involved 3 generations in which the  $t_3$  and chromosome 25 were segregating (Fig. 5-1). The pedigree chart has been modified to allow for the multiple matings involved in the  $F_2$ . Pedigree 2 involved the heterozygous progeny of a  $t_3$  homozygous ram mated to  $t_3$  heterozygous ewes (Fig. 5-2). In this pedigree the inheritance of the Ag-NORs of the C25 from the dams could also be determined.

### 5.2.2 Establishment of mating groups

The above pedigrees involved a  $t_3$  chromosome with a high Ag-stainability. Three further mating groups were established to test the inheritance of Ag-NORs with different degrees of Ag-stainability. Pedigree 3 involved 2 rams with a staining chromosome 25 (121/5 and 37/5), while pedigree 4 involved a ram (60/5) with a non-staining chromosome 25. These rams were mated to  $t_3 t_3$  ewes and the heterozygous ( $t_3/C25$ ) progeny were screened for the stainability of chromosome 25.

In addition, a screening programme was carried out to find a ram with a non-staining  $t_3$  chromosome to use in a mating group. In addition to the rams previously screened as part of the first pedigree groups, and for other purposes in this research, 8 further rams were screened. At least 10 cells were counted from each animal. Only 176/6 (50,XY, $t_1 t_2 t_3 t_3$ ) was found to have a low frequency of staining of the  $t_3$  chromosome. This ram was mated to normal ewes and all progeny were screened for the stainability of the  $t_3$  chromosome.

For details of the management of the mating flocks see Appendix VII.

### 5.2.3 Determination of karyotypes of lambs

The karyotypes of the lambs of these 3 flocks were determined 1-3 weeks after birth in Giemsa-stained preparations. Five cells were counted in each case. It was necessary in some cases in pedigrees 3 and 4 to determine whether a submetacentric chromosome was a  $t_1$  or  $t_3$ . This was accomplished by Ag-staining and, in one

case, G-banding. The G-banding technique is described in Appendix V.

#### 5.2.4 Culture, Staining and Analysis Methods

The lymphocyte culture and Ag-staining techniques were the same as those previously described (section 2.2). In pedigrees 1 and 2, 30 Ag-stained cells were analysed from each animal. At least 60 cells were analysed from the sires in pedigrees 3, 4 and 5. Ten cells were counted from each of the lambs in these pedigrees. Size polymorphism data were collected from all animals in pedigrees 3, 4 and 5, and on the four dam-progeny pairs in pedigree 2.

#### 5.2.5 Notation

To show whether a chromosome had an Ag-NOR or not, a + or - notation was used. A chromosome was designated + if it had an Ag-NOR in more than 50% of metaphases. Thus an animal with  $t_3 + C25-$  has a staining  $t_3$  chromosome and a non-staining C25.

### 5.3 Results

#### 5.3.1 Pedigrees from mating records

##### 5.3.1.1 Pedigree 1

Fig. 5-1 shows the pedigree, karyotype and Ag-NOR frequency of the animals which were silver-stained from pedigree 1. The pedigree involves the segregation of a  $t_3$  chromosome through 2 generations. The  $t_3$  and C25 chromosomes are designated + or - as described above. Full details of the frequencies of all NO-chromosomes in all the pedigrees are given in Appendix XII. The Ag-stainability of the  $t_3$  chromosome has been retained through two generations.

### 5.3.1.2 Pedigree 2

In this pedigree (Fig 5-2) the sire had 2 Ag-positive  $t_3$  chromosomes. The Ag-stainability of the  $t_3$  chromosome has been inherited by the five  $F_1$  sheep studied. This ram was crossed with  $t_3$  heterozygous ewes so that each of the  $F_1$  heterozygous animals must have received their chromosome 25 from the dam. Three of these dams were still available for study, and in each case the Ag-stainability of the C25 in the dam was inherited by the progeny.

### 5.3.2 Results of mating experiments

#### 5.3.2.1 Lambing results of pedigree 3

This flock involved the mating of 2 rams, 121/5 and 37/5, both with a karyotype of  $51,XY,t_1t_2t_3$ , to 7  $t_3t_3$  ewes. Both rams had closely similar staining frequencies for their chromosome 25 and  $t_3$  chromosome (1.0 for  $t_3$  in 37/5, 0.9 for  $t_3$  in 121/5 and 1.0 for C25 in both rams). An Ag-stained karyotype of 121/5 is shown in Fig. 5-3. The lambing results are shown in Table 5-1. Only lambs heterozygous for the  $t_3$  chromosome were suitable for study as the origin of the  $t_3$  chromosome was known to be maternal in these. Lambs with a karyotype of  $52,t_2t_3$  or  $53,t_3$  could be determined to be suitable for Ag-staining on the basis of Giemsa-stained preparations. Lambs with 3 submetacentric chromosomes, and therefore homozygous for the  $t_3$  chromosome ( $51,t_1t_3t_3$  and  $50,t_1t_2t_3t_3$ ), could be rejected on the basis of Giemsa-staining. Lambs containing two submetacentric chromosomes had to be analysed further as the  $t_3$  and  $t_1$  chromosomes can not be distinguished in Giemsa-stained preparations. In each of the lambs with 2 submetacentric chromosomes (145/8, 83/8; 127/8, 47/8; 82/8) the identity of the second submetacentric was determined by silver-staining. If a small acrocentric had Ag-NORs, the lamb was determined to be a  $t_3$  heterozygote, and in other cases, the presence of Ag-NORs on both submetacentric chromosomes identified a  $t_3$  homozygote. Three of the 6 surviving lambs had suitable karyotypes for study. Chromosome 25 had Ag-NORs in each of these lambs. The NO-chromosomes of two of these lambs are shown in Fig. 5-4.

Table 5-1: Lambing and Ag-staining results of pedigree 3

Ewe No.	Lamb No.	Karyotype	Suitability	Ag-stainability $t_3$ C25	
166/1	145/8	51,XY, $t_2t_3t_3$	x		
49/3	Dead Lamb				
13/5	127/8	52,XY, $t_3t_3$	x		
88/5	85/8	52,XX, $t_1t_3$	✓	+	+
88/5	92/8	53,XX, $t_3$	✓	+	+
128/5	47/8	51,XY, $t_1t_2t_3$	✓	+	+
108/5	Dry				
202/5	82/8	51,XX, $t_1t_3t_3$	x		

Fig. 5-3: Karyotype of ram 121/5 showing Ag-NORs on 5 metacentric chromosomes, C4, t<sub>3</sub> and C25 (Ag-I)

Fig. 5-4: Karyotype of NO-chromosomes of 2 progeny of pedigree 3 showing inheritance of dark Ag-staining C25

- a. Lamb 85/8: NORs on C1, both C2s, C3, C4, t<sub>3</sub> and C25
- b. Lamb 92/8: NORs on C1, both C2s, C3, C4, t<sub>3</sub> and C25

(Ag-I)



**a**



**b**



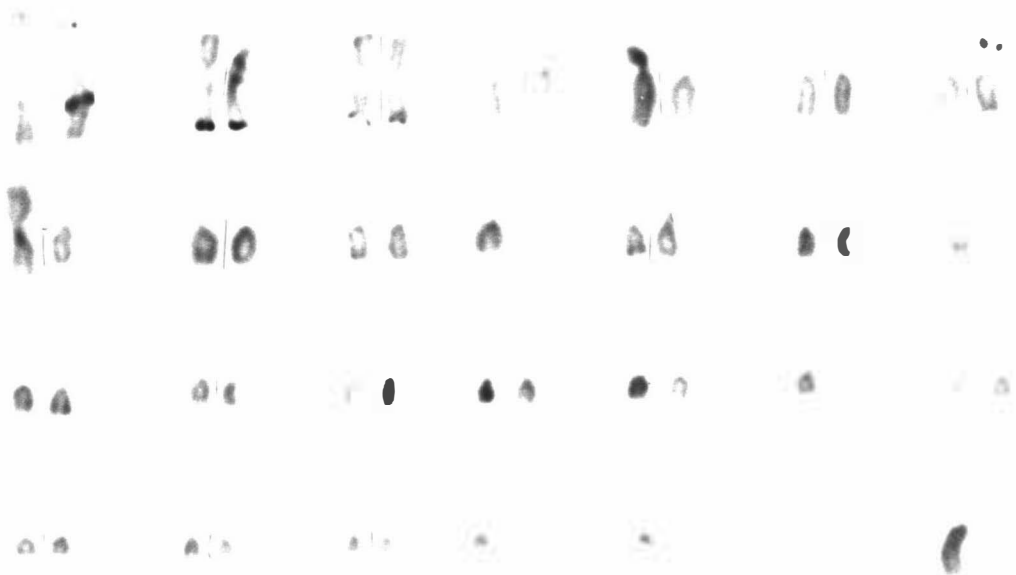
## 5.3.2.2 Lambing results of pedigree 4

In this flock 8  $t_3t_3$  ewes were mated to ram 60/5 (51,XY, $t_1t_2t_3$ ) which had a staining  $t_3$  chromosome and a non-staining C25. A karyotype of this ram is shown in Fig. 5-5. The lambing results are shown in Table 5-2.

Table 5-2 Lambing and Ag-staining results of pedigree 4

Ewe No.	Lamb No.	Karyotype	Suitability	Ag-stainability $t_3$	C25
107/3	130/8	52,XY, $t_2t_3$	✓	+	-
124/3	122/8	52,XX, $t_2t_3$	✓	+	-
124/3	123/8	51,XX, $t_1t_2t_3$	✓	+	-
164/3	54/8	51,XX, $t_2t_3t_3$	x		
164/3	55/8	53,XY, $t_3$	✓	+	+
48/5	60/8	52,XX, $t_1t_3$	✓	+	+
48/5	61/8	51,XX, $t_1t_3t_3$	x		
64/5	96/8	51,XX, $t_2t_3t_3$	x		
64/5	97/8	50,XY, $t_1t_2t_3t_3$	x		
100/5	16/8	52,XX, $t_2t_3$	✓	+	+
170/5	112/8	52,XY, $t_1t_3$	✓	+	+
115/5	184/8	53,XX, $t_3$	✓	+	+

Fig. 5-5: Karyotype of ram 60/5 showing  
Ag-NORs on both C1s, both C2s, both C3s  
and  $t_3$   
(Ag-I)



x y

Fig. 5-6: G-banded karyotype of lamb 123/8  
showing identification of  $t_1$  and  $t_3$   
chromosomes

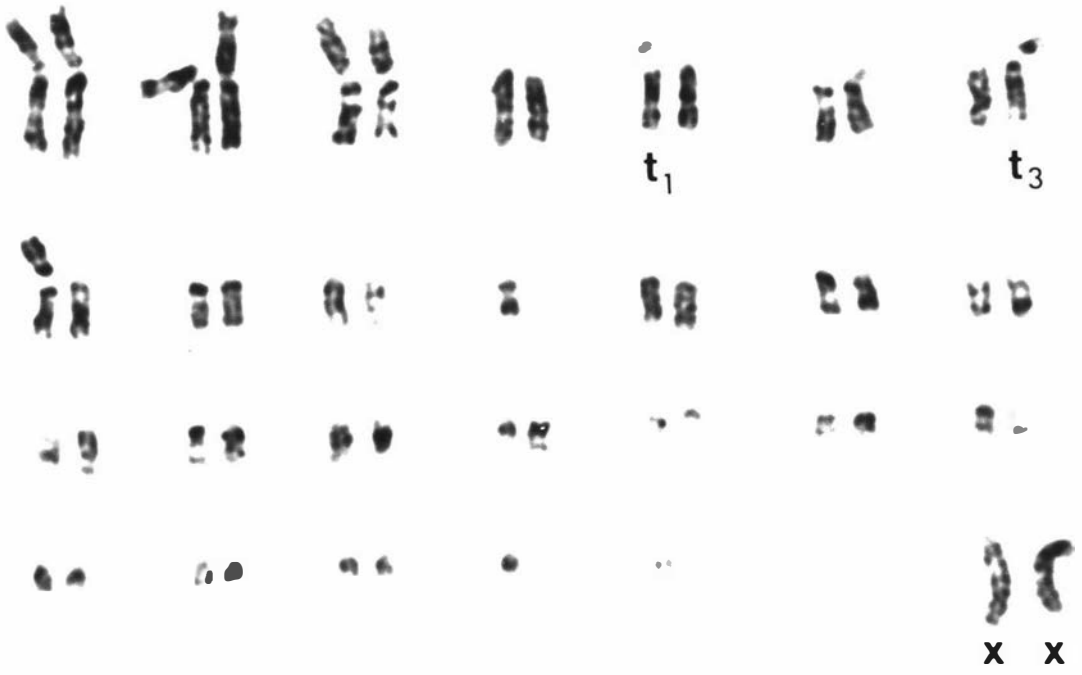


Fig. 5-7: Karyotype of NO-chromosomes of 4 progeny of pedigree 4 showing 2 progeny which inherited an Ag-staining C25 and 2 which did not.

- a. Lamb 123/8: NORs on both C1s, both C2s, both C3s, C4 and  $t_3$
- b. Lamb 112/8: NORs on C1, both C2s, both C3s, C4,  $t_3$  and C25
- c. Lamb 122/8: NORs on both C1s, both C2s, C3, C4 and  $t_3$
- d. Lamb 184/8: NORs on both C1s, C2, both C3s,  $t_3$  and C25

(Ag-I)

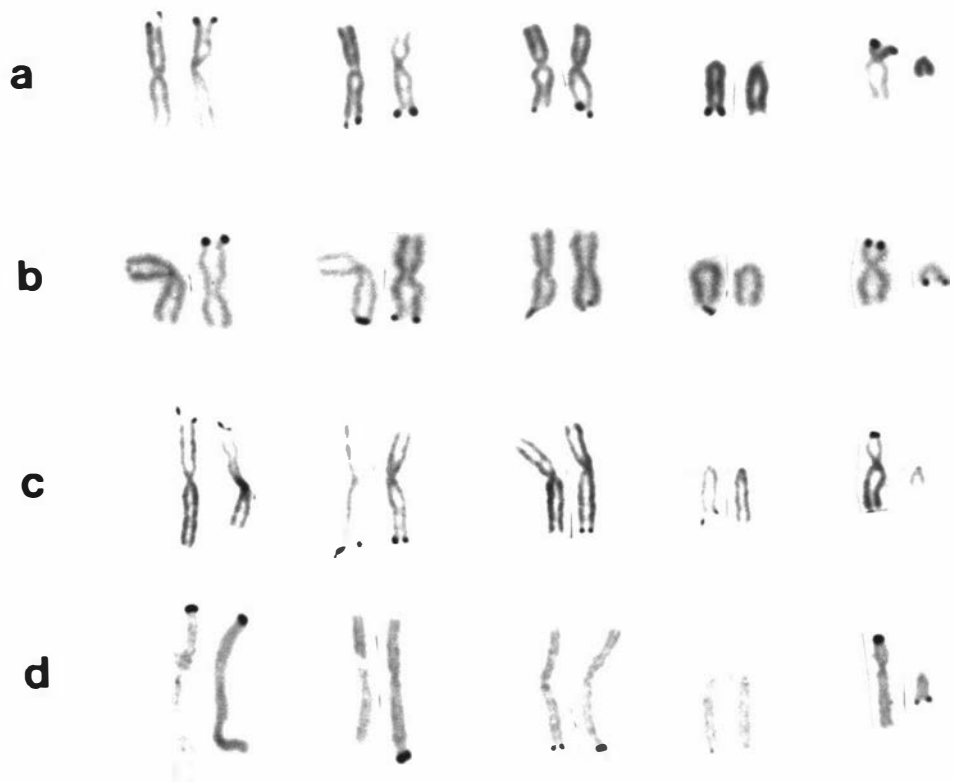


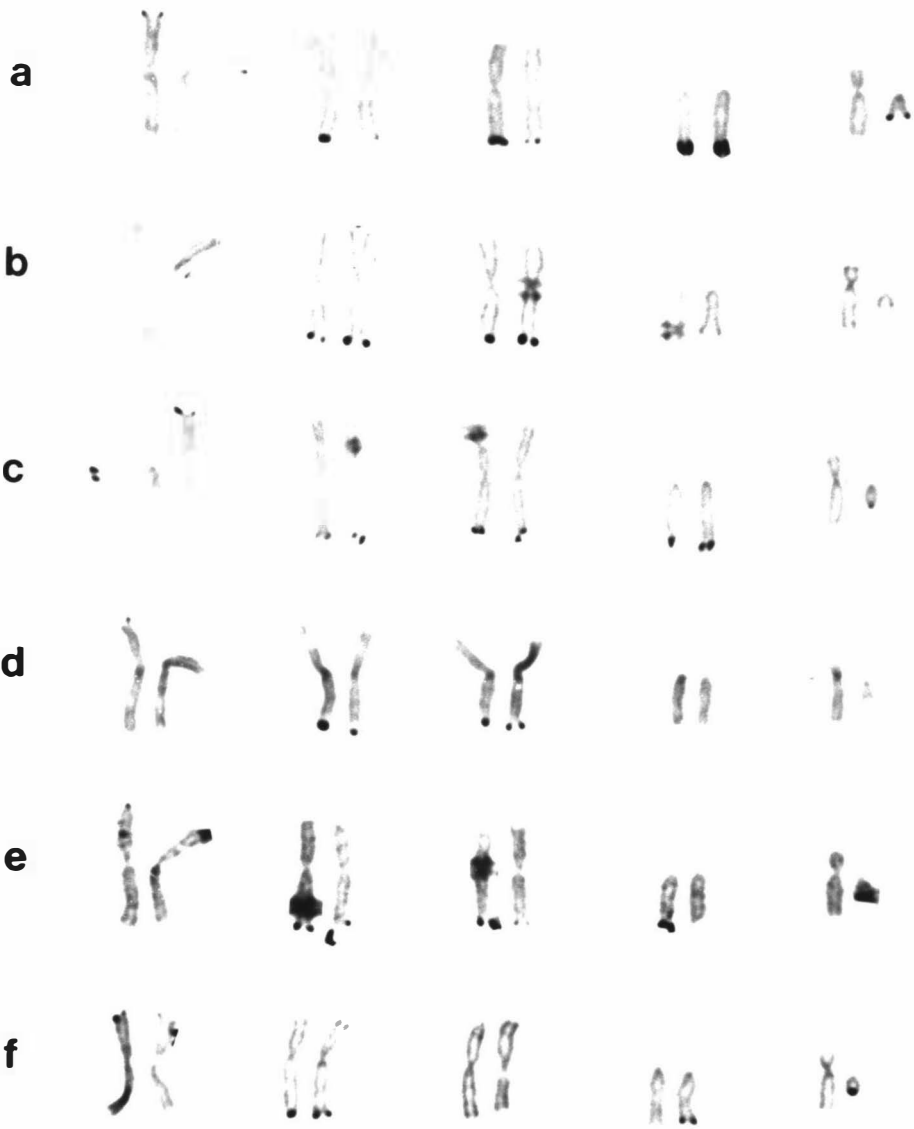
Fig. 5-8: Karyotype of sire of pedigree 5  
(176/6) showing Ag-NORs on 6 metacentrics  
and a C4  
(Ag-I)



Fig. 5-9: Karyotype of NO-chromosomes of  
6 progeny of pedigree 5 showing inheritance  
of non-staining  $t_3$

- a. Lamb 156/8: NORs on both C1s, both C2s, both C3s  
both C4s and C25
- b. Lamb 164/8: NORs on both C2s and both C4s
- c. Lamb 155/8: NORs on both C1s, both C2s, both  
C3s, both C4s and C25
- d. Lamb 64/8: NORs on C1, both C2s, and both  
C3s
- e. Lamb 152/8: NORs on both C1s, both C2s, both C3s,  
C4 and C25
- f. Lamb 150/8: NORs on both C1s, both C2s, C4 and  
C25

(Ag-I)



As for flock 3, the 5 lambs with 2 submetacentric chromosomes were silver-stained to determine their identity. In all cases but one (123/8) the presence of silver dots on both submetacentric chromosomes, or the presence of an Ag-NOR on a small acrocentric chromosome differentiated  $t_3t_3$  animals from  $t_1t_3$  double heterozygotes. Lamb 123/8 had one staining  $t_3$ , but no Ag-NORs could be seen either on the other submetacentric chromosome or on a small acrocentric chromosome. It was therefore necessary to identify the submetacentric chromosome by G-banding. This showed that the animal had a karyotype of  $51,XX,t_1t_2t_3$  (Fig. 5-6).

In this flock, 8 lambs were suitable for study. Five lambs had inherited an Ag-positive C25 and in 3 lambs the chromosome 25 was Ag-negative. The NO-chromosomes from 4 of these lambs is shown in Fig. 5-7.

#### 5.3.2.3 Lambing results of pedigree 5

This mating involved ram 176/6 ( $50,XY,t_1t_2t_3t_3$ ) by 20 normal ewes ( $54,XX$ ). This ram had 2 predominantly non-staining  $t_3$  chromosomes as shown in Fig. 5-8. The results of this mating are shown in Table 5-3. 24 lambs were born in this group and all were suitable for study as they had inherited a  $t_3$  chromosome from the ram and a C25 from their dams. Only 4 cells were counted from one lamb and so this lamb was excluded from the analysis. Two lambs had a  $t_3$  chromosome staining in half the metaphases, and one lamb had a  $t_3$  chromosome staining in the majority of its metaphases. The NO-chromosomes of 6 of the lambs are karyotyped in Fig. 5-9.

#### 5.3.3 Recombination of Ag-NORs

Three animals were heterozygous for the Ag-stainability of the C25 and  $t_3$  chromosomes. In these cases, recombination would result in changes in the Ag-stainability of the  $t_3$  chromosome or chromosome 25 inherited by their progeny. The Ag-stainability of their progeny are shown in Table 5-4. Five of the 10 lambs show a difference from their parental chromosome of origin in the Ag-stainability of the  $t_3$  chromosome or C25.

Table 5-3 Lambing and Ag-staining results of pedigree 5

Ewe No.	Lamb No.	Karyotype	Ag-stainability t <sub>3</sub>	C25
178/6	150/8	52,XX,t <sub>1</sub> t <sub>3</sub>	-	+
178/6	151/8	51,XY,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	-	+
4/2	51/8	52,XY,t <sub>1</sub> t <sub>3</sub>	-	+
5/2	144/8	53,XY,t <sub>3</sub>	-	+/-**
13/2	64/8	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	-	+
13/2	65/8	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	-	-
16/2	95/8	53,XX,t <sub>3</sub>	-	+/-
22/2	68/8	51,XY,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	-	+
26/2	81/8	52,XX,t <sub>1</sub> t <sub>3</sub>	-	+
36/2	155/8	52,XX,t <sub>1</sub> t <sub>3</sub>	-	+
36/2	156/8	52,XY,t <sub>2</sub> t <sub>3</sub>	-	+
60/2	157/8	53,XX,t <sub>3</sub>	-	-
60/2	158/8	52,XY,t <sub>2</sub> t <sub>3</sub>	-	-
98/2	148/8	52,XY,t <sub>2</sub> t <sub>3</sub>	-	-
103/2	39/8	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	*	*
107/2	175/8	53,XY,t <sub>3</sub>	+/-	-
120/2	56/8	52,XX,t <sub>2</sub> t <sub>3</sub>	-	+/-
121/2	152/8	52,XX,t <sub>1</sub> t <sub>3</sub>	+/-	+
140/2	109/8	52,XX,t <sub>1</sub> t <sub>3</sub>	-	-
143/2	67/8	51,XX,t <sub>1</sub> t <sub>2</sub> t <sub>3</sub>	-	+
63/2	163/8	52,XX,t <sub>2</sub> t <sub>3</sub>	-	-
63/2	164/8	52,XX,t <sub>2</sub> t <sub>3</sub>	+	-
154/2	185/8	52,XY,t <sub>2</sub> t <sub>3</sub>	-	+/-
174/2	160/8	52,XY,t <sub>1</sub> t <sub>3</sub>	-	+
178/2	Dry Ewe			

\* Only 4 cells counted so results were not analysed

\*\* +/- refers to chromosomes which were stained in half the metaphases counted

Table 5-4 Inheritance of Ag-stainability in sheep heterozygous for Ag-stainability

Pedigree	Dam/Sire	Ag-stainability	Lamb	Ag-stainability
1	116/2	$t_3^+ c25^-$	73/4	$t_3^+$
2	34/3	$t_3^+ c25^-$	98/5	$c25^-$
4	60/5	$t_3^+ c25^-$	180/8	$c25^-$
	60/5		122/8	$c25^-$
	60/5		123/8	$c25^-$
	60/5		55/8	$c25^+$
	60/5		60/8	$c25^+$
	60/5		16/8	$c25^+$
	60/5		112/8	$c25^+$
	60/5		184/8	$c25^+$

#### 5.3.4 Inheritance of frequency of Ag-NORs and size polymorphism

The correlation of Ag-staining frequency in 5 rams (73/4, 106/3, 121/5, 37/5, 176/6) and 2 ewes (67/3, 74/2) and their 39 offspring was computed from the data in Appendix XII. Only sheep which were non-heterozygous in Ag-stainability of their  $t_3/c25$  were used. Both  $t_3$  chromosomes in 106/3 and 176/6 were assumed to have the same frequency of staining (0.97 for 106/3 and 0.25 for 176/6). This assumption is valid for 106/3 where in 30 metaphases counted, one metaphase had neither  $t_3$  staining and in the rest, both  $t_3$  chromosomes had Ag-NORs. However a different situation occurs in 176/6 where in 124 metaphases, 6 had 2  $t_3$  chromosomes staining, 53 had one  $t_3$  chromosome staining and 65 had no  $t_3$  staining. Assuming that in the

metaphases with one  $t_3$  staining, it is the same chromosome staining in each case, then one  $t_3$  chromosome has a staining<sup>frequency</sup> of 0.48 and the other has a frequency of 0.05. It is not possible to determine which of these chromosomes has been inherited in each of the progeny. Therefore, pooling of the results will produce an approximation only.

The correlation co-efficient between Ag-stainability for the  $t_3$ /C25 in the dam/sire and that in their progeny was found to be 0.93 (Fig. 5-10).

A similar analysis was made of the correlation of size polymorphism of the Ag-NORs between two successive generations. For this analysis the three lambs of pedigree 3, all lambs in pedigree 5 and the lambs of 67/3 and 74/2 in pedigree 2 were analysed. The data on size polymorphism of the C25 or  $t_3$  chromosome is presented in Table 5-5. Again, an approximation is involved due to the difference in Ag-NOR size of the two  $t_3$  chromosomes in 176/6 (pedigree 5) and the slight difference in size (2.6 and 2.8) of the deposit score of the  $t_3$  in 121/5 and 37/5 (pedigree 3). The results indicate a high correlation of deposit size in parent and progeny ( $r = 0.97$ ) (Fig. 5-11).

## 5.4 Discussion

### 5.4.1 Inheritance of Ag-stainability

This study provides evidence for the heritable nature of the Ag-NORs. The Ag-stainability of 49 chromosomes was compared in 2 successive generations and in 44 cases it was found to be consistent between generations. In the other five animals sired by a ram heterozygous for Ag-stainability on his  $t_3$ /C25, the difference in Ag-stainability in the C25 in the progeny can be accounted for by recombination. In flock 5 in which the ram had a low stainability of the  $t_3$ , one individual (164/8) was found to have a  $t_3$  staining in 70% of the metaphases, but in all cases the deposit was faint (deposit score of 0.7). Another two animals (152/8, 175/8) had a  $t_3$  staining in 50% of their metaphases, but again, the deposit was faint in every case. In no case was a large deposit found in the progeny of a parent with a small Ag-NOR, or vice versa.

Table 5-5 Size of Ag-NORs in five animals and their progeny

Parental No.	Chromosome Involved	Parental deposit size	Progeny No.	Progeny deposit size
<u>Pedigree 2</u>				
67/3	C25	1.6	157/5	1.1
74/2	C25	1.5	198/5	1.6
74/2	C25	1.5	199/5	1.5
<u>Pedigree 3</u>				
121/5 } 37/5 }	C25	2.7	47/8	2.5
			85/8	2.6
			92/8	2.8
<u>Pedigree 5</u>				
176/6	t <sub>3</sub>	0.2	150/8	0
			151/8	0.2
			51/8	0
			144/8	0.2
			64/8	0
			65/8	0.3
			95/8	0.3
			68/8	0
			81/8	0.1
			155/8	0
			156/8	0
			157/8	0
			158/8	0
			148/8	0
			175/8	0.5
			56/8	0
			152/8	0.5
			109/8	0
			67/8	0.4
			163/8	0
			164/8	0.7
			185/8	0
			160/8	0.2

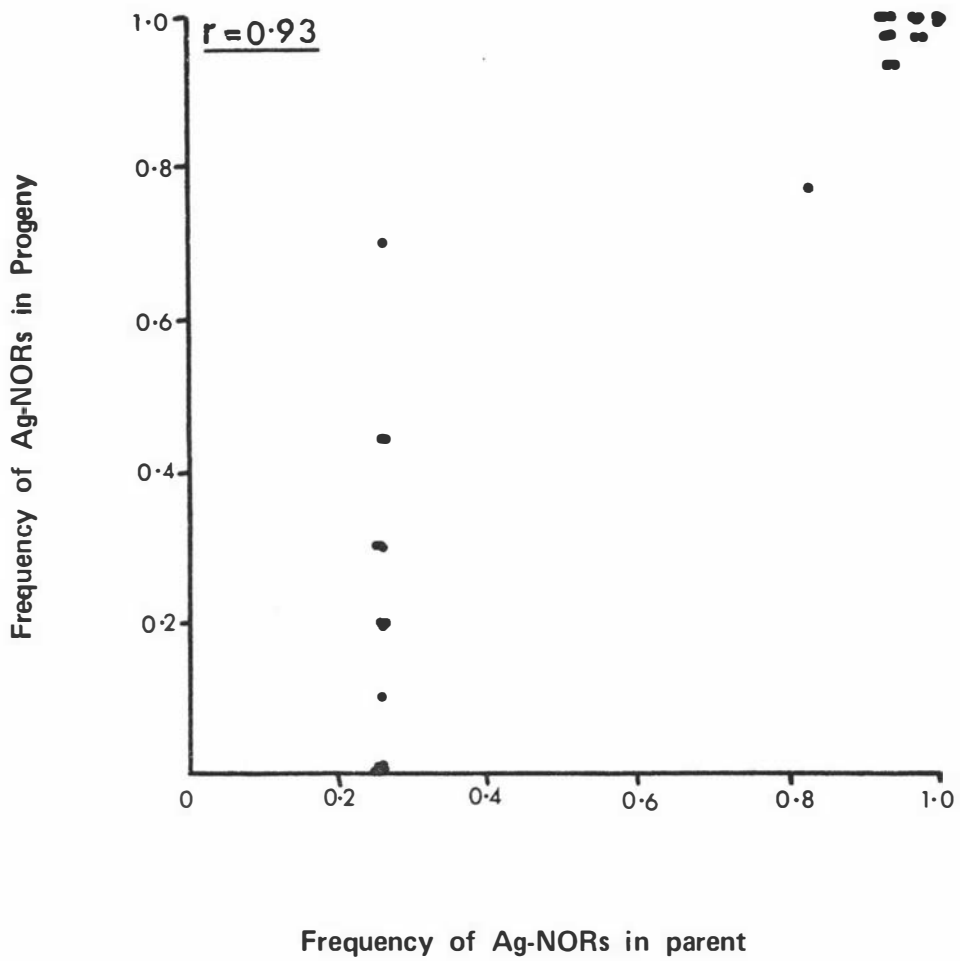


Fig. 5 – 10 : Scattergram showing correlation between Ag-NOR frequency in parent and progeny

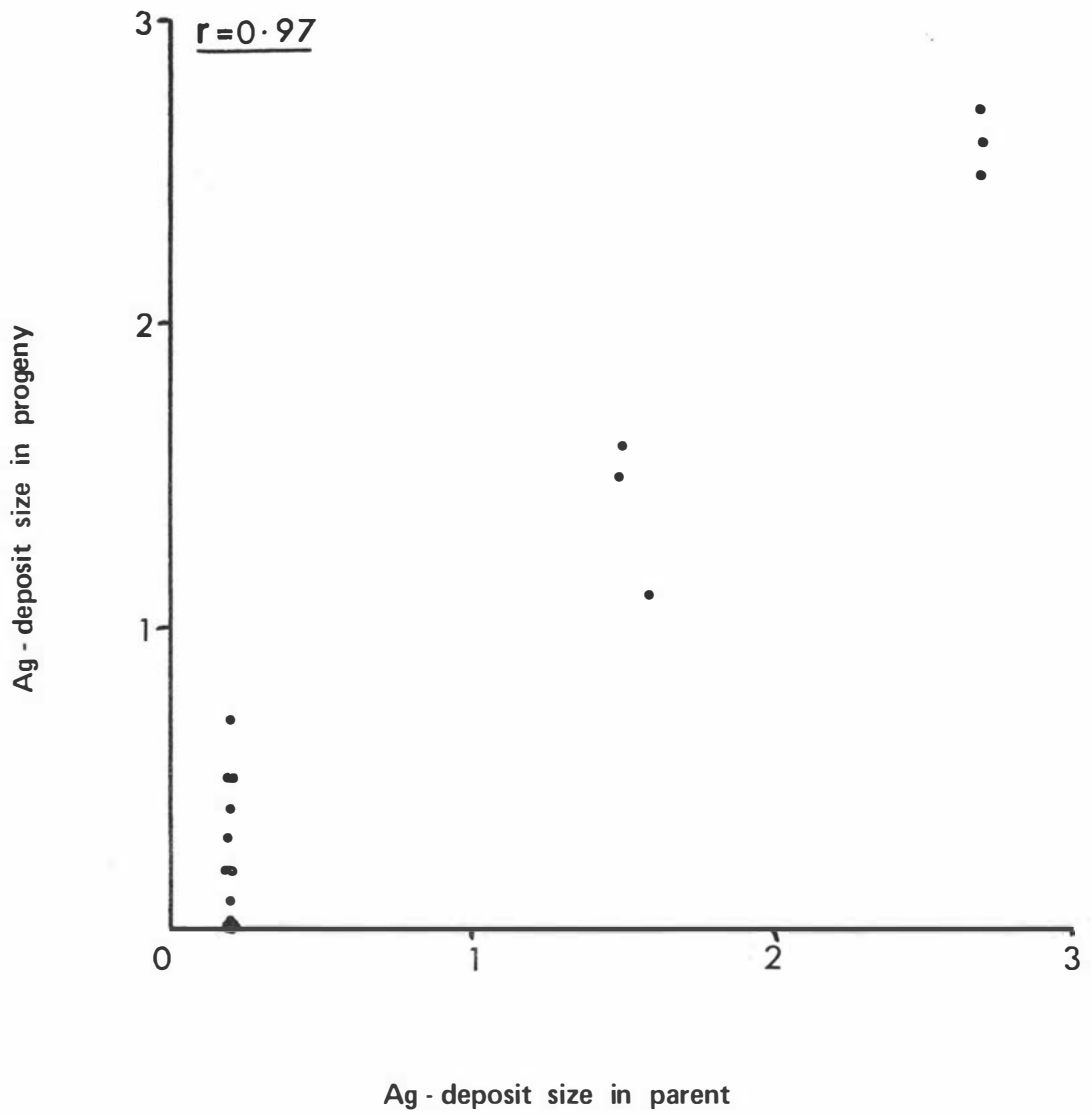


Fig. 5 - 11 : Scattergram showing relationship of Ag-NOR size polymorphism in parent and progeny

The correlation between Ag-stainability and Ag-deposit size in successive generations was high.

Variation in Ag-NOR frequency between cells in a culture, and between replicate cultures from an animal have been found (section 3.3). The slight variation in Ag-NOR frequency found between successive generations in some of the animals in this study is therefore probably similar to this variation. As discussed in Chapter 3, this variation is probably due to technical variation in culture and slide preparation, although it may possibly reflect slight differences in activation state of the NORs in different cells or cultures.

These results are in close agreement with those of Mikelsaar et al. (1977b) and Markovic et al. (1978). Mikelsaar et al. studied 7 families and scored the Ag-NORs as positive if seen in more than 50% of metaphases, and negative otherwise. In 11 chromosome pairs individual homologues could be identified by quinacrine markers. In all of them the Ag-staining was consistent in parent and child. In the other pairs where individual homologues could not be identified the child showed combinations of the Ag-NORs which were compatible with a direct chromosomal transmission. The small deviations they observed in some cases could also be accounted for by intra-individual variation. The degree of intra-individual variation in their study is smaller than that found in this study. Only 3-11 metaphases were scored in each of their subjects, and it is possible those chosen were more uniform in staining intensity than those in this study. No attempt to categorise the deposit size was made in their study.

Markovic et al. (1978) studied the size of Ag-NORs in individual acrocentric chromosomes in two successive generations in 6 Down's syndrome children and their parents. A strong correlation was found between the NOR score in the parent and child in the 31 chromosomes which could be traced by fluorescence polymorphisms.

The present study and these two recent reports therefore indicate that the Ag-NOR stainability and size polymorphism is a heritable characteristic. However there are three reports that indicate that the Ag-stainability of an Ag-NOR may be modifiable. In 2 mouse cell lines, some evidence was found that changes can take place in the amount of Ag-stain on a particular chromosome (Dev et al., 1977). The number 15 chromosome in one cell line had a large Ag-NOR, whereas the 15's in two other cell lines of the same origin were unstained.

In humans a change of Ag-stainability was found in a family in which a balanced translocation involving a NOR is segregating (M. Parslow, pers.comm.). Some family members with this translocation chromosome have positive Ag-NORs and some are Ag-negative. This may be accounted for by differences in the activation of NORs in different family members, or it may reflect a further chromosomal change (e.g. deletion of NOR) in some family members. Another possibility is that the Ag-stainability of a NOR could be altered by exchange events between NORs (e.g. either sister chromatid, between non-homologues or between homologues in meiosis), resulting in changes in the rDNA levels. Such exchanges are suggested to account for differences in rDNA level between homologous chromosomes (Henderson et al., 1976b; MacGregor et al. 1977 and see section 3.4.4). If the ability to be active in nucleolus organization, as shown by silver staining, is a function of rDNA content, such exchange events could result in a modification of Ag-stainability between generations.

In another study, the NORs were studied in a proband with a 13/13 translocation, her parents, and her sister (Zankl and Hahmann, 1978). The 13/13 translocation resulted in the loss of two NORs so the study aimed to determine whether other previously inactive NORs would be activated to compensate for this loss. They found that the chromosome 22 appeared to be more heavily stained in the proband than in the parents. They suggested that this could have been due to increased activity in this NOR, or it could have been due to variation of the intensity of staining. The chromosomes 22 in the proband were found to have a higher association frequency

than in the parents which was suggested to reflect a higher degree of activity of these NORs. However, since the homologues were not differentiated, this could possibly be accounted for by the segregation and combination of the two stronger Ag-positive NORs from the parents. The normal sister also had a higher association frequency for chromosome 22 than the parents. Therefore this paper, while indicating that a change in Ag-NOR activity may occur, can not be taken as strong evidence for this.

#### 5.4.2 Recombination of Ag-NORs

In this study, the Ag-stainability of ten progeny of animals heterozygous in Ag-stainability of their  $t_3/C25$  NORs was scored. In five of these the Ag-stainability was the same in both the parent and progeny and in the other five the Ag-stainability was consistent with the homologue not inherited by the progeny. Although these numbers are small, they can be used to provide an estimate of the genetic map length of chromosome 25. Thus the recombination frequency of 0.5 indicates that chromosome 25 is at least 50 genetic map units long (or  $\frac{1}{2}$  a morgan (Renwick, (1971)). This frequency agrees with cytological observations on the number of chiasmata in  $t_3$  heterozygous rams. In a study of meiosis in Robertsonian translocation rams (Chapman, 1974; Chapman and Bruere, 1977) the majority of trivalents in  $t_3$  heterozygous rams were found to have 3 chiasmata including one interstitial or terminal chiasma between the short arms. Although the mean number of chiasmata was not stated, "commonly" one chiasma per short arm was seen and no trivalent was found with more than one chiasma on its short arms (Chapman, 1974). Since bivalents with no chiasma do not normally occur, the mean number of chiasmata per short arm can be assumed to be 1.0, corresponding to a map length of 50 genetic units (White, 1961; Renwick, 1971). This is consistent with the minimum estimate of 50 genetic units based on recombination frequency.

The occurrence of NORs in Ag-staining and non-staining forms should provide a useful marker for genetic mapping studies. This, of course, applies not only to the NORs on the  $t_3$  chromosomes but

also to the other NO-chromosomes. The usefulness of genetic mapping to animal breeding studies has been mooted previously (Bruère, 1975b; Logue, 1978). Thus, in addition to the potential application of the segregation of the  $t_1, t_2$  and  $t_3$  chromosome polymorphisms in genetic mapping of sheep, the assignment of the rRNA cistrons to five chromosomal loci in sheep provides a starting point for linkage studies.

In humans the frequency of chiasma in the acrocentric short arms is zero for the G group chromosomes and 0.12 for each D group chromosome (Hultén, 1974), and so recombination does not pose a problem in the interpretation of data in inheritance studies. In the present study the conclusion that Ag-stainability of a particular NOR is an inherited characteristic was based on observations in individuals homozygous for their Ag-NOR deposit size and frequency. The results from individuals heterozygous for their Ag-NORs was consistent with direct transmission of Ag-stainability. While heterozygous individuals were useful in obtaining data on recombination frequencies, they have a limited usefulness in determining the heritability of Ag-NORs in this species.

## CHAPTER 6

### COMPARISON OF SILVER-STAINING PATTERNS IN FIBROBLASTS AND LYMPHOCYTES

#### 6.1 Introduction

As shown and discussed in Chapter 3, each NOR appears to have a characteristic ability to be involved in nucleolus organisation, as shown by the presence of a silver deposit. The factors determining whether a NOR is either active or not are not yet understood. It was therefore important to determine whether this ability to be involved in nucleolus organization was determined early in development, in a manner similar to the X-chromosome inactivation phenomenon or whether it is a heritable property. To investigate these alternatives, the heritability of the NORs was tested (Chapter 5) and the frequency and chromosome distribution of NORs in different tissues was determined. If the ability to express the genes for rRNA is due to an activation/inactivation phenomenon then different tissues might have different patterns of silver staining.

In addition to determining whether the pattern of silver staining was constant between tissues, another aim was to determine the frequency of the Ag-NORs in different tissues. Different cell types may show differential activity in rRNA production. In differentiated cells such as neurons and Sertoli cells a single nucleolus per nucleus is predominant. While this has been interpreted as a change in the orientation of NORs in highly differentiated cells (Hsu et al., 1975), it is possible that this is in fact related to a lower activity of the NORs and a lower number of active NORs. Different states of activation might exist in different tissues and this could be reflected in the frequencies of Ag-NORs.

Recently, two reports have been published on the frequency of NORs in different tissues. Mikelsaar and Schwarzacher (1978) found little difference in the silver-staining of NORs in human lymphocytes and fibroblasts. However, a comparative study of patterns of silver-staining in cells of six day blastocyst and kidney fibroblasts of the domestic rabbit indicated a difference in frequency between these two tissue types (Martin-DeLeon et al., 1978).

## 6.2 Materials and Methods

### 6.2.1 Tissue Source

Tissues and blood were collected from three rams during student surgery. Blood, fascia and muscle were collected from 90/6 (53,XY,t<sub>2</sub>); blood, fascia and gut were collected from 155/6 (52,XY,t<sub>1</sub>t<sub>2</sub>) and blood, muscle and fascia were collected from 165/6 (51,XY,t<sub>2</sub>t<sub>2</sub>t<sub>3</sub>). Lymphocyte cultures were prepared as described earlier (Section 2.2.1).

### 6.2.2 Fibroblast culture technique

The technique used was an explant culture method using plasma clot embedding, which is in routine use in our laboratory. This was originally adapted from the technique described by Hyman (1968). The culture materials are described in Appendix IV.

Small pieces of tissue were collected in medium TC199 with 1% penicillin, streptomycin and kanamycin (PSK). For each animal 10 ml of blood was collected in a vacutainer with Alsever's solution. The blood was centrifuged and the serum drawn off. The tissue was cut up with fine scissors into small pieces (about 1 mm square) and floated in media TC199 with 8% foetal bovine serum (FBS) and 1% PSK. Nine pieces of tissue were planted on the floor of each 250 cm<sup>2</sup> Falcon flask using a siliconised pipette bent at one end. The excess fluid was removed. The tissue was attached to the flask by making a plasma clot. 0.2 ml of 2% calcium chloride solution was mixed with 1 ml of autologous serum (in Alsever's) and one drop of this mixture was placed on each piece of tissue. The excess fluid was removed with a pasteur pipette. This was dried for 20 minutes in an incubator at 37°C and then about 5 ml of culture medium (TC199 plus 8% FBS plus 1% PSK) was added carefully so as not to disturb the clot. The cultures were incubated at 37°C.

### 6.2.3 Subculturing

The media was changed either every 4-7 days or when the phenol red indicated a change in pH. When the cultures showed good growth (about 7-10 days after start) they were subcultured. This involved pouring off the old media, washing the flask with phosphate buffered saline (PBS), then incubating the flasks with 2 ml ATV (containing antibiotic, about 2% trypsin and versene) for 10-15 minutes until the cells could be seen to be floating freely when observed under phase contrast. The contents of the flask were then divided, media was added and the flasks were reincubated.

### 6.2.4 Harvesting of fibroblast cultures

The medium was changed in the flask 24 hours before the cells were harvested. 0.1 ml of colchicine (0.001 mg/ml) was added to each flask 10-12 hours before the cells were harvested. All media from the flasks were poured into centrifuge tubes. The flasks were rinsed out with PBS and this rinsing was also retained. The flasks were incubated with 2 ml ATV for 10-15 minutes until the cells were removed. This cell suspension was added to the supernatant which was then spun for 5 minutes at 1000 rpm. The supernatant was removed and the cells treated with 10 ml of 0.075 M KCl for 20 minutes at 37°C. Following spinning at 1000 rpm for five minutes, the pelleted cells were fixed (3 alcohol : 1 acetic acid) and allowed to stand in the fridge for one hour. The cells were recentrifuged, resuspended in 0.5 ml fixative, spread on ice-chilled slides and flame-dried.

### 6.2.5 Ag-staining

The preparations were treated by the Ag-I technique as described earlier (Section 2.2.4). Twenty-five cells were scored from each of three tissue types in the three animals. The frequency, chromosome distribution and size of the Ag-NORs were scored in each case.

### 6.3 Results

#### 6.3.1 Frequency of Ag-NORs in different tissues

The frequency of Ag-NORs in the three tissues from each of the three animals is shown in Table 6-1.

Table 6-1: The frequency of Ag-NORs in lymphocyte and fibroblast cultures from three animals

Animal No.	Tissue	Frequency of Ag-NORs			C25	Overall
		Meta	C4	t <sub>3</sub>		
90/6	Blood	3.9	1.2	-	1.9	7.0
	Fascia	3.6	1.6	-	1.8	7.0
	Muscle	2.3	1.2	-	1.6	5.1
155/6	Blood	5.4	2.0	-	1.6	9.0
	Fascia	5.1	1.8	-	1.4	8.3
	Gut	5.2	1.8	-	1.7	8.7
165/6	Blood	4.8	1.7	1.0	0.6	8.1
	Fascia	3.4	1.7	1.0	0.2	6.1
	Muscle	3.2	1.7	1.0	0.2	6.1

In two instances the overall frequency of Ag-NORs differs markedly from the other tissues in the animal. The average number of Ag-NORs per metaphase in the muscle of 90/6 is considerably lower than for blood and fascia, and in the lymphocyte culture from 165/6 the frequency is considerably higher than for the two fibroblast cultures. The chromosomal distribution of the Ag-NORs is shown in Fig. 6-1. It can be seen that the lower frequency of Ag-NORs in the

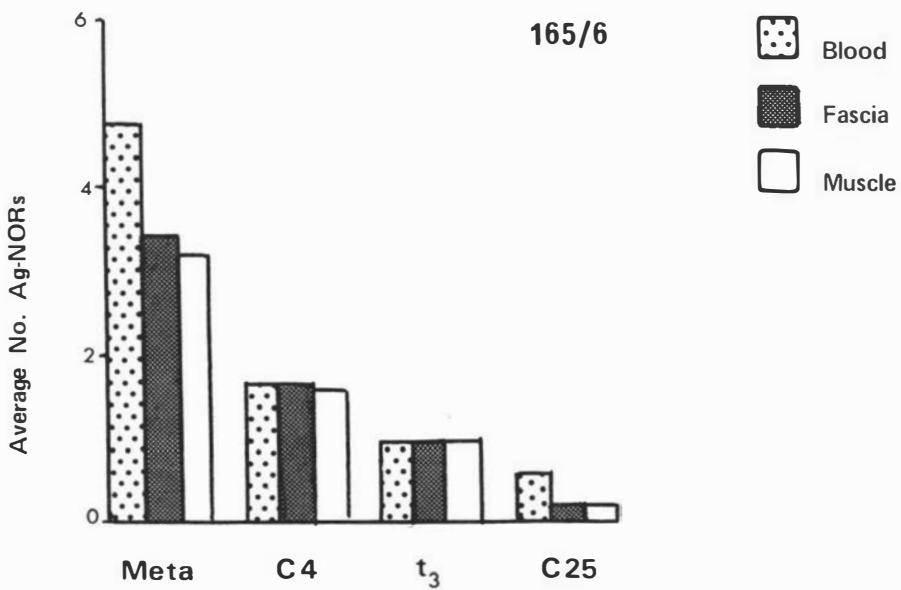
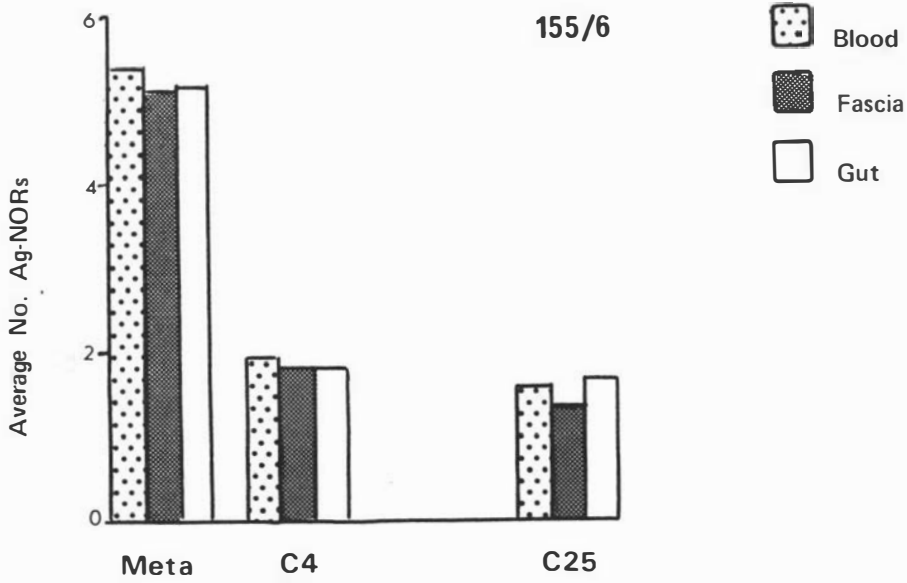
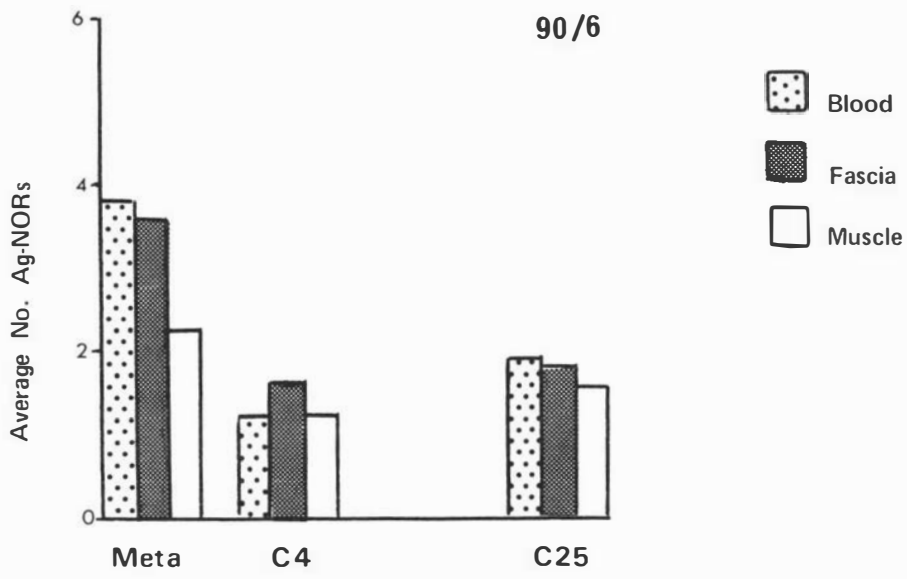


Fig. 6 - 1 : Histograms of average number of Ag-NORs per metaphase in different tissues in 3 animals.

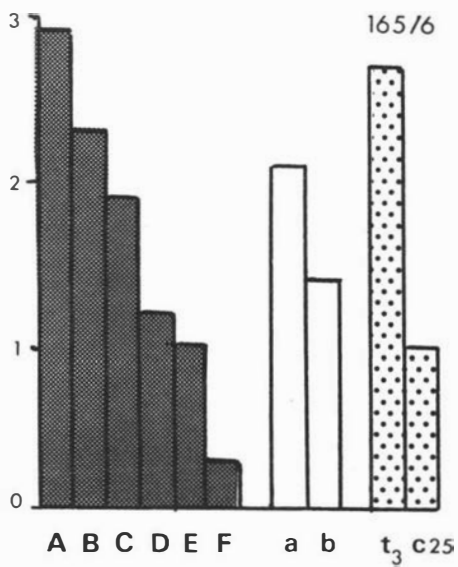
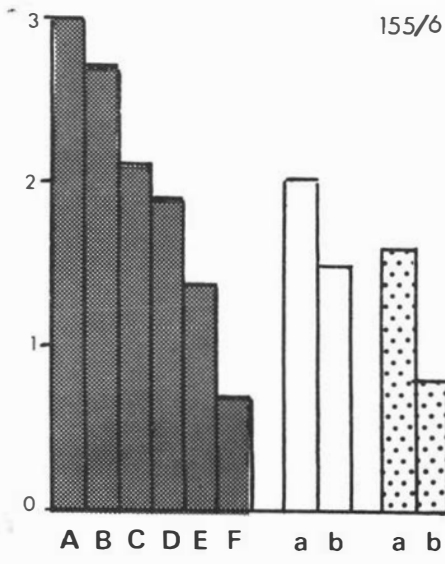
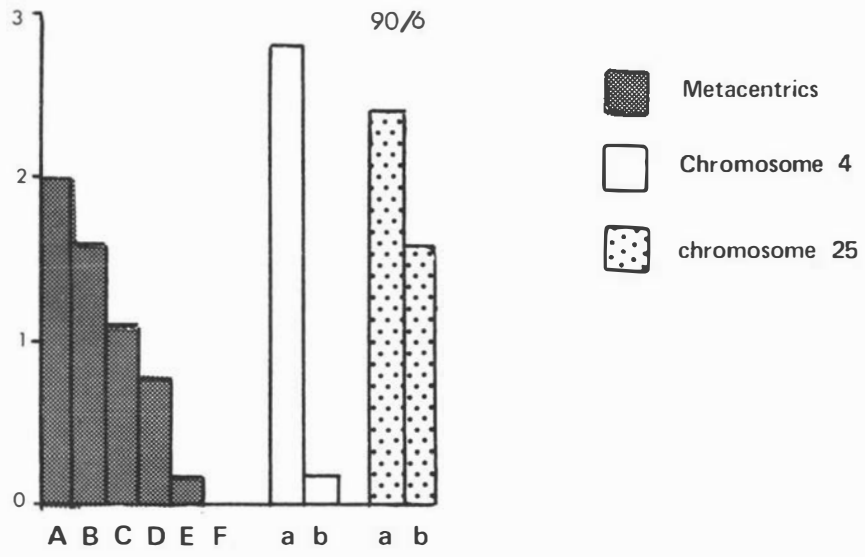


Fig. 6 - 2 : Sizes of Ag-NORs in lymphocyte cultures in 3 animals

muscle of 90/6 is due mainly to a lower frequency of Ag-staining of the metacentrics. The higher frequency of Ag-NORs in the lymphocytes of 165/6 is due predominantly to a higher staining frequency of the metacentrics, and to a lesser extent to the higher staining frequency of chromosome 25.

### 6.3.2 Size polymorphism of Ag-NORs in different tissues

The sizes of the Ag-NORs in the lymphocyte cultures are shown in Fig. 6-2. It can be seen that the Ag-NORs which showed the greatest variation between tissue types are those which are the smallest. Thus several of the metacentric chromosomes in 90/6 and 165/6 have small deposits and show variability in frequency between cultures.

The relative sizes of the Ag-NORs tended to be fairly constant between tissues. Thus a chromosome which had dark Ag-NORs in one tissue, had dark Ag-NORs in the other tissues. Data on the size polymorphism in each of the tissues is presented in Table 6-2. Tissues which had the lowest overall frequency of Ag-NORs also tended to have a lower total Ag-deposit size. For example, the deposit size on chromosomes in the muscle culture of 90/6 was lower for each Ag-NOR, with the exception of the second chromosome 4, than the deposit size in the lymphocyte culture. The deposit sizes on the NORs of gut and fascia chromosomes are smaller than those from the lymphocyte cells.

Fig. 6-3 is a karyotype of the Ag-NORs in the three tissues from ram 155/6. This shows the similar distribution of Ag-NORs on the NO-chromosomes of the three tissues, and also the difference in intensity of staining between the tissues.

## 6.4 Discussion

In each of the animals studied the tissues showed the characteristic individual pattern in the chromosomal distribution and size of Ag-NORs. In some cases, the intensity and frequency of Ag-deposits varied between tissues.

Table 6-2: Size polymorphism of Ag-NORs in each tissue from three animals

Animal No.	Tissue	A	B	C	<u>Metacentrics</u>		F	<u>C4</u>		<u>C25</u>	
					D	E		a	b	a	b
90/6	Blood	2.0	1.6	1.1	0.8	0.2	0.0	2.8	0.4	2.4	1.6
	Muscle	1.3	0.9	0.6	0.2	0.0	0.0	2.1	0.6	1.8	0.9
	Fascia	1.8	1.2	0.9	0.6	0.2	0.0	2.9	1.0	2.6	1.3
155/6	Blood	3.0	2.7	2.1	1.9	1.4	0.7	2.0	1.5	1.6	0.8
	Gut	2.5	2.0	1.6	1.3	0.9	0.5	1.7	1.1	1.4	0.7
	Fascia	2.8	2.5	1.7	1.4	0.9	0.5	2.0	1.2	1.2	0.5
165/6	Blood	2.9	2.3	1.9	1.2	1.0	0.3	2.1	1.4	2.7	1.0
	Fascia	2.5	1.5	1.0	0.5	0.0	0.0	1.6	0.8	2.2	0.2
	Muscle	2.3	1.3	0.9	0.4	0.1	0.0	1.6	0.8	2.0	0.1

Fig. 6-3: Karyotype of NO-chromosomes from  
3 tissues of 155/6

- a. Lymphocyte culture: NORs on both C1s, both C2s,  
both C3s, both C4s and both C25s
- b. Gut culture: NORs on both C2s, both C3s, both  
C4s and both C25s
- c. Fascia culture: NORs on C2, both C3s and both  
C4s  
(Ag-I)

**a**



**b**



**c**



Although the average frequency of Ag-NORs on chromosomes derived from lymphocytes tended to be higher than in fibroblast cultures, the relationship was not consistent. Thus the two fibroblast cultures and the lymphocyte culture of 155/6 have similar Ag-NOR frequencies. In 90/6 the lymphocyte culture and the fibroblast culture derived from the fascia had similar Ag-NOR frequencies but the culture from muscle was considerably lower. In 165/6 both fibroblast cultures had similar Ag-NOR frequencies which were considerably lower than the frequency of Ag-NORs found in the lymphocyte culture.

Differences in Ag-NOR frequency could be due either to differences in the state of activation of NORs between tissues, or to technical variation. There may be no difference in the state of activation of the NORs in fibroblasts and lymphoblasts since these are undifferentiated and undergoing rapid growth. They would therefore be expected to be in a maximum activation state. In view of the inconsistent relationship between frequency of Ag-NORs in fibroblasts and lymphocytes technical variation is more likely to account for variation in Ag-NOR frequency in different tissues. One possibility is that trypsin was used in the harvesting of the cell cultures and it is known that the Ag-staining material is an acidic protein and therefore trypsin-sensitive (Section 2.1.6). However it is uncertain whether live cells are as susceptible to the proteolytic action of trypsin as dead cells, and whether trypsin exposure during the harvesting process was sufficient to remove the Ag-staining material. It has been shown (Chapter 3) that differences in Ag-NOR frequency between replicate lymphocyte cultures from an animal and between the metaphases of a culture are due to variation in staining of the smaller Ag-NORs. This also applies to the variation in Ag-NOR frequencies between tissue cultures and suggests technical variation may be involved. Factors which could contribute to differences in the Ag-stainability of small NORs are discussed in more detail in Section 3.4.1.

Whether differences in the activation state exist in different tissues could not be confirmed by this study. However, differing

patterns of Ag-NORs have been found in 2-cell, 4-cell and 8-cell stages of mouse embryos (Engel et al., 1977; Hansmann et al., 1978).

The results of this study are similar to those of Mikelsaar et al. (1978) in man. They found minor differences in silver-staining frequency and pattern between replicate lymphocyte cultures and between lymphocyte and fibroblast cultures from the same individual.

Different results were obtained by Martin DeLeon et al. (1978) in rabbit blastocysts and fibroblasts. They reported different patterns of staining and frequency of Ag-NORs in blastocysts and fibroblasts. Their results were obtained from 25 cells from five blastocysts and 50 fibroblasts from 'at least' six animals. The number of cells from each animal and blastocyst were not given. The Ag-NOR counts and patterns were therefore not obtained from the same individual. Therefore it is impossible to determine the importance of individual differences in the chromosome distribution of Ag-NORs to the differences reported in chromosomal distribution of Ag-NORs between fibroblasts and blastocysts. This is particularly important in view of the small number of fibroblasts and blastocyst cells counted (50 and 25 respectively) where individual counts are not given and these could greatly bias the results. On the basis of their data, Martin-DeLeon et al. (1978) suggested the higher frequency of Ag-NORs in blastocysts than fibroblasts could be due to differential activation. The possibility remains that, as in this study the differences in frequencies could be due either to technical variation or to the trypsinisation of the fibroblast cultures.

In conclusion, the present evidence suggests that the ability of NORs to be active is a heritable property, rather than due to an inactivation or specific regulation event in different tissues.

## CHAPTER 7

### HOMOLOGY OF NORs IN THE SHEEP, GOAT, AOUADAD, BHARAL AND CATTLE

#### 7.1 Introduction

##### 7.1.1 Chromosomal evolution in the Bovidae

The major mechanism of chromosomal evolution in the super-family Bovoidea is believed to be by Robertsonian translocation. This hypothesis was first proposed on the basis of the small variation in the fundamental number despite a variation from 30 to 60 in the diploid number among these species (Wurster and Benirschke, 1968). In support of this hypothesis, G-band homology was found to occur in cattle (2n=60), goat (2n=60) and sheep (2n=54) (Evans et al., 1973; Schnedl and Czaker, 1974). All the chromosome arms in sheep were found to correspond to the goat acrocentrics. All but chromosomes 11 and 12 of cattle also had homologous chromosomes in the goat. More detailed studies of members of the Capridae have also shown G-band homologies between the aoudad, Ammotragus lervia (2n=58); the goat, Capra hircus (2n=60); domestic sheep, Ovis aries (2n=54); and various species of wild sheep including O.vignei (2n=58); O.orientalis (2n=54), O.musimon (2n=54), O.canadensis (2n=54) and O.nivicola (2n=52) (Nadler et al., 1973; Nadler et al., 1974; Bunch et al., 1976; Bunch, 1978). The bharal, Pseudois nayaur has a chromosome number of 54. It also shows G-band homology but the three metacentric pairs differ from the sheep metacentrics (T.D. Bunch, pers. comm.). On the basis of the cytogenetic evidence of chromosomal evolution by Robertsonian translocation, a hypothesis of the pathway of evolution in the caprids has been proposed (Bunch et al., 1976). It is commonly believed that the ancestral chromosome number in the Bovidae was 60. This is retained by goats and cattle. The aoudad has undergone one centric fusion resulting in a diploid number of 58. Its karyotype is identical to the Urial sheep, O.vignei. Further centric fusion events during the evolution of sheep have produced species with diploid numbers of 56, 54 and 52.

A more extensive G-banding study of 12 species of Rovidæ and members of the related superfamilies, the Giraffoidea and Cervoidea, revealed a large degree of chromosome-arm homology (Buckland and Evans, 1978a). This strongly indicates the importance of Robertsonian translocation type rearrangements in producing interspecies karyotype differences, with a minor contribution from inversions, and reciprocal and tandem translocations. It is believed that reciprocal translocation accounts for the non-homology with the goat karyotype of the cattle 11 and 12 chromosomes mentioned earlier.

Other aspects of chromosomal evolution in the Bovidæ have been studied. Sheep and goats have closely similar satellite DNAs (Curtain et al., 1973). Satellite I of the goat is identical to the sheep satellite I. The satellite II components are not identical (Kurnit et al., 1978). These are located at the centromere in the acrocentric chromosomes and the sheep and goat satellite DNA components cross-hybridize (A.R. Mitchell, pers. comm.; Kurnit et al., 1978). Cattle have five satellite DNA fractions (Kurnit et al., 1978). Divergence in the satellite DNA composition during evolution is indicated by the inability of the medium cattle satellite to hybridize with either sheep or goat chromosomes, and the absence of hybridization between two goat satellites and cattle chromosomes (A.R. Mitchell, pers. comm.). However restriction enzyme analyses indicate common ancient species may persist in sheep, goat and cattle (Kurnit et al., 1978).

Goat and sheep nuclei have a similar DNA content, whereas cattle have 14% more DNA (Sumner and Buckland, 1976). In view of the similar chromosome banding patterns in these species, these authors suggested that numerous minute interstitial deletions or additions of DNA could account for the higher DNA content of cattle nuclei. Work in their laboratory on the proportion of satellite DNA in the genome in the sheep and ox has found similar values (unpublished data quoted in Buckland and Evans, 1978b). <sup>This conflicts with the data published by Kurnit et al., 1978.</sup> In agreement with Curtain et al. (1973), they found the satellite I and II components of sheep and goat were about 12% and 2.5% respectively of the genome. The percentage of total DNA accounted for by the cattle satellites I, II, III and IV was 6-9%, 2%, 4% and 8% respectively. The percentage for satellite V was not given. These authors suggested therefore that some, and possibly most, of the variation in total DNA between cattle and goat was due

to the contributions of satellites III, IV and V in cattle. If this is correct, then the suggestion of Buckland and Evans that chromosomal deletions or additions have occurred during evolution, may be incorrect.

#### 7.1.2 Studies on the evolution of NORs

Comparative studies on the location and number of NORs have now been published for the Djungarian and Syrian hamster (Bigger and Savage, 1976), human, chimpanzee, gorilla, orangutan and gibbon (Tantravahi et al., 1976) for 22 species of toads and tree-frogs, (Schmid, 1978a), in 12 frog species of the suborder Diplasiocoela (Schmid, 1978b) and for inbred lines of mice (Dev et al., 1977).

The Djungarian hamster, Phodopus sungorous has four pairs of Ag-AS staining chromosomes whereas the Syrian hamster Mesocricetus auratus has five pairs (Bigger and Savage, 1976). The G-band idiograms of these species do not appear to show obvious homology so that it is impossible to compare changes in location of the NORs during evolution in these species.

In contrast, the human, chimpanzee, orangutan and gorilla have very similar banding patterns, so that it has been possible to recognise presumptive homologies for the entire complement of the human, chimpanzee, gorilla and to a great extent the orangutan chromosomes (Dutrillaux et al., 1973; Grouchy et al., 1973; Warburton et al., 1973; Miller et al., 1974; Paris Conference Supplement, 1975). A study on the location of the NORs in these species, and that of the gibbon has shown a variation in the number and position of the NORs (Tantravahi et al., 1976). A table on the location of the NORs in these species compared with the homologous human chromosomes, based on their data, is presented below (Table 7-1). The nomenclature is that set out in the Paris Conference Supplement, 1975. The two unidentified silver-stained chromosomes in the orangutan are probably chromosomes 11 and 12 which are homologous to the p and q arms of HSA2. These chromosomes have been shown to have ribosomal DNA sites (Gosden et al., 1978).

Table 7-1: Comparison of location and number of NORs in man, chimpanzee, orangutan and gorilla, based on banding homology with the human karyotype

	HSA1	HSA13	HSA14	HSA15	HSA18	HSA21	HSA22
Human	-	+	+	+	-	+	+
Chimpanzee-		+	+	-	+	+	+
Orangutan *	-	+	+	+	+	+	+
Gorilla	+	-	-	-	-	+	+

\*plus 2 unidentified chromosomes

Whether the direction of evolution has been from a large number of sites as in the orangutan, to a lower number as in the gorilla, or vice versa, cannot be determined, although the authors suggest that the larger number of sites in the orangutan may be characteristic of a primitive primate ancestor. NORs may have been lost by Robertsonian translocation, pericentric inversions and deletions. They point out that this would represent a very extensive change from the less advanced primates such as the gibbon and old world monkeys which have a single pair of chromosomes with rRNA genes. The mechanisms which have produced the different chromosomal locations in these primates are not known but in at least one case, that of the telomeric NOR on chromosome 1 of the gorilla, it appears that only the rRNA genes have been translocated. Loss of rRNA genes have also occurred in two pairs of acrocentrics in the gorilla without other chromosome changes occurring (Gosden et al., 1978).

In the 47 bufonids (toads) and hylids (tree-frogs) studied by Schmid (1978a) there has been conservation of the number of NORs but not of location. All species studied had only two NORs and between individual differences were not present, apart from variation

in the size of the Ag-NORs. In the genus Bufo, chromosome breaks occur preferentially in constitutive heterochromatin. The NORs of the bufonids are always associated with constitutive heterochromatin and Schmid suggests that breaks in these adjoining regions, rather than in the nucleolar constriction, leading to inversions and translocations, have produced the variation in location while retaining the original NOR number.

In the Diplasiocoela the number and chromosomal positions of the NORs vary quite strongly between species and between families (Schmid, 1978b). In the genus Rana the morphology of the chromosomes has remained constant. It is thought that translocations and inversions are not responsible for the change in number and location of the NORs. It is speculated that extrachromosomal rDNA molecules which are formed by amplification during amphibian oogenesis might become reintegrated at various additional sites on the chromosomes.

### 7.1.3 NOR studies in the Bovidae

Comparative studies on the G-band and C-band patterns of the chromosomes of some Bovidae, the satellite DNA composition and localization, the DNA content and the 5-methylcytosine content (Schnedl et al., 1976) (see section 7.1.1), have been made. The G-band patterns, the amount and location of satellite DNA and the 5-methylcytosine content of sheep, goat and cattle chromosomes are similar. However, cattle differ from sheep and goats in their satellite DNA properties and DNA content. Comparison of satellite DNA between species is useful because even closely related species may differ markedly in satellite DNA (Hennig and Walker, 1970). The sheep and goat satellites are remarkable in their similarity.

It appears likely that rDNA regions may also undergo a high rate of change during evolution. A high variation of rRNA gene content was thought to be generated over 50 generations of selection for high and low bristle number in Drosophila (Frankham et al., 1978). The phenomena of magnification reported in Drosophila where rRNA genes are either gained or lost over a few generations (see section 3.1.2.1), also demonstrates this capacity for rapid change. It is possible the NORs may undergo rapid change as a result of unequal

crossing over. Different strains of mice differ in the chromosomal location of rDNA (Henderson et al., 1974a, Elsevier and Ruddle, 1975) and in the location of the Ag-NORs (Dev et al., 1977). This suggests that the NORs have undergone changes in Mus while the banding patterns have remained identical.

In view of this possibility that a rapid rate of evolution occurs in the NOR region, it was thought that a comparative study of the Ag-NORs in various species of Bovidae would make an important contribution to the accumulating data on chromosomal evolution in these species. This is especially important in view of the confusion which has existed in studies of the phylogeny of the Caprini (Curtain and Fudenberg, 1973; Hight and Nadler, 1976). The taxonomy of the sheep and goats and the relationship of these genera to the aoudad has been debated. The Caprinae fall into two groups on the basis of the cross-reactivity of their  $IgG_1$ ,  $IgG_2$ ,  $\alpha$  and  $\mu$  antigens, with the thar, Hemitragus and Capra on one hand and Ammotragus and Ovis on the other (Curtain and Fudenberg, 1973). However, a study of serum proteins by an immunodiffusion technique demonstrated a closer relationship between Ovis and Capra than between either of those and Ammotragus (Hight and Nadler, 1976). It was hoped the silver-staining patterns in these genera might help in elucidating these relationships.

A preliminary report on silver-staining in cattle reported centromeric and telomeric staining regions in this species (Diamond et al., 1977). The telomeres of 'long, medium-sized and short acrocentrics' were reported to show silver deposits although these were not identified. It was reported that 'about' four to six of these deposits were 'usually' found in each metaphase spread, although no data was presented on this. This paper suggested that the location of the NORs in cattle was telomeric, as in sheep but it was unclear whether the number or chromosomal locations were similar.

Five species were used in this study: domestic sheep Ovis aries; goat, Capra hircus; cattle, Bos taurus; aoudad, Ammotragus lervia;

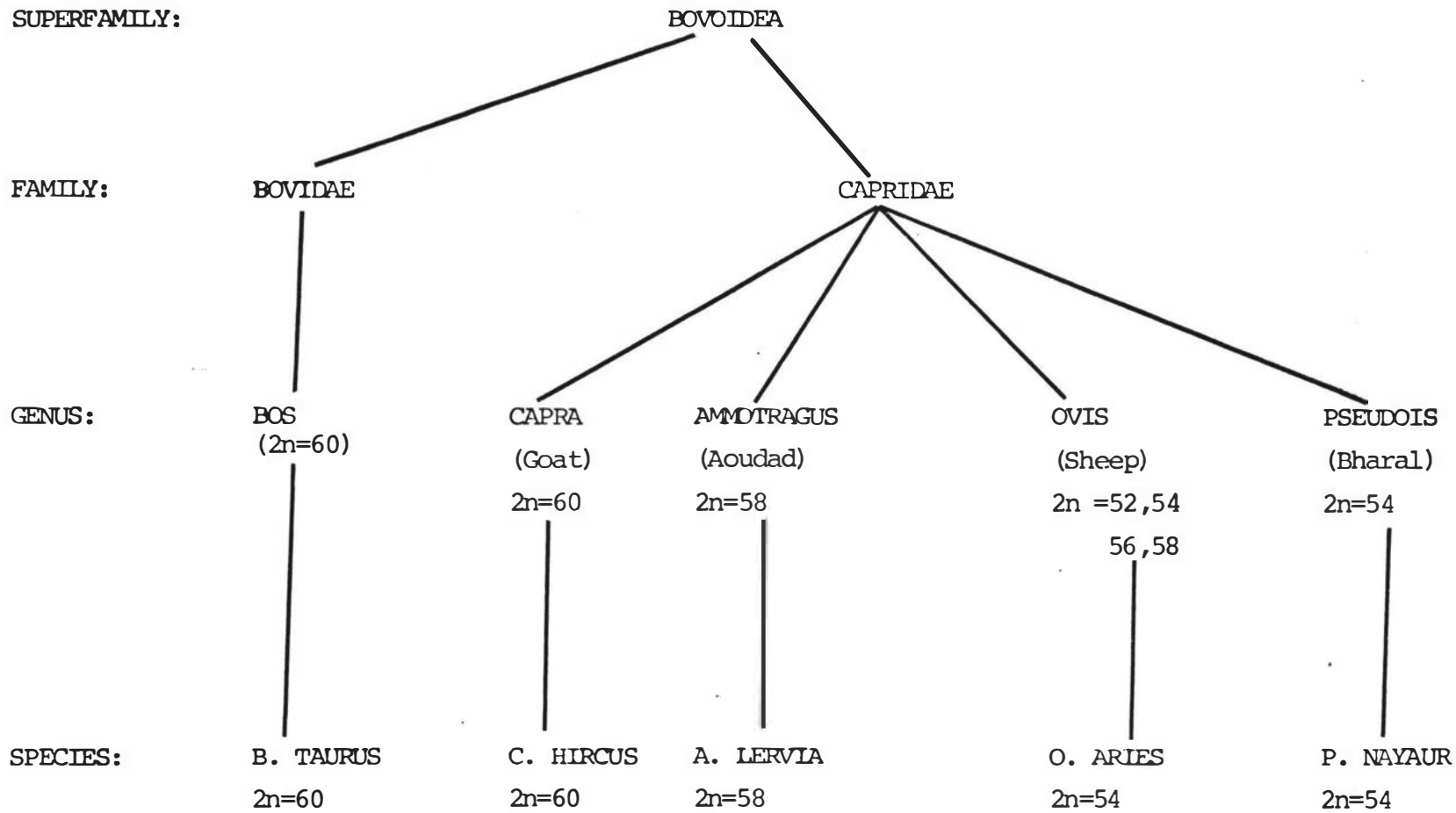


Fig. 7-1: Taxonomy of bovid species used in this study

and bharal, Pseudois nayaur. Their taxonomic relationships and chromosome numbers are shown in Fig. 7-1. (For details on the taxonomy refer to Walker, 1975).

## 7.2 Materials and Methods

### 7.2.1 Animals

Venous blood samples were obtained from five Friesian calves with normal karyotypes; a Charolais bull (Bos taurus) and a Red Poll bull which were both presumptive 1/29 translocation carriers; four feral goats (Capra hircus) which were probably of Saanen and Toggenburg derivation; and five aoudads (Ammotragus lervia) kept at the Auckland and Wellington Zoological Parks. Fixed cells from a bharal (Pseudois nayaur) were supplied by Dr T D Bunch of the International Sheep and Goat Institute in Utah. The suspended cells were several months old, and had been kept at room temperature due to a 4 month delay in the mail before the slides were made.

### 7.2.2 Karyotyping and Comparative Studies

Identification of the NO-chromosomes in the goat, cattle, aoudad and bharal was based on karyotypes of Ag-I preparations. For the comparative studies the goat and cattle chromosomes homologous to the sheep NO-chromosomes were determined from the data of Evans et al. (1973). The aoudad chromosomes homologous to the sheep NO-chromosomes were determined from the data of Nadler et al. (1974). The chromosome homologies of the bharal and sheep were determined by Dr Bunch (pers. comm.). Although Dr Bunch stated the 3p arm was homologous to the smallest acrocentric of goat and sheep, the G-banded karyotype he supplied indicated that this was homologous to chromosome 25 in sheep. The above information is combined in Table 7-2.

Table 7-2: G-banding homology between sheep, aoudad, cattle, goat and bharal autosomes based on data of Evans et al. (1973), Nadler et al. (1976), and Dr T D Bunch (pers. comm.).

Sheep NO- chromosomes	Homologous chromosomes			
	Aoudad	Cattle	Goat	Bharal
1p	1p	3q	3q	3q
2q	2q	2q	2q	4
3q	3q	4q	4q	1q
4q	4q	5q	5q	5q
25q	27q	28q	28q	3p

### 7.3 Results

#### 7.3.1 Location of NORs

In cattle, four of the largest acrocentric chromosome pairs and one of the smallest acrocentric pairs had Ag-NORs (Fig. 7-2). The non-involvement of both chromosome 1 and 29 was indicated by the lack of staining of either arm of the 1/29 translocation chromosome (Fig. 7-3). Most of these metaphases had two small acrocentric chromosomes staining, proving the non-involvement of the translocation chromosome short arm. Unfortunately no metaphases with more than five large acrocentric chromosomes with Ag-NORs were found in either of these bulls. Therefore it cannot be ruled out that chromosome 1 does have a NOR but it is inactive in both these animals. In animals with normal karyotypes, the four large chromosome pairs with Ag-NORs consistently karyotyped at positions 2, 3, 4 and 5. The small Ag-stained acrocentric pair was placed as chromosome 28, corresponding to sheep chromosome 25.

As in the cattle, in the goat four large acrocentric chromosome pairs and one of the smallest acrocentric pairs were silver-stained (Fig. 7-4). The X chromosomes could not be identified by their short arms in Ag-I preparations. The X chromosome of the goat has been variously identified as the longest, second longest and third longest chromosome (Hansen, 1973). The longest acrocentric pair was never silver-stained. Four of the five following pairs had Ag-NORs. It was believed that the non-staining pair which was ranked third in the prepared karyotypes is the X chromosome. The small acrocentric pair showing silver-staining was karyotyped as chromosome 28.

In the aoudad, the short arm of chromosome 1, three large acrocentric pairs and a small acrocentric pair which is probably chromosome 27, showed Ag-NORs. The longest acrocentric pair is the X chromosome (Bunch et al., 1977) which sometimes has distinct short arms visible in Ag-stained preparations. The three following acrocentrics, chromosome 2, 3 and 4 had Ag-NORs (Figs. 7-5 and 7-6).

Fig. 7-2: Karyotype of Ag-stained metaphase from cattle showing location of NORs on both C2s, both C3s, C4, both C5s, and both C28s  
(Ag-I)

Fig. 7-3: Metaphase cell from 1/29 bull showing Ag-NORs on 2 small acrocentric chromosomes in association  
(Ag-I, x 3,500)

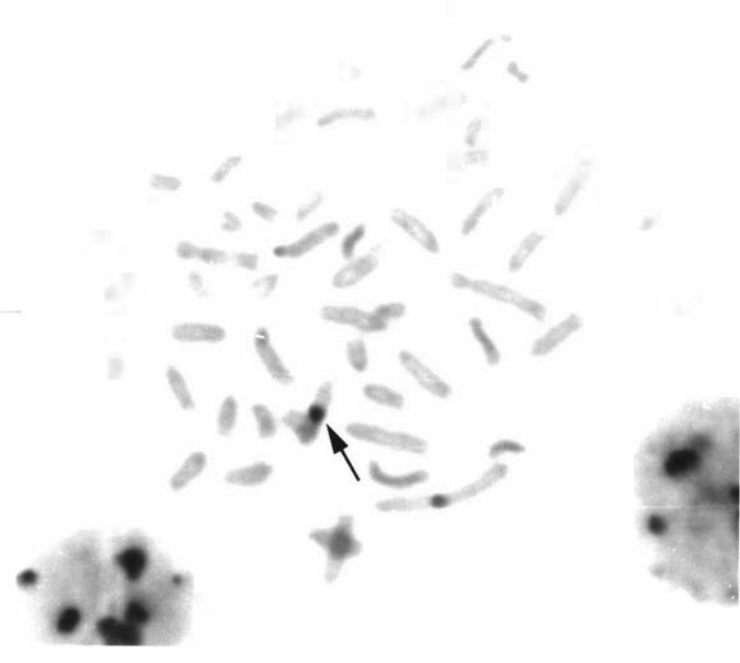


Fig. 7-4: Karyotype of Ag-stained metaphase  
from goat showing location of 10 NORs on the C2,  
C3, C4, C5 and C28 pairs  
(Ag-I)

1000	1000	1000	1000	1000	1000
1000	1000	1000	1000	1000	1000
1000	1000	1000	1000	1000	1000
1000	1000	1000	1000	1000	1000
1000	1000	1000	1000	1000	1000

x y

Fig. 7-5: Karyotype of Ag-stained metaphase of an aoudad showing location of 7 NORs on both C2s, both C3s, both C4s and C27  
(Ag-I)

Fig. 7-6: Partial metaphase from an aoudad showing an Ag-NOR on chromosome 1  
(Ag-I, x 2,650)

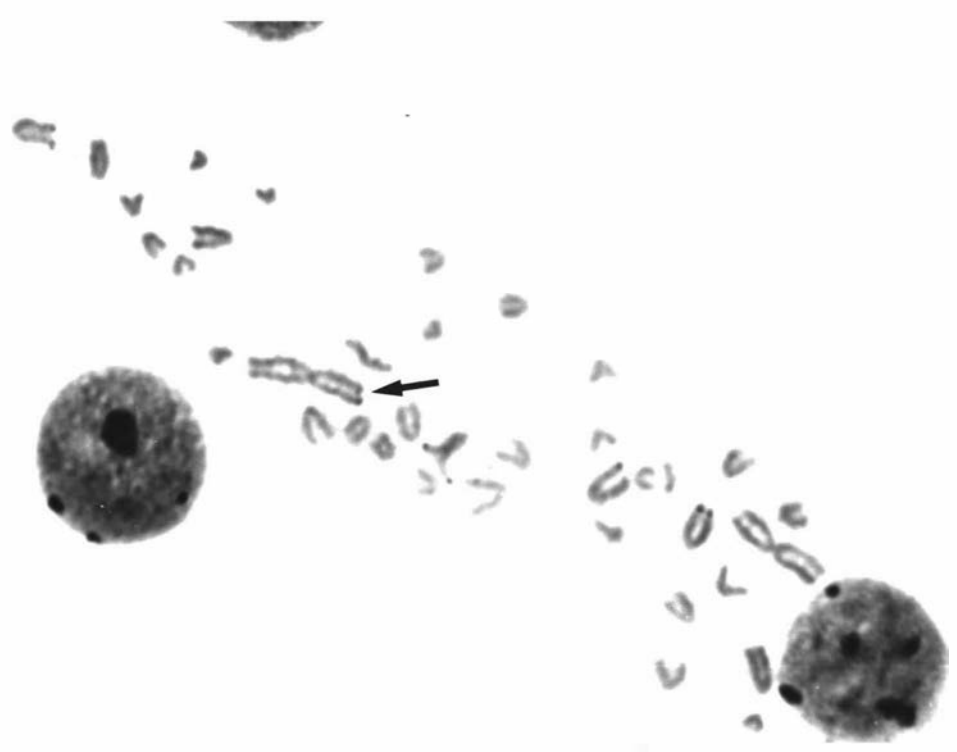
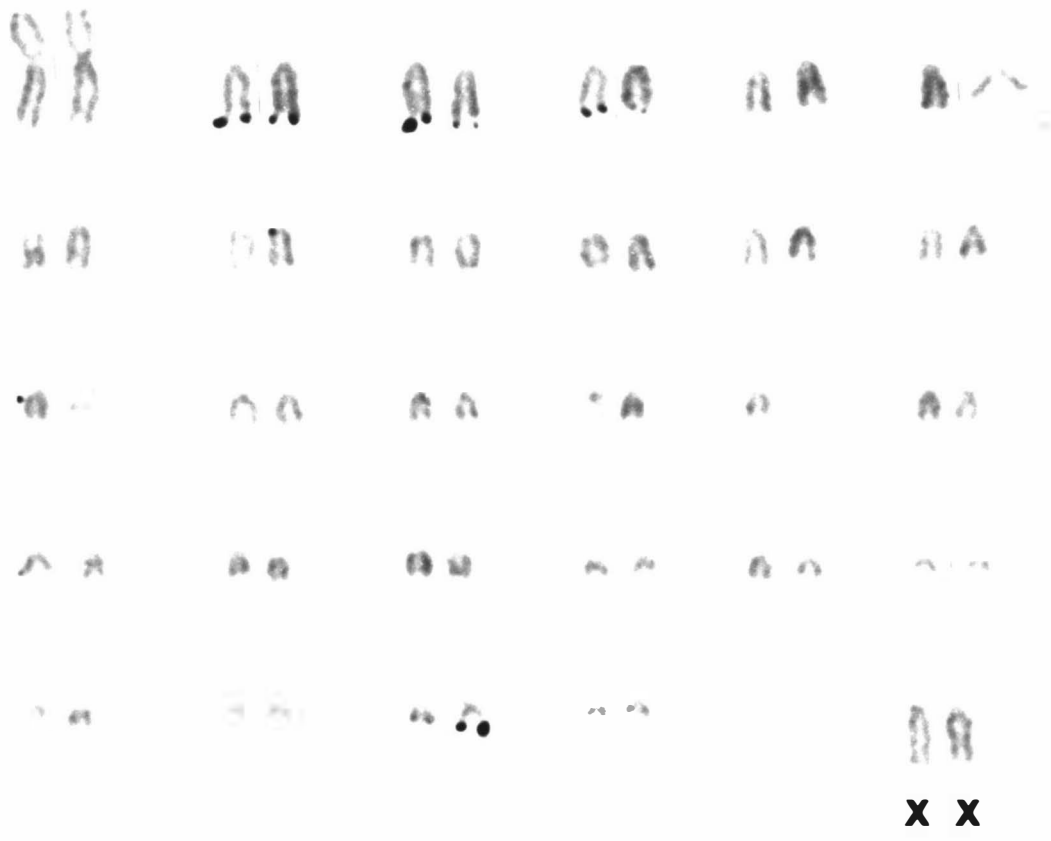
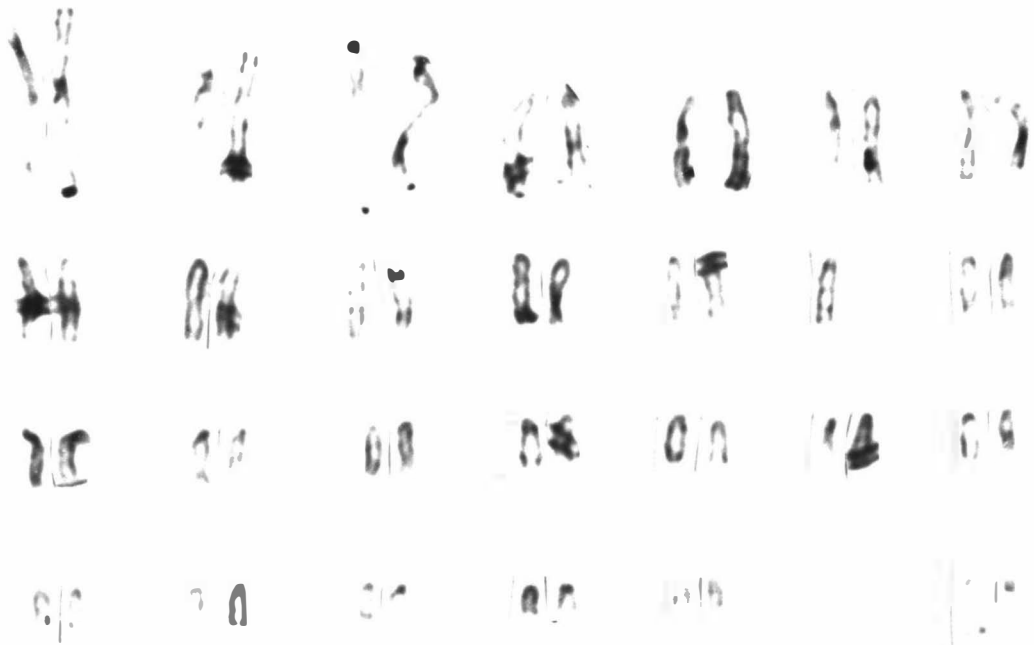


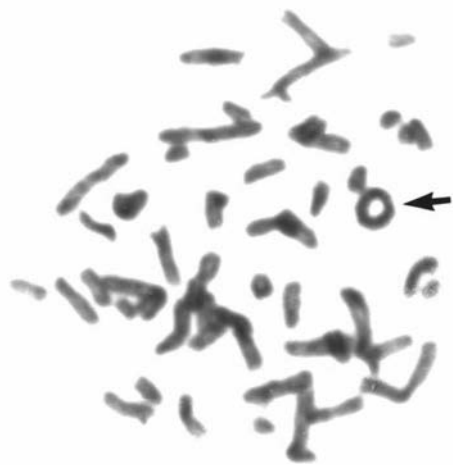
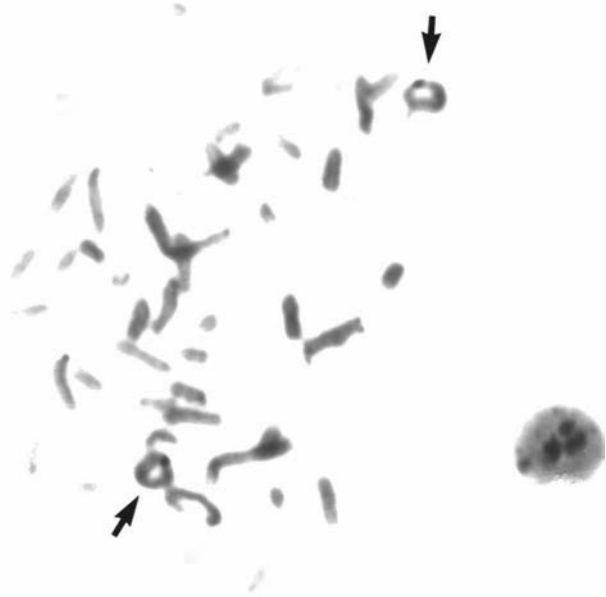
Fig. 7-7: Metaphase cell from a bharal showing Ag-NORs on both telomeres of 2 submetacentric chromosomes, the long arm of C1 and 2 large acrocentric chromosomes  
(Ag-I, x 3,400)

Fig. 7-8: Karyotype of an Ag-stained metaphase cell from a bharal showing the location of NORs on C1, both arms of both C3s, and both C4s  
(Ag-I)



x y

Fig. 7-9: Two metaphase cells from a bharal  
showing 'ring' chromosomes  
(Ag-I, x 3,500)



In the bharal the long arm of the metacentric chromosome, both telomeres of one of the submetacentric pairs and at least one large acrocentric pair had Ag-NORs (Figs. 7-7 and 7-8). In one metaphase from this animal there did appear to be three large acrocentrics staining but this metaphase was unfortunately fairly indistinct. In some metaphases Ag-NORs on the p and q arms of the submetacentric chromosome were seen to be in association, forming 'ring chromosomes' (Fig. 7-9).

### 7.3.2 Variation in number and size of Ag-NORs

With the exception of the bharal, in each of the Bovidae studied, a maximum of five chromosome pairs were found to stain with silver. The modal numbers and range are shown in Table 7-3. Although no attempt was made to study the frequency of Ag-NORs in individual animals in this study, individual chromosomes did appear to have a characteristic ability to stain in individual animals, as reported for sheep in Chapter 3.

The size of the Ag-deposits varied from small, faint deposits to large, dark deposits. In some of the species studied individual NO-chromosome pairs are distinguishable, including chromosome 1 in the aoudad, chromosome 4 and 25 in sheep and the smallest acrocentric pair in the other species. In these cases it was possible to observe that the size of the deposit and the presence or absence of stain on a particular chromosome pair was relatively constant in an animal, but varied from individual to individual. For example the aoudad karyotyped in Fig. 7-6, rarely had an Ag-deposit on chromosome 1, whereas 2 other aoudads consistently had large deposits on both chromosomes 1.

## 7.4 Discussion

### 7.4.1 Assignment of NORs to specific chromosomes

Ranking the acrocentric chromosomes according to size can result in errors. However consideration of the karyotypes of the

Table 7-3: Modal value and range of number of Ag-NORs per metaphase in sheep, aoudad, cattle and goat

	No. of animals	No. of cells	Modal value of Ag-NORs	Range of Ag-NORs
Sheep *	15	428	8	3-10
Aoudad	5	41	6	4-10
Cattle	5	73	6-7	3-10
Goat	4	71	8	4-10

\* Data of Henderson and Bruere, 1977

normal and translocation animals studied shows that several NORs can be unambiguously identified. Duplicate G-banding and Ag-I has confirmed the chromosome locations of the NORs in sheep to 1p, 2q, 3q, 4q and 25q (section 2.3.4). The metacentric chromosome pair in the aoudad is homologous to the first metacentric pair in sheep and it also has a NOR occurring terminally. Other unambiguous assignments can be made in the bharal where the metacentric long arm is known to be homologous in G-band pattern to sheep 3q, and the smaller submetacentric chromosome has arms homologous to the sheep 1p chromosome arm and chromosome 25.

In the absence of duplicate G-banding and silver-staining, assignment of NORs to the other chromosomes is not unequivocal. It is believed that an error of several chromosome positions may occur in karyotypes based only on chromosome arm length measurements. Four large chromosomes stain in cattle and these were karyotyped as chromosomes 2, 3, 4 and 5. Chromosome 1 probably does not have a NOR as shown by the results from the two 1/29 translocation bulls. Assuming that an error of placement of up to 2 chromosome positions may have occurred, 3 of the NO-chromosomes in cattle can be identified as chromosomes 2, 3 and 4. Consideration of the other karyotypes leads similarly to an assignment of NORs to chromosomes 2 and 3 in the aoudad and 3 and 4 in the goat. Uncertainty applies to the fourth largest pair in cattle and aoudad, the first and fifth largest pair in the goat and the small acrocentric pair in the cattle, goat and aoudad. Evidence in support of the assignment of NORs to the fifth chromosome pair and the second smallest chromosome pair in the goat is the observation that these chromosomes (identified by G-banding) may occasionally be satellited (Bunch *et al.*, 1977). The homology in NOR location in sheep, cattle, goat and aoudad is supported by the study on the bharal. In this species the chromosome homologous to the chromosome 25 in sheep forms the short arm of a submetacentric so that the presence of an Ag-NOR on this chromosome is indisputable evidence on conservation of NO location in the evolution of sheep and bharal.

A drawback of the Ag-staining techniques in the identification of NORs is that they only stain active regions, so that it is necessary to pool the data from several animals to be confident all NOR sites have been observed. This drawback has been discussed

as it related to the lack of silver-staining on the 1/29 translocation in cattle. It also applies to the study on the bharal where only one animal was available for study. In this species two large acrocentric chromosomes showed Ag-NORs. The two largest acrocentric pairs correspond to two NO-chromosomes in the sheep (2q and 4). It is impossible to say on the basis of arm-length whether the two silver-stained acrocentrics observed in the bharal belong to either one pair or two pairs. A G-banding study or a study of other specimens which may have a different Ag-staining pattern might elucidate this. Unfortunately the bharal cells were not of a high quality for G-banding. Further samples from the bharal are unobtainable at present, as the animal has since died. The bharal is maintained in only two other zoological parks throughout the world, and these are not available for analysis. (Bunch et al., 1978).

Considering the homology of some of the NO-chromosomes, the evidence from the prepared karyotypes and the support from the observation of satellites in the goat, it is very likely that all NO-chromosomes in the sheep have homologous NO-chromosomes in the goat, cattle, aoudad and bharal.

#### 7.4.2 Nomenclature and Presumptive homologies of the chromosomes of the Bovidae

During the course of this comparative study many problems were encountered in interpreting which chromosomes were being referred to in other publications. The need for a standard idiogram and nomenclature system became obvious. Determination of homologous chromosomes by comparison of published karyotypes is sometimes erroneous. It is felt that the numbering system of Evans et al. (1973) is too cumbersome for routine use. In this system, chromosomes are numbered according to their homologous chromosomes in the goat. Thus the three metacentric chromosomes are called  $1/3$ ,  $2/8$  and  $4/9$ . It is suggested that a numbering system based solely on chromosome morphology and size should be used in non-comparative studies. In comparative studies where there is a need to determine homology a system similar to the nomenclature system for the chromosomes of Hominoidea is desirable (Paris Conference, (1971), Supplement (1975)).

GOAT



GOA 2



GOA 3



GOA 4



GOA 5



GOA 28

CATTLE



CAT 2 (GOA 2)



CAT 3 (GOA 3)



CAT 4 (GOA 4)



CAT 5 (GOA 5)



CAT 28 (GOA 28)

SHEEP



SHE 1p (GOA 3)



SHE 2q (GOA 2)



SHE 3q (GOA 4)



SHE 4 (GOA 5)



SHE 25 (GOA 28)

AOUDAD



AOU 1p (GOA 3)



AOU 2 (GOA 2)



AOU 3 (GOA 4)



AOU 4 (GOA 5)



AOU 27 (GOA 28)

BHARAL



BHA 1q (GOA 4)



BHA 2



BHA 3q (GOA 3)



BHA 4 (GOA 2)



BHA 5 (GOA 5)

BHA 3n (GOA 28)

Figure 7-10: Homologies in NO-chromosomes in Sheep, Goat, Cattle, Aoudad and Bharal.

A standard karyotype on which to base the determination of homology is required. Chromosomes can then be designated by their numbering system in their own species karyotype, and their homologous chromosome in the standard karyotype. The goat karyotype would be best for the standard karyotype as it contains all acrocentric chromosomes and it has already been used as a standard karyotype by Evans et al. (1973). As an example of the use of this system, the sheep metacentrics could be written as SHE1 (GOA 1,3), SHE2 (GOA 2,8), SHE3 (GOA 4,9). The bharal chromosomes could be written as BHA1 (GOA 4,13), BHA2 (GOA 1,27) BHA 3 (GOA 3,28).

The homologies of the NO-chromosomes identified in this study are shown in Fig. 7-10 and this uses the system proposed above.

#### 7.4.3 The ring chromosome of the bharal

The 'ring' chromosome frequently observed in the bharal as a result of association of NORs on the same chromosome is a previously unreported phenomenon. True 'ring' chromosomes result from deletion of both telomeric regions of a chromosome and a rejoining of the ends.

#### 7.4.4 Frequency of Ag-NORs

In each of the species studied, except the bharal, the maximum number of Ag-NORs observed per metaphase was 10. The modal values of the number of Ag-NORs per metaphase (see Table 7-3) are similar for each of the species and the small variation between them may be largely due to individual variation between animals, regardless of the species. The modal number for cattle reported here is higher than the modal value of 'about' 4-6 reported by Diamond et al. (1975) from an unstated number of cells or animals. This difference is more likely to be due to technical variation than variation in the individuals sampled as discussed in Section 3.4. The total number and modal values of Ag-NORs in the Bovidae is similar to that in humans where there are 10 NO-chromosomes and the modal values of Ag-NORs per metaphase have been reported as 6-9 (Bloom and Goodpasture, 1976), 5-9 (Miller et al., 1977) and 4-7 (Varley, 1977).

#### 7.4.5 Homology of NORs in sheep, goat, cattle, aoudad and bharal

The present study strongly suggests that the NORs of the sheep, aoudad, goat, cattle and bharal occur on chromosomes which have previously been shown to have homologous banding patterns (Evans et al., 1973; Nadler et al., 1974; Bunch, pers. comm.). It appears that there has been a conservation of the number and location of NORs during the evolution of these species. The conservation in the location of the NORs between these species reinforces the evidence from the homologous banding patterns that no major chromosome rearrangements have occurred in these species. Differences in location of NORs in primates (Tantravahi et al., 1976) and toads and tree-frogs (Schmid, 1978a) have arisen as a result of chromosomal rearrangements during evolution. In primates some changes, such as Robertsonian fusions and pericentric inversions in acrocentric chromosomes have apparently resulted in loss of NOR material (Gosden et al., 1978). In the Bovidae species studied here, the only major chromosomal rearrangements apart from Robertsonian fusions is a reciprocal translocation in the chromosomes homologous to 11 and 12 of cattle. These chromosomes are not involved in nucleolus organization. Robertsonian fusions do not affect the nucleolus organizer regions. In the gorilla it is thought that the NOR on chromosome 1 may have arisen by a translocation of the NOR only (Tantravahi et al., 1976). If this sort of change occurred in the Bovidae it would not be detectable by G-banding. However no such changes appear to have occurred.

Apart from the homology in location of the NORs in sheep, cattle, goat, aoudad and bharal, the stability in number of NORs is even more remarkable. The rRNA genes appear to be capable of rapid change (see Section 7.1.3) and evidence has been produced for a wide range of rDNA content between different chromosomes of an individual in several species including human (Evans et al., 1974) rhesus monkey (Henderson et al., 1974b), mouse (Atwood et al., 1976) and the salamander (MacGregor et al., 1977). Highly inbred strains of mice may differ in the location of their rRNA genes (Henderson et al., 1974a; Elsevier & Ruddle, 1975) and in the location of their Ag-NORs (Dev et al., 1977). In the primates loss

of rRNA genes appears to have occurred on a chromosome in a species without concomitant chromosomal rearrangement (Gosden *et al.*, 1978). Various types of exchange have been suggested to be involved in these changes of the rDNA region e.g. sister chromatid exchange, unequal meiotic crossing over and exchange between non-homologous rDNA regions (Henderson *et al.*, 1976b; MacGregor *et al.*, 1977). The exchange mechanisms producing rDNA variation are likely to be universal but it is possible that bovid rDNA regions may be more stable and less likely to undergo these exchange events. Such mechanisms may be less likely to occur in the Bovidae because of the terminal nature of the NORs. The apparent stability of the NORs in these species could also be related to the absence of adjacent heterochromatin, since exchange events may involve heterochromatin regions. This has been suggested to be a factor in the distribution of the NORs in toads and tree-frogs (Schmid, 1978a). A final possible explanation of the stability of the rDNA regions in these species may be that there is a strong selection for 10 active NORs (which may only be required in some tissues or at some stages of development).

In conclusion, NORs, which may show diversification between closely related species, are apparently homologous in number and location in cattle, goat, sheep, aoudad and bharal. This strongly supports the contention that the Bovidae karyotype tends to be fairly stable apart from the gross morphological changes occurring by centric fusion.

In the introduction, it was stated that one of the aims of this comparative study was to determine whether the pattern of Ag-NORs might have an application in the solving of problems concerning the taxonomy of the caprids. The homology of NORs in species as different as the goat and cattle prevented such an application. Other markers which have undergone a greater change during evolution will be more useful in this respect.

#### 7.4.6 Relationship of NORs to Robertsonian translocations in the Bovidae

As discussed in Chapter 1, one of the original aims of this research project was to determine whether NORs had a causative role in Robertsonian fusions in sheep, as has been suggested in man (Ohno et al., 1961). The terminal location of the NORs in sheep suggested that this was not the case. However a consideration of the karyotypes of the bovids used in this study, and of the case reports of occurrence of Robertsonian fusions in these species, seemed to indicate a high number of chromosomes with NORs being involved in Robertsonian fusions. A list was compiled of the chromosomes involved in karyotypic evolution and of those which have been reported in the literature as being involved in centric fusions (Table 7-4). Only chromosomes which were identified by banding techniques were included. Without a standard karyotype it is not certain that the chromosome numbers assigned to the fused acrocentrics are homologous to the corresponding chromosome number in goat based on the karyotype of Evans et al. (1973). There are 29 autosomal chromosome pairs in the ancestral karyotype, and 5 of these contain NORs. On the basis of random involvement of chromosomes in centric fusions, the expected proportion of chromosome arms having NORs is  $\frac{5}{29}$ . If it is assumed that each centric fusion has arisen only once, 12/32 of the chromosomes which have undergone centric fusion have NORs. The numbers involved are too low to be statistically significant but it is possible that NO-chromosomes may be predisposed to be involved in centric fusions. Several translocations have been reported in the goat (e.g. Popescu, 1972) and cattle (e.g. Darre et al., 1974) which have not been banded. There is a need for banding studies to be done on these translocations to increase the data. Additional data could be obtained from a study of the chromosomes involved in centric fusion in cell lines. If it is real, it is possible that the increase in involvement of the NO-chromosomes is related to their size or some factor other than the presence of a NOR.

Table 7-4: Involvement of chromosomes in centric fusion during karyotypic evolution and within species

Species	Chromosome Number	Homologous Goat Number	NO-Chromosome	Reference
<u>Ovis aries</u>	1	1-3	3	Evans <u>et al.</u> , 1973
	2	2-8	2	"
	3	4-9	4	"
<u>O. nivicola</u>	4	7-17	-	Bunch, 1978
<u>O. aries</u>	t <sub>1</sub>	6-29	-	Bruere <u>et al.</u> , 1974
	t <sub>2</sub>	11-14	-	"
	t <sub>3</sub>	10-28	28	"
<u>Bos taurus</u>	-	1-29	-	Gustavsson <u>et al.</u> , 1976
	-	3-4	3,4	Popescu, 1977
	-	2-4	2,4	Pollock and Bowman, 1974
	-	14-20	-	Logue, 1978
	-	6-16	-	"
<u>Capra hircus</u>	-	5-15	5	Evans <u>et al.</u> , 1973
<u>Pseudois nayaur</u>	1	4-13	4	Bunch, 1978
	2	1-27	-	"
	3	3-28	3,28	"

It is of interest that an increase in involvement of NO-chromosomes in Robertsonian translocations in mouse cell lines has been reported (O.J. Miller et al., 1978a). The observation that usually only one NOR-bearing chromosome was seen in any banded chromosome led the authors to suggest that although the nucleolus plays an important role, nucleolar fusion is relatively unimportant in the origin of Robertsonian translocations in the mouse. However the centromeric location of the NORs in mice makes these more likely to have a role in centric fusion in mice than in sheep.

## CHAPTER 8

### CONCLUSION

#### 8.1 Relation of Ag-staining to NOR activity

It is commonly believed that the presence of an Ag-NOR indicates a NOR which has been active in the preceding interphase. This belief is based primarily on two types of evidence. Firstly in hybrid cells where rRNA of only one species is produced, only the chromosomes of that species show Ag-NORs (D.A. Miller et al., 1976; O.J. Miller et al., 1976; and see section 3.1.2.). The second evidence is the observation that the onset of Ag-staining coincides with the onset of rRNA synthesis in early mouse embryos (Engel et al., 1977; Hansmann et al., 1978). These observations provide an interpretation of the lack of Ag-staining of some regions known to contain the genes for rRNA, while other NORs in the same metaphases are Ag-positive. It is commonly assumed that variation in Ag-stainability of the NORs within a cell reflects differential activity of these NORs (e.g. Tantravahi et al., 1976; Varley 1977).

While there is no doubt that the presence of an Ag-deposit on a NOR shows that it was active in the preceding interphase, the evidence should be taken as less conclusive that the lack of an Ag-deposit on a NOR shows that it was inactive. Some observations indicate that some regions involved in nucleolus organization do not have Ag-deposits in the subsequent metaphase. On several occasions during the course of this study, chromosomes with no detectable Ag-staining were seen in association with other chromosomes with Ag-NORs (see Fig. 4-5). Without the presence of an interconnecting silver-thread these NORs would have been scored as Ag-negative. The association of NO-chromosomes with almost no Ag-staining has also been reported by others (see Fig. 3 (c) in Miller et al., 1977; Schwarzacher et al., 1978). Association of NORs is evidence of activity in the preceding interphase. Schwarzacher et al. showed that in the electron microscope very small silver granules can be seen on some associating chromosomes that are presumably invisible in the light microscope. Further information on the nature of the Ag-staining differences can be obtained from consideration of the changes in the amount of silver-staining material during the cell cycle. The observation has been made that the Ag-deposits are

much larger in interphase and prophase than in metaphase (section 2.3.6; and Martin-Deleon et al., 1978; Schwarzacher et al., 1978). It appears that the amount of Ag-staining material starts to decrease during prophase as transcription ceases and the nucleolus disintegrates so that only a remnant remains at metaphase. It is probable therefore that some regions which were active in rRNA transcription in the preceding interphase will fail to have Ag-deposits at metaphase. The regions which will be affected the most will be those which had a lower amount of Ag-staining material associated with them. It has also been noted (sections 3.4.1 and 6.4) that regions with small Ag-deposits show the greatest variation in staining frequency between cells of an individual, between replicate cultures and between different tissues. While this could be due to differences in the activation of these smaller NORs, it may also be due to technical variation.

The evidence therefore suggests that in a cell showing differential Ag-staining some NORs which are Ag-negative were active in interphase. It is not possible to distinguish whether all NORs which are Ag-negative under these conditions were active or whether some Ag-negative NORs were active but had lost their Ag-staining material, while others were inactive. Some acrocentric chromosomes in humans, do not have Ag-staining material when viewed by electron microscopy (Schwarzacher et al., 1978) which may indicate that the individual differences in Ag-NOR presence, seen by light microscopy, reflect inactivity of some NORs, or the lack of Ag-staining material around these chromosomes may be due to its loss during prophase and the cell preparation procedures.

Thus the Ag-staining patterns of an individual may result partly from absolute differences in the activity or inactivity of the NORs, and partly from differences in the amount of Ag-staining material accumulated around each NOR.

## 8.2. The nature of the Ag-staining polymorphisms

The Ag-staining of NORs demonstrates an example of a polymorphism in a genetic locus. NORs, as shown by Ag-staining, differ in the presence or absence of Ag-staining material, and in the size of this material. There is an increasing number of cases in which genetic polymorphisms are known to affect quantitative variation in the levels of proteins or enzymes (Snyder, 1978). There may also be polymorphisms in regulatory loci as has been suggested to account for variation in the levels of haemoglobin synthesis in the deer mouse, Peromyscus maniculatus (Snyder, 1978). It has been suggested that regulatory loci may affect the level of activity of their associated structural loci.

The Ag-staining polymorphisms could be due to the presence or absence of NORs. However, it has been shown that Ag-staining may be absent on regions which contain rDNA, since all ten acrocentric chromosomes in humans have rDNA but only 3-10 have Ag-NORs (Varley, 1977). In situ hybridization of rDNA has not been done on sheep chromosomes, but extrapolation between species is probably valid in this case. These experiments do not rule out the possibility that Ag-staining may be related to the presence or absence of a control element regulating transcription.

The polymorphism in Ag-staining of the NORs could be related to a polymorphism in rDNA content of the NORs. A large variation in the rDNA content of different NORs within an individual has been found in several species (section 3.4.4). Within a population of the salamander, P. cinereus, a 7.5 times range in the rDNA content of different individuals within a population was found, indicating the actual range in the size of the nucleolus organizers might be 15 times. In two studies in man, the variation in rDNA contents between 2 homologues was found to be 4 times in one case (Evans et al., 1974) and 9 times in the other case (Warburton et al., 1976a). These values will give underestimates of the extent of polymorphism which exists in the rDNA content of NORs in man. Polymorphisms in rDNA content of NORs have also been demonstrated in the onion and mice, and may therefore be a widespread phenomenon.

As yet, the relationship of rDNA content of NORs and Ag-staining is not clear. There is evidence from 10 cases studied sequentially with hybridization in situ and silver staining that the amount of Ag-staining material is related to the rDNA level of a NOR (D. Warburton, pers.comm.). In addition there is indirect evidence to support this hypothesis. It has been shown that the size of a secondary constriction is positively correlated with its rDNA content (Evans et al., 1974; Henderson et al., 1976b; MacGregor et al., 1977) and also that the size of the secondary constriction is positively correlated with the size of its Ag-NOR (Dev et al., 1977), suggesting a positive relationship between rDNA content and Ag-staining. Also the frequency of association of NORs has been found to be positively correlated to the amount of rDNA (Dittes et al., 1975; Henderson and Atwood, 1976; Warburton et al., 1976a) and to the frequency and size of the Ag-NOR (Henderson and Bruère, 1977; Miller et al., 1977; and Chapter 4 of this thesis). Again, this indicates a positive relationship between the amount of rDNA and the Ag-stainability of a NOR.

However none of the above reports have demonstrated unequivocally the relationship of rDNA amount to Ag-staining. In the study of 10 individuals mentioned above, some exceptions to the rule of the positive relationship of rDNA was found (D. Warburton, pers.comm.). These largely involved chromosomes with very large quantities of rDNA but normal amounts of silver-staining material. A report on a marker chromosome with a large amount of rDNA but normal Ag-staining has also recently been published (D.A. Miller et al., 1978b).

### 8.3. A model of NOR regulation and Ag-staining in diploid organisms

It has been shown that polymorphisms exist in the presence and amount of the Ag-staining material on NORs in diploid organisms. Arguments have been presented that lack of Ag-staining within a diploid cell may represent the loss of a small amount of Ag-staining material rather than the inactivity of a NOR. Also in situ hybridization experiments show that Ag-staining is related to rDNA

content except at high levels of rDNA, which may have normal levels of Ag-staining material.

The polymorphism of NORs found in this and other Ag-staining studies may be largely explained in terms of the "gene competition" hypothesis proposed by Schwartz (1971) for the two alleles of the alcohol dehydrogenase gene in maize. In detail the main proposals of the model which are relevant to the present problem are:

1. The amount of enzyme synthesized in a particular cell is limited by the concentration of some specific factor.
2. The limited factor acts at gene level to control activity (Schwartz suggests two possibilities - either the limited factor may displace histones on the gene to allow the region to uncoil and become active or the limited factor might be an initiator of mRNA transcription such as a polymerase or a cofactor).
3. Unrepressed genes within a group compete with each other for the limited factor. The sum total of the activity of a set of genes is determined by the concentration of the limited factor, but the activity of each gene within the group is determined by its capacity to compete with other genes in the same group for the limited factor.

Such a competition model may be applied to the differential Ag-staining of NORs. It is proposed that:

- (1) Different NORs compete for a limiting factor.
- (2) The ability to compete for this factor is a function of the rDNA content of a NOR. This may be mediated by the promotor regions.
- (3) The amount of the limiting factor in a particular cell or tissue determines the rRNA transcriptional activity of that cell or tissue.

The limiting factor postulated in the model could be RNA polymerase I as the level of this enzyme has been found to differ

in cells with different rates of rRNA synthesis (Schwartz and Roeder, 1974). Alternatively, the limiting factor could be a factor involved in the unwinding of the chromatin of the NOR or a regulatory protein which interacts with the template. Each promotor may have an equal competitive ability and may be associated with a given number of 18S and 28S rRNA genes, so that the overall activity of a NOR will be dependent on its rDNA content.

The model is based primarily on the following observations:

- (1) In situ hybridization experiments have indicated that Ag-staining is related to rDNA content of a NOR.
- (2) The total rRNA transcriptional activity of a cell or organism is not related to the dosage of the NORs (section 3.1.4).
- (3) Suppression of one NOR by another apparently occurs within a diploid cells as seen in interspecific crosses in Drosophila (Bicuodo and Richardson, 1977).

In this study on the inheritance of NOR-stainability, only slight differences in Ag-stainability were found between generations. If the total rDNA content of different genomes shows a large variation in sheep (and this is testable by in situ hybridization), then the competition model would predict that the Ag-stainability of a NOR could be modified between generations. For example, a large Ag-deposit in an individual with a relatively low total rDNA level might show a lower Ag-stainability when incorporated in a genome with a high rDNA level. The similar frequencies and sizes of Ag-NORs between generations found in this study may indicate either that each NOR has an intrinsic stainability, regardless of the other NORs in the genome, or that the level of rDNA multiplicity does not show sufficient variability between individuals to result in changes in Ag-staining of individual NORs.

The model proposed here interprets the variation in size of Ag-deposits on different NORs as differences in the number of genes active in rRNA transcription. The variation in the frequency of

Ag-NORs on the metacentrics, chromosome 4 and chromosome 25 would reflect differences in rDNA levels of the different chromosome types and this is testable by in situ hybridization. Different tissues with the same demands for rRNA transcription would have similar staining patterns, as was found in this study. Tissues with different rRNA transcription rates would differ in the amount of Ag-staining material, but the relative sizes of the Ag-NORs should be constant.

The model does not explain the observations of the normal amount of Ag-staining material around NORs with large amounts of rDNA. Since only isolated cases have been reported it is of interest to determine whether the level of activity of all NORs with large amounts of rDNA is regulated, since this may be associated with the activity of a regulatory loci, possibly of a promotor-type region. Alternatively, there may be some other form of restraint on the overall level of activity of a NOR.

#### 8.4. The stability of NORs

The study of Ag-staining patterns in various tissues of an individual and the study of the inheritance of the Ag-stainability of NO-chromosomes has shown that the Ag-stainability of a NOR tends to be fairly stable between generations and between different tissues. Furthermore, in the comparative study of 5 species of Bovidae the location and number of NORs appears to have remained stable despite the large time interval since these animals have diverged. In this study only one presumptive de novo event involving a NOR was found when a single cell was found containing a chromosome 1 with a terminal NOR on its long arm (section 2.3.5).

Yet it has often been stated that NORs might be genetic loci which fairly frequently undergo change. This has been suggested to account for the polymorphism in the number of rRNA genes per NOR which has been found in several species (Evans et al., 1974; Henderson et al., 1974b; MacGregor et al., 1977 and see section 7.4.5). The mechanisms involved in producing these changes have been suggested to be unequal mitotic crossing-over during association

of NORs, unequal meiotic crossing-over or sister chromatid exchanges involving unequal exchange (Henderson et al., 1976b; MacGregor et al., 1977). There is some experimental evidence for changes in rDNA content of a NOR. For example, the 'magnification' phenomena (Ritossa, 1968) and a rapid change of rRNA gene number under selection in Drosophila (Frankham et al., 1978) have been described. In the mouse, Dev et al. (1977) reported the Ag-stainability of a NOR of one chromosome differed in a cell line and its derivative. And in humans, a change in the Ag-stainability of a translocated chromosome with a NOR appears to have occurred in different generations of a family (M Parslow, pers.comm.; see section 5.4.1).

In the studies on the inheritance and tissue distribution of Ag-NORs which have been reported to date (Mikelsaar et al., 1977b; Markovic et al., 1978; and Mikelsaar and Schwarzacher, 1978) no evidence of de novo changes in Ag-stainability has been found. This is similar to the observation that large scale family studies have failed to detect changes in C-band polymorphisms in man although a high frequency of crossing-over has been suggested to account for the extent of C-band polymorphism (Comings, 1978).

The maintenance of rDNA in five loci on human chromosomes has been discussed by Henderson et al. (1972). Since the number of rRNA genes may vary in different NORs this could lead to irreversible losses of rDNA loci. That is, under the simplest assumptions, a multilocus system is unstable. They suggested a system may have evolved to regulate rRNA production according to the number of loci inactivated. Alternatively, as discussed here, rRNA production might be regulated by the number of active rRNA genes throughout the NORs rather than by the number of active NORs. A multilocus system could be required if all rRNA genes in the genome were required to be active simultaneously to produce enough rRNA at some stage of development. At this stage, for mechanical reasons, it may be advantageous to have the NORs spread throughout the genome so that the nucleoli are formed on different chromosomal loci.

## 8.5 Summary of advances made in this study

The application of silver-staining to sheep mitotic chromosomes allowed the identification of the nucleolar organizer chromosomes in this species. This showed that the NORs were unrelated to either Robertsonian translocation events or acrocentric associations in this species. Both of these are therefore suggested to be related to heterochromatin attraction and satellite DNA homologies. The determination of the NO-chromosomes in sheep provided another chromosome identification technique in this species which has already found an application in the simple differentiation of the  $t_1$  and  $t_3$  chromosomes.

A detailed study of the frequency and distribution of the Ag-NORs was made. Ag-NORs were found to be polymorphic in the staining frequency and size of the Ag-deposits. These were an individual characteristic and stable between cells derived from fibroblast and lymphocyte cultures. The Ag-stainability of a NOR was found to be heritable.

The association frequency of NORs was found to be positively correlated to the frequency of staining and size of the Ag-NOR. The combinations of chromosomes in association were determined by the individual staining frequencies of the NO-chromosomes.

The location of the NORs was found in 4 other species of the family Bovidae and this provided evidence of the stability of the NORs during evolution in these species. The identity in number and location of the NORs in these species prevented their application to the problem of the phylogenetic relationships of sheep, goat and aoudad.

The existence of loci polymorphic for Ag-NORs on 5 chromosome pairs provides a starting point for linkage studies and chromosome mapping in sheep. The potential application of this was demonstrated in an estimate of crossing-over on the  $t_3$  chromosome from 10 offspring of parents heterozygous for Ag-NORs on their

$t_3$  chromosome and chromosome 25.

It has been assumed that lack of Ag-staining of a NOR within a diploid cell reflects inactivity of that NOR. The hypothesis is discussed here that this is due to undetectable levels of Ag-staining material rather than inactivity. A model is presented on the regulation of NOR activity. Further studies on the nature of Ag-stainability and the regulation of NORs within diploid cells are required. These should determine the relationship of Ag-staining to rDNA content.

APPENDIX I.Personal Communications

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## Appendix II. Leucocyte culture technique

### a. Collection of blood samples

Ten ml samples of blood were collected aseptically from the jugular vein into a 10 ml venoject tube containing 143 I.J. sodium heparin.

### b. Leucocyte culture technique

Leucocytes were grown in universal jars containing 7 ml Tissue Culture media 199 (Reconstituted 10 x concentrated dried powder, Wellcome Reagents Ltd., Berkenham, England), 0.2 ml phytohaemagglutinin (Wellcome Reagents Ltd., Wellcome Research Laboratories, Beckenham, Kent, England), 0.75 - 1 ml of whole blood and 1.5-2 ml of autologous serum. The serum was prepared by centrifuging the whole blood at 1000 rpm for 10 minutes. Cultures were incubated for 48-66 hours at 37°C.

### c. Harvesting of cultures

0.05 cc of 24 ug/ml colchicine (Bio-cult Laboratories Ltd., Glasgow, Scotland) was added to each culture 3 hours before harvesting. The cultures were transferred to centrifuge tubes and spun for 5 minutes at 1000 rpm and the supernatants were discarded. The cell deposit was resuspended in 0.075 M KCl and incubated for 5 minutes in a 37°C water bath. The tubes were spun again at 1000 rpm for 5 minutes and the supernatant discarded. The cells were resuspended in cold, freshly prepared fixative (3:1 methanol: glacial acetic acid), and kept in the refrigerator for 1 hour. The tubes were then centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The cell deposit was resuspended in cold fixative to make the total volume up to 0.5- 0.75 ml.

### d. Slide preparation

Slides were immersed in alcohol for at least 3 hours. They were washed individually under filtered tap water, covered with distilled water and chilled in the refrigerator for several hours. Cells from the prepared suspension were dropped on to the chilled

slides, the slides were passed briefly through a bunsen flame without igniting the suspension, and air-dried on a hot plate.

e. Mounting slides

Coverslips (Matsunami 22 x 64 No.2, Watson Victor Ltd., Wellington, New Zealand) were immersed for at least several hours in alcohol and dried with a swab. Several drops of D.P.X. mounting medium (G.T. Gurr Ltd., London, England) were placed on each coverslip. The slide to be mounted was inverted over the coverslip and gently pressed until the D.P.X. covered the area of the coverslip and all air bubbles had been removed. Slides were left to dry overnight.

Appendix III. Giemsa stain

- (i) Dissolve 0.5 g Giemsa powder (Allied Chemical Corporation New York) in 33 ml glycerine at 55°C for 1.5 to 2 hours.
- (ii) Remove from heat and add 33 ml of methanol to make the stock solution.
- (iii) For use, a 2% solution was made with pH 6.8 buffered water (Buffer tables pH 6.8, Geo. W. Wilton and Co. Ltd., Lower Hutt, New Zealand: 1 tablet to 1 L distilled water).

Appendix IV. Fibroblast Culture Materials

- a. Foetal bovine serum: Laboratory Supplies, Auckland.
- b. PSK: 10g streptomycin, 10 l - megal vials of penicillin and 10g kanamycin made up in 1000 mls of PBS and sterilized by filtration.
- c. PBS (phosphate buffered saline):
  - 8.0 NaCl
  - 0.2 g KCl
  - 1.15 g Na<sub>2</sub> HPO<sub>4</sub>

Appendix IV. continued0.2gKH<sub>2</sub> PO<sub>4</sub>

Made up to 1000 ml of deionized water

Final pH 7.2-7.4.

d. ATV (antibiotic - trypsin - versene):

Trypsin (Difco 1:250)	0.5 g
Versene (EDTA)	0.2 g
NaCl	8.0 g
KCl	0.4 g
Dextrose	1.0 g
NaHCO <sub>3</sub>	0.58 g
PSK	10 ml
Phenol red	0.02 g

Made up to 1000 cc with water, sterilized by filtration and stored at -20<sup>0</sup>C in 20 ml aliquots.

Appendix V. G-banding technique

The technique used was a modification of that used by M. Parslow (pers. comm.).

1. Incubate slides in 2 x SSC for 1.5 hr at 60<sup>0</sup>C.
2. Rinse slides in normal saline for 1-5 min.
3. Flood with 0.25% trypsin (Difco 1:250) made up in saline for 15-60 sec. The trypsin is made up about 30 minutes before use.
4. Rinse slides in saline and then in 0.06 M Sorensen's buffer (pH 6.8)
5. Stain slides in 5% Giemsa in Sorensen's buffer for 10-20 min.
6. Rinse in deionized water.

2 x SSC: NaCl 8.77 gm  
Tri-sodium citrate 4.41 gm  
in 500 ml deionized water

Sorensen's buffer:

KH <sub>2</sub> PO <sub>4</sub>	4.08 g
Na <sub>2</sub> HPO <sub>4</sub>	5.34 g

Made up to 1000 ml with deionized water

Normal Saline Solution: The National Dairy Association Limited  
Auckland, New Zealand

Appendix VI. Washing of laboratory glassware

All glassware was soaked for 24 hours in Decon 75 (approximately 5 ml in 10 litres), rinsed in tap water, washed in Pyroneg, rinsed, drained and dried. Universal jars and lids used in blood leucocyte cultures and bijou bottles used for the silver solutions were rinsed in 1M HCl and then rinsed 3 times in distilled water before being dried. The culture bottles were then autoclaved.

Appendix VII. Photography techniques

- (a) Camera Wild Mka. 5 "Photoautomat".
- (b) Film Copex panrapid (Agfa).
- (c) Developing film
  - (i) The film is developed in D76 (Kodak) or 1 D 11 (Ilford) for 6 minutes at 20°C.
  - (ii) The film is then fixed in Universal fixer for 2-5 mins, and washed in running tap water for at least 30 mins.
  - (iii) Finally, the film is rinsed with a wetting agent and hung up to dry.
- (d) Enlarger: Focomat 11c, Leitz, Germany.
- (e) Developing prints
  - (i) The paper used was Ilfospeed No 3,4 or 5 depending on the contrast between Ag-deposits and background.
  - (ii) The prints were developed in 1 part Dektol diluted with 2 parts water at 20°C for 2 minutes.
  - (iii) The prints were briefly immersed in a stop bath of a very weak acetic acid solution.
  - (iv) The prints were then put in the hypo bath for 5 minutes, with occasional agitation
  - (v) The final step is washing in the water bath for at least 5 minutes, and then drying.

Appendix VIII. Experimental Animals

(a) History

The sheep used in this study were from flocks carrying 3 Robertsonian translocations which have been established by

Appendix VIII. continued

Prof. A.N. Bruère at Massey University (Bruère and Mills 1971; Bruère et al., 1972; Bruère, 1973). The  $t_1$  and  $t_2$  translocations were originally found in Romney sheep while the  $t_3$  chromosome was polymorphic in Drysdale sheep. Mating experiments have been conducted since 1969 in order to study the fertility effects of these translocations and to breed sheep with various combinations of the  $t_1$ ,  $t_2$  and  $t_3$  chromosomes (Bruère, 1974; 1975a).

(b) Management

The sheep used in this study were maintained on the Massey University No.2 Sheep Unit. The flocks were closely supervised during tugging and lambing. For details of the husbandry practices refer to Bruère (1974).

Appendix IX: Distribution of counts of number of Ag-NORs  
per metaphase

Animal No.	Number of cells having this number of Ag-NORs						
	≤ 4	5	6	7	8	9	10
61/6	1	0	4	8	14	6	0
174/6			4	16	23	9	7
124/3			1	5	5	6	19
169/1		1	4	15	9	6	1
166/1		4	4	10	3	3	2
176/6			4	61	58	9	10
45/6			1	3	5	19	22
67/5		6	10	5			
18/6					5	3	10
166/7			1	3	6	5	1
156/7				1	1	5	2
60/5		2	5	21	2	0	0
73/4	3	2	1	12	12	0	0
106/3	1	8	4	8	6	3	1
34/3	1	0	5	6	13	5	0
74/2			1	3	8	10	8
157/5	1	2	1	5	8	7	6
67/3	4	0	1	9	10	0	0
80/3		1	0	2	2	4	1
114/5			2	5	3	0	0
121/5	1	4	3	14	5	2	1
91/5		1	5	7	10	7	0
19/6		4	9	15	2		
128/7				2	6	16	6
68/5			1	0	7	22	
116/2			2	9	12	5	2
199/5		1	2	1	2	7	17
98/5			2	3	12	10	3
198/5			2	3	5	8	12
155/6				2	5	10	8
90/6		1	8	8	4	4	0
165/6		2	3	2	5	10	3

Appendix X: Calculation of F statistic by two-level nested ANOVA for the determination of variance due to between-replicate and between-animal differences in number of Ag-NORs.

This computation is based on the data in Table 3-4

$$a = 6 \text{ animals}$$

$$b_1 = 2-5 \text{ replicates per animal}$$

$$n_{ij} = \text{cell count for } k\text{th cell in } j\text{th replicate}$$

$$i = 1, \dots, a$$

$$j = 1, \dots, b$$

ANOVA Table

Source of Variation	d.f.	Sum of Squares	Mean Square	$F_s$
Among groups (animals)	5	65	13	3.69
Among subgroups within groups	13	45	3.46	4.29
Within subgroups	403	325	0.806	

Significance test for  $MS_{\text{subg}}$ :

$$F_s = \frac{MS_{\text{subg}}}{MS_{\text{within}}} = 4.29$$

To test the  $MS_{\text{groups}}$  it is necessary to compute the coefficients of the variance components,  $n'_o$ ,  $(nb)_o$  and  $n_o$

$$n'_o = 22.39$$

$$n_o = 21.8$$

$$(nb)_o = 65.83$$

Solving for estimates of the three unknown variance components:

$$MS_{\text{groups}} = S^2 + n'_o S_{B \subset A}^2 + (nb)_o S_A^2 = 13$$

$$MS_{\text{subg}} = S^2 + n_o S_{B \subset A}^2 = 3.46$$

$$MS_{\text{within}} = S^2 = 0.806$$

$$\text{Therefore } S^2 = 0.806$$

$$S_{B \subset A}^2 = 0.1217$$

$$S_A^2 = 0.1439$$

$$F_s = \frac{MS_{\text{groups}}}{MS_{\text{subg}}}$$

$$= 3.69$$

$$F_s(5,13) = 3.69 > F_{0.05}(5,12) = 3.11$$

Appendix XI. Example of size polymorphism data from one animal (199/5)

METACENTRICS						CHROMOSOME 4		CHROMOSOME C25		
A	B	C	D	E	F	a	b	t <sub>3</sub>	C25	
3	3	3	3	1	1	2	2	3	2	
3	3	3	3	2	2	1	1	3	3	
1	1	1	1	1	1	1	0	3	1	
2	2	2	1	1	0	2	0	1	0	
1	1	1	1	0	0	0	0	2	1	
3	2	2	2	2	2	2	0	2	2	
3	3	2	1	0	0	1	1	3	1	
3	2	2	2	2	2	2	1	3	2	
3	2	2	2	0	0	0	0	3	1	
3	2	2	2	2	1	1	1	3	2	
3	3	3	2	2	1	1	1	2	1	
3	3	2	2	2	1	1	0	2	1	
3	2	2	2	2	1	2	1	3	2	
3	3	2	2	1	1	1	1	3	2	
3	3	3	3	1	1	2	1	3	2	
3	3	3	2	1	1	1	1	3	2	
3	3	2	2	1	0	2	1	3	2	
3	2	2	1	0	0	0	0	2	0	
3	3	3	2	2	1	2	1	3	2	
3	3	3	2	2	2	1	0	2	1	
3	2	2	1	1	1	2	1	3	2	
3	3	3	3	2	2	1	1	3	2	
3	3	3	3	2	2	1	1	3	2	
3	3	3	3	2	1	2	1	3	1	
3	3	3	3	1	0	2	2	3	1	
3	3	3	3	1	0	1	1	3	1	
3	3	3	3	1	1	2	1	3	2	
3	3	3	2	2	1	1	1	3	1	
3	3	3	3	2	1	2	1	3	2	
<b>TOTAL</b>	82	75	71	62	39	27	39	23	79	44
<b>AVERAGE</b>	2.8	2.6	2.5	2.1	1.3	0.9	1.3	0.8	2.7	1.5

Appendix XII. Data on Ag-NOR frequency in animals involved in inheritance study

Animal No.	Frequency of Ag-NORs				TOTAL
	Meta	C4	t <sub>3</sub>	C25	
<u>Pedigree 1</u>					
116/2	5.9	0.6	1.0	0.4	7.9
73/4	5.0	0.1	0.9	0.8	6.8
60/5	5.6	0.1	1.0	0.1	6.8
68/5	5.8	1.0	1.0	0.8	8.6
91/5	4.8	0.8	1.0	1.0	7.6
121/5	4.2	0.8	0.9	1.0	6.9
101/6	4.5	1.0	1.0	0	6.5
<u>Pedigree 2</u>					
67/3	5.4	0.3	0.8	0.8	7.3
74/2	5.8	1.2	0.8	0.9	8.7
34/3	5.3	1.1	1.0	0.1	7.5
106/3	4.1	0.4	1.9	-	6.4
37/5	4.3	1.3	1.0	1.0	7.6
98/5	5.7	1.3	1.0	0.3	8.3
157/5	5.6	0.7	1.0	0.8	8.0
198/5	5.3	1.6	1.0	1.0	8.8
199/5	5.6	1.6	1.0	0.9	9.1
<u>Pedigree 3</u>					
37/5	4.3	1.3	1.0	1.0	7.6
121/5	4.2	0.8	0.9	1.0	6.9
47/8	4.7	0.1	1.0	1.0	6.8
85/8	4.6	0.8	0.9	1.0	7.3
92/8	4.9	1.2	1.0	1.0	8.1
<u>Pedigree 4</u>					
60/5	5.6	0.1	1.0	0.1	6.8
16/8	4.9	1.2	1.0	1.0	8.1
55/8	5.3	1.2	1.0	1.0	8.5
60/8	5.1	0	1.0	1.0	7.1
112/8	5.0	0.8	1.0	0.9	7.7
122/8	5.5	1.1	1.0	0.1	7.7
123/8	5.8	1.2	1.0	0	8.0
130/8	5.9	0.6	1.0	0	7.5
184/8	5.0	0.5	1.0	1.0	7.5

continued ...../2

Appendix XII. continued

Animal No.	Frequency of Ag-NORS				TOTAL
	Meta	C4	t <sub>3</sub>	C25	
<u>Pedigree 5</u>					
176/6	5.9	1.0	0.6	-	7.5
51/8	5.5	1.0	0	1.0	7.5
56/8	5.0	2.0	0	0.5	7.5
64/8	5.4	0	0	0.7	6.1
65/8	5.6	1.4	0.3	0.3	7.6
67/8	5.4	1.8	0.3	0.9	8.4
68/8	5.6	2.0	0	1.0	8.6
81/8	6.0	1.1	0.1	0.8	8.0
95/8	5.4	1.1	0.3	0.5	7.3
109/8	5.4	1.3	0	0.4	7.1
144/8	5.8	1.6	0.2	0.5	8.1
148/8	5.6	1.0	0	0	6.6
150/8	5.3	1.9	0	1.0	8.2
151/8	5.7	1.1	0.2	1.0	8.0
152/8	5.9	1.0	0.5	0.7	8.1
155/8	5.8	1.8	0	1.0	8.6
156/8	5.3	1.9	0	1.0	8.2
157/8	5.6	0.8	0	0	6.4
158/8	6.0	1.0	0	0	7.0
160/8	5.6	1.8	0.2	0.6	8.2
163/8	5.6	1.1	0	0.1	6.8
164/8	5.9	0	0.7	0	6.6
175/8	5.8	0.1	0.5	0	6.4
185/8	6.0	1.9	0	0.5	8.4

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## CHROMOSOMAL EVOLUTION IN SHEEP, GOATS, BHARAL, AOUADAD AND CATTLE

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The major mechanism of chromosomal evolution in the Bovidae is believed to be by Robertsonian fusions. The ancestral bovid karyotype is thought to have contained 58 acrocentric autosomes plus the sex chromosomes. In karyotypes with a lower chromosome number there is an increase in the number of metacentrics so that the number of chromosome arms remains constant. Domestic and wild sheep (*Ovis*), domestic goats (*Capra hircus*), the bharal (*Pseudois nayaur*), the aoudad (*Ammotragus lervia*) and domestic cattle (*Bos taurus*) have homologous G-banding patterns. The arms of the metacentric and submetacentric chromosomes in sheep, aoudad and bharal are homologous in banding patterns to acrocentric chromosomes in goat and cattle. This confirms the hypothesis of karyotypic changes due to centric fusion in these species and further indicates a structural stability of their chromosomes.

Sheep and goat nuclei have a similar DNA content. The satellite DNA fractions of these species have very similar properties and can cross-hybridise. This is remarkable in view of the wide differences in satellite DNA reported in other closely related species.

The regions containing the genes for rRNA may have a high frequency of involvement in exchange events. Deletions of these genes may occur during evolution. Therefore a study was made of the nucleolus organising regions (NORs) of sheep, goat, cattle, bharal and aoudad to determine whether the location and number of the genes for rRNA had undergone changes during evolution.

Treatment of metaphase chromosomes with a silver solution (the Ag-I technique) results in staining of the regions containing the genes coding for 28S and 18S rRNA. The NORs were found to occur terminally on 5 chromosome pairs in each of the species studied. These chromosomes are homologous in G-banding patterns. The NORs of these species have therefore remained stable during evolution. This contrasts with the situation in the human, chimpanzee, gorilla, orangutan and gibbon where differences in number and location of NORs occur. The conservation of NOR patterns in these species supports the evidence for the relative stability of the chromosomes in the Bovidae.

The process of karyotypic evolution by centric fusion allows an interpretation of the pathway of evolution in these species. Domestic and wild sheep species have chromosome numbers of 52, 54, 56 and 58. It has been proposed that the common ancestor of sheep and goats had a chromosome number of 60. The goats evolved from this ancestor without further karyotypic changes. Following a centric fusion resulting in a chromosome number of 58, the aoudad and a wild sheep species (*O. vignei*) evolved. These species have identical karyotypes although they belong to different genera. Three further centric fusion events produced sheep with chromosome numbers of 56, 54 and 52.

An analysis of the evidence from the karyotypic changes, present-day distribution of the wild sheep species and fossil and paleontological evidence enables a hypothesis to be made on the evolution of these species.

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