NUCLEOLUS ORGANIZERS IN CHROMOSOMES OF THE DOMESTIC DOG, *CANIS FAMILIARIS*

by

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APPROVAL BY THESIS DIRECTOR

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Date

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Lastly, I owe special thanks to my parents for their love and support, and for helping me believe I can do anything I set out to do.

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ABSTRACT

The chromosomes from six breeds of domestic dog were analyzed for identification and characterization of nucleolar organizing regions (NOR's). Peripheral lymphocytes of whole blood were cultured using the standard 72 h method. Sequential staining with quinacrine mustard followed by NOR staining with a modified Ag-I technique was performed to identify NOR-bearing chromosomes. Telomeric (q arm) NOR's were found in 6 chromosomes (3 pair) in females and 7 chromosomes (3 pair + 1) in males. Chromosome assignments of NOR's were 7, 17, 30 or 31 and the Y based on comparison of the Q-bands to a G-banded standard karyotype. The observation that the Y is an NO-chromosome is significant as this condition is unusual in mammals.

Telomeric associations among NO-chromosomes were observed in 43.25 percent of cells in females and 50.5 percent of cells in males. Chromosomes involved in associations appear to be at random. Data suggest that NOR size is a heritable property.

INTRODUCTION

The chromosomes of Canidae have been studied, yet no synthesis of these data in an attempt to reconstruct their phyletic evolution has taken place. Wurster and Benirschke (1968) compiled a general review of the chromosomes of a large portion of the Carnivora group and Todd (1970) has proposed a theory for karyotypic evolution in Canids. Recently Wurster-Hill and Centerwall (1982) studied interrelationships of banding patterns in Canids. Conventionally prepared karyotypes of Canis lupus, the wolf, C. latrans, the coyote, and C. familiaris, the domestic dog, appear to be the same (Chiarelli, 1975). While diploid chromosome numbers, early G-band and C-band studies showed no obvious differences between these three species (Wurster and Benirschke, 1968), Wurster-Hill and Centerwall (1982) recently observed some differences in the G-banded small chromosomes of wolf and coyote. Distinct banding on these small chromosomes is difficult to achieve, and "interpretation is open to debate" (Wurster-Hill and Centerwall, 1982).

In this study the chromosomes of the dog were analyzed for identification and characterization of the nucleolar organizing regions (NOR's) for use as a potential tool in identification of interspecific hybrids between dogs, wolves, and coyotes.

Canis familiaris, the domestic dog, has a diploid number of 78 (N.F.=80) (Minouchi, 1928; Makino, 1949; Takayama, 1958). The autosomal complement consists of 38 acrocentric chromosomes, ranging in

size from about 5 um to 1 um. The X is a large submetacentric chromosome and the Y is a small sub- or metacentric chromosome. No qualitative variation in chromosome complement has been observed between different breeds of dogs based on conventional karyotypes (Ahmed, 1941; Gustavsson, 1964; Borgaonkar et al., 1968; Chiarelli, 1966; Moore and Lambert, 1963; Todd, 1970). Giemsa-banded karyotypes have been published by Selden et al. (1975), and Manolache, Ross, and Schmid (1976). Q-bands and 33258 Hoechst banded karyotypes have been described by Manolache et al. (1976), and Howard-Peebles and Pryor (1980) have published R-bands. C-banding in Canids is hard to obtain, and extreme variation exists in the family suggesting unique properties of repetitive DNA (Pathak and Wurster-Hill, 1977).

Nucleolus organizer regions (NOR's) on chromosomes can be demonstrated in the light microscope by the use of silver staining techniques (Goodpasture and Bloom, 1975; Howell, Denton, and Diamond, 1975; Bloom and Goodpasture, 1976). Ammoniacal silver nitrate selectively stains proteins associated with the genes for the 18S and 28S ribosomal subunits (Goodpasture and Bloom, 1975; Howell et al., 1975; Howell, Hsu, and Block, 1977). These genes have been shown to consist of highly repetitive DNA that is rich in G-C base pairs (Goodpasture and Bloom, 1975). Silver deposits seen in metaphase chromosomes reflect transcription of the NOR's in the previous interphase (Howell et al., 1977). Proteins associated with the transcribed rRNA at the NOR site are entrapped around the NOR as the chromosomes condense and

enter prophase. It is these proteins that are still present in the metaphase chromosomes that selectively reduce the silver and become stained (Miller et al., 1977; Howell et al., 1977).

Identification of NOR-bearing chromosomes has been accomplished by sequential staining techniques. Other workers have stained chromosomes with silver nitrate and followed with trypsin-Giemsa banding in order to identify NOR location. This technique is unsatisfactory as trypsin often removes silver staining proteins (Henderson and Bruère, 1979; or see for example Kopp, Mayr, and Schleger, 1982). Lau et al. (1978), have devised a more effective method of sequential staining. By following silver staining with quinacrine mustard, NOR-bearing chromosomes can be identified by their Q-banding patterns.

NOR-bearing autosomes have been identified in many mammals, but few mammals have been shown to demonstrate NOR's on the Y chromosome. To date, only 5 mammalian species have been reported with NOR-bearing Y chromosomes: Hylobates (Symphalangus) syndactylus, a primate (Ledbetter, 1981); Carollia castanea, a fruit bat (Hsu, Spiritu, and Pardue, 1975); Nyctereutes procyonoides, the raccoon dog (Mäkinen and Fredga, 1980); Ammospermophilus harrisii, the antelope squirrel (Elder et al., 1980); and Canis familiaris, the domestic dog (Pathak, Van Tuinen, and Merry, 1982).

Ranges for the number of NOR-bearing chromosomes in the domestic dog have been found to be 3-8 (Pathak et al., 1982).

Inheritance of Ag-stainability has been studied in humans (Mikelsaar et al., 1977; Markovic, Worton, and Gerg, 1978; Taylor and

Martin-DeLeon, 1981) and in domestic sheep (Henderson and Bruère, 1980). In each case the data support a Mendelian inheritance pattern for silver deposits on the NOR-bearing chromosomes.

The purpose of this study was: 1) to determine the modal number and ranges of NOR-bearing chromosomes in both male and female domestic dogs, and 2) to identify and characterize the NOR-bearing chromosomes of the genome. During this study other questions as to the nature of the NOR's were generated and additional data were accumulated on: 1) the associations between the telomeres of NOR-bearing chromosomes, and 2) heritability of NOR's.

MATERIALS AND METHODS

Cell Source and Culture Procedure

Whole blood, collected from adult domestic dogs in heparinized syringes, was obtained from the Animal Science Laboratory at the Arizona Health Sciences Center. The apparent breed and the sex were recorded for each sample. Peripheral blood lymphocytes were cultured to obtain metaphase spreads. The culture medium was Gibco RPMI 1640 media supplemented with 20% fetal calf serum, 2.5% Phytohemagglutinin (M-form), 2% Penicillin/Streptomycin (5000 units/cc each), and .25% sodium heparin (5000 units/cc) was used. For culture, conical 15 ml centrifuge tubes containing 10 ml of the medium were each inoculated with whole blood, usually 0.5-1.0 ml, containing 1×10^4 white blood cells. The tubes were tightly capped and incubated in an inclined position at 37° C for 72 hrs.

Cytological Preparation

One hr before harvest Colcemid (Gibco) was added to each culture tube in a final concentration of .08 ug/ml. The tubes were inverted to mix and returned to 37° C for one hr. At the end of the incubation time the cultures were spun down in a centrifuge for 5 min. All centrifugations were at 200XG. After centrifugation the supernant was removed and the cells were resuspended by gentle agitation. Hypotonic, .075M KCl, was added slowly to a volume of 5-8 ml, depending on

the pellet size, and the suspensions were incubated at 37° C for 8 min. The tubes were centrifuged 5 min, the supernant was removed, and the pellets were resuspended in fresh cold fixative (3 parts methanol: 1 part glacial acetic acid). The fixation procedure was begun by displacing and resuspending the pellet in a volume of fixative approximately equal to the volume of the pellet: the suspension was immediately drawn up in a Pasteur pipette and repipetted into the culture tube, after which fixative was added to a volume of 3-5 ml. This quick exposure of the pellet to fixative reduced clumping of cell proteins and led to cleaner, unprecipitated preparations. Cell suspensions were fixed at 4° C for 15 min, after which they were centrifuged and resuspended twice more in fresh fixative. After the last centrifugation the supernant was removed and the cells were resuspended in 0.5-1.0 ml of fresh fixative and stored or used for slide preparation.

Experiments were performed to increase chromosome length using an actinomycin D (AMD) method of Mankinen (pers. comm. letter dated 1981; Denton, Texas). According to this technique, 5 ug of AMD (Sigma A-4262) were dissolved in 5-10 drops of acetone, and the solution was diluted to 50 ml with RPMI. For use, aliquots of AMD were added to the culture media (final concentration of 3.5 ug/ml) with the Colcemid solution one hr before harvest. Following this treatment, cells were exposed to .075 M KCl hypotonic treatment for 10 min at 37° C. The AMD technique resulted in longer chromosomes in which about one in 7-10 spreads contained chromosomes of optimal length for banding. Many

chromosomes exhibited gaps and breaks, a characteristic of AMD treatment also noted by Mankinen, which complicated identification of chromosomes by banding analysis.

Slide Preparation

Commercial slides were cleaned by dipping in a Coplin jar of acetone and flushing with deionized water. Excess water was tipped off of the slide and 5-6 drops of the cell suspension were dropped from a Pasteur pipette onto the wet surface from a height of about 1 m. Each drop was allowed to spread to its maximum and contract before another was added. One short explosive breath of air was given to the wet slide, and the slide was allowed to air dry.

Chromosome Staining Techniques

Staining of metaphase chromosomes for identification of NOR's was accomplished by: 1) fluorescent staining using quinacrine mustard or 33258 Hoechst, 2) silver staining using an Ag-I technique modified from Bloom and Goodpasture (1976), and 3) trypsin-Giemsa banding.

Fluorescent Staining

Quinacrine Mustard (QM). Quinacrine mustard, 100 ug/ml, was made up in McIlvaine's buffer (McIlvaine, 1921) at pH 4.5. The QM solution was added to a fresh slide and a coverslip applied. The slide was placed in the dark for 20 min, and the coverslip was then rinsed off in deionized water. The slide was washed in a Coplin jar containing deionized water (pH 4.5) for 3 min. Wash time was critical in adjusting the amount of stain in the chromosomes; a short wash resulted

in uniform brightness without bands, a long one in loss of fluorescence. After the wash, the slide was mounted in either McIlvaine's buffer (McIlvaine, 1921) with glycerol or in pH 4.5 water and sealed using clear fingernail polish.

If a QM stained slide was to be followed by silver staining, all the buffers in the QM procedure were replaced with deionized water. This reduced any precipitation formed on the slide by exposure of chloride ions in the buffers to the $AgNO_3$ solution; however, brightness and stability of the QM stain was reduced.

33258 Hoechst. 33258 Hoechst is a benzimidazole compound used as a fluorescent stain (Jamal, Markvong, and Hsu, 1974). The 33258 Hoechst stain, in a working solution of .05 ug/ml in deionized water, was not sensitive to pH and the solution was very bright. The 33258 Hoechst solution was placed on a freshly prepared slide and covered with a coverslip. The preparation was stained for 15-20 min in the dark, washed in deionized water for 3 min, mounted in deionized water, and sealed with a coverslip. If the slides were observed immediately after staining, the brightness was generally poor; storage for one day stabilized the stain and improved the brightness. Wash time was critical and needed to be adjusted to produce optimal differentiation in the chromosomes. Hoechst stain was excellent for use in sequential staining for, as no chloride-containing buffers were required, no silver chloride precipitate formed on the slide to interfere with NOR identification.

Silver Staining of Nucleolar Organizer Regions (NOR's)

The technique used was modified from that of Bloom and Goodpasture (1976). A fresh 50% (w/v) silver nitrate ($AgNO_3$) solution was prepared for each group of slides to be stained using 1 g of $AgNO_3$ in 2 ml of deionized water. The water quality is important in silver staining as chloride ions will cause precipitation. One-day-old slides stain optimally; however, freshly prepared slides will stain acceptably. For treatment slides were soaked in deionized water at 37° C for 20 min and air dried. Slides were placed on rubber washers in humidifying chambers consisting of square plastic Petri dishes containing a layer of damp paper toweling. The silver nitrate solution was placed on the slide after filtering through a disposable 0.2 um filter (Acrodisc, #4192). A coverslip was placed on the slide and the chamber was placed in a 37° C incubator for 30 min. Staining in our laboratory was rapid. With older slides or those slides for which the 20 min deionized water pretreatment was omitted, a longer staining time was needed. The incubation time was judged sufficient when the chromosomes appeared golden in color with distinct black nucleolar organizing regions.

Sequential Staining

In order to identify NOR-bearing chromosomes it was necessary to induce chromosome bands on the same preparations which were to be used for NOR detection. For this purpose a sequential staining technique was employed. QFQ or QFH banding was carried out, after which

the preparation was photographed, rinsed, and restained with silver nitrate for NOR detection. Photographs of fluorescent spreads were compared with silver stained spreads and the locations of NOR's on chromosomes were marked directly on the photograph. NOR-bearing chromosomes were then identified by QFQ band patterns and compared to the G-banded standard karyotype (Figure 4).

Trypsin-Giemsa Banding

The staining method used to prepare a standard Giemsa banded karyotype for this study was that of Grouchy and Turleau (1977). One-week-old slides were used after hardening by overnight exposure to 65° C. The slides were dipped into a Coplin jar containing a 0.05% (w/v) trypsin solution of Difco (1:250) trypsin in Phosphate Buffered Saline (PBS) maintained at 37° C. Usual exposure times ranged from 10-30 sec. The slides were immediately rinsed by dipping 10 times in each of 2 Coplin jars of PBS, air dried, and stained 2-5 min in a Giemsa solution. The Giemsa solution consisted of the following: Harleco Giemsa, 1.5 ml; methanol, 1.5 ml; 0.1 M citric acid, 2 ml; .02 M Na_2HPO_4, 4 ml; and deionized water to 50 ml. The slides were then rinsed briefly in deionized water and air dried.

Photography

Fluorescent Preparations

Cells were photographed on Kodak 35mm Panatomic X film using a 50X oil immersion objective and a 10X photoeyepiece. Exposure times varied from 3 to 3.5 min. Care was taken to assure stability of the

camera as the slightest movement with exposures of such length results in blurred images. Film was developed 8 min at 68° F in full strength Kodak D76 developer and negatives were printed on Kodabromide F4 or F5 paper. Exposure time was usually 6-8 sec at f11-f16.

Silver Stained and Giemsa Banded Preparations

Cells were photographed on Panatomic X film using a 50X oil immersion objective and a 10X photoeyepiece. A dark green filter was used to improve contrast. Exposures were made automatically using a Wild Photoautomat MKA2. Film was developed 8 min at 68° F in D76. Negatives were printed on Kodabromide paper as above. Exposure time was 3-5 sec at f8-f11.

Scoring NOR's

The method used to score chromosomes bearing NOR's was adapted from techniques by D. A. Miller et al. (1977), and Taylor and Martin-DeLeon (1981). Slides stained by the Ag-I method were scored as follows:

a. The number of NOR's in each cell was recorded after counting the number of chromosomes bearing telomeric silver grains.

b. Chromosomes bearing NOR's were categorized according to their relative length: long, chromosomes 1-10 in the standard canine karyotype (Selden et al., 1975); medium, chromosomes 11-21; short, chromosomes 22-32; and very short, chromosomes 32-38.

c. NOR's on the above chromosomes were quantified on the basis of size relative to the other NOR's in the cell. Silver deposits, representing NOR's were scored as: 0 (absent), 1 (small), 2 (medium), or 3 (large).

d. Cells were scored for telomere-telomere NOR associations. The numbers of associations and the chromosomes involved in each, i.e., medium to long, short to very short, etc., were recorded.

e. Data from 20 cells/slide were tabulated and modal numbers and association frequencies were calculated and recorded.

RESULTS

The chromosomes of 15 domestic dogs, eight males and seven females, were studied for: 1) the number of NOR-bearing chromosomes, 2) the identification of these chromosomes, 3) the distribution of the size of the silver deposits on the NOR's, 4) the frequency of telomeric associations between NOR-bearing chromosomes, and 5) the inheritance of NOR's. Of the 15 dogs studied, five were German Shepherds, two were Labrador Retrievers, five were mixed breeds. The remaining three were an Irish Setter, a Cairn Terrier, and a Greyhound.

In each of the females studied a maximum of six chromosomes exhibited silver stained NOR's (Table 1). Males exhibited seven NOR-bearing chromosomes, including a small chromosome identified as the Y (Table 1 and Figure 1). Most frequently, five of seven observations in males and six of seven observations in females, the modal number for each individual was the highest number of the range (Table 1). In Table 2 are presented the frequencies that the chromosomes in the classes long, medium, short, and very short (Y) exhibited NOR's in 20 cells per individual. No significant difference was found between males and females in the frequency of expression of NOR's on each of the three pairs of autosomes. The Y chromosome NOR's were the most variable in expression and exhibited the lowest frequency; in some individuals this expression was as low as 25%.

The distribution of the relative size of silver deposits on NOR's in long, medium, short, and very short chromosomes in the 14 individuals is shown in Table 3. These data support identification of the smallest NOR-bearing chromosome as the Y, as none of the females studied ever exhibited this small, unpaired NOR (Figure 2).

Sequential staining of metaphase spreads with QM followed by $AgNO_3$ was performed to identify NOR-bearing chromosomes (Figure 3). The Q-banding patterns of chromosomes exhibiting silver deposits on their telomeric ends were compared to the G-banded standard karyotype (Figure 4). The NOR's of domestic dogs were found to be terminally located on chromosomes identified as numbers 7, 17, 30, or 31 and the Y (Figure 5).

Table 4 represents the data accumulated on telomeric associations between NOR-bearing chromosomes. Frequencies were calculated by totaling the number of cells whose chromosomes were involved in telomeric association and dividing by the total number of cells. No significant differences were found between frequencies of cells with associations in males and females. The males studied had a range of 30-80% cells with associations and the females had a range of 20-70%. The Y chromosome in males exhibited a frequency of association that was about one-half the frequency in the long, medium, and short chromosomes. This was probably because there is only one Y chromosome per cell which can be involved in an association, while the others all have two chromosomes which can be involved. Telomeric associations in a metaphase spread of a female German Shepherd are shown in Figure 6. The most common nucleolar associations involve two chromosomes (Table 5).

Information on the relative size distribution of NOR's from the study of a family of mixed breed dogs are presented in Table 6. Individuals D40♀ and D41♂ were the parents; individuals D42♀, D43♀, D44♂, D45♂ the progeny. No polymorphic markers for chromosomes 7, 17 or 30-31 were found. The Y chromosome, however, had to be inherited by the male progeny from the male parent. D41♂ had an extremely large silver deposit on the Y chromosome, as did the male offspring. Chromosome 31 or 30 in both male and female parent exhibited large NOR's on each member of the pair of chromosomes; the progeny also exhibited this character.

Table 1. Modal value and range of number of Ag-NOR's in metaphase chromosomes in male and female dogs.[1]

CODE	MODE	RANGE
D3-1 ♂	6	5-7
D1-1 ♂	7	5-7
D2-2 ♂	7	6-7
D14 ♂	7	5-7
D33 ♂	7	6-7
D41 ♂	7	5-7
D45 ♂	6	4-7
D38 ♀	6	5-6
D13 ♀	5	4-6
D35 ♀	6	4-6
D24 ♀	6	5-6
D40 ♀	6	4-6
D42 ♀	6	3-6
D43 ♀	6	5-6

[1] 20 cells/individual were studied.

Table 2. Frequency of chromosomes exhibiting NOR's in male and female dogs.

SEX	NO. ANIMALS	TOTAL NO. OF NO-CHROMOSOMES SCORED	FREQUENCY OF CHROMOSOMES EXHIBITING[1]			
			LONG	MEDIUM	SHORT	VERY SHORT[2]
MALE	7	280	.93	.91	.92	.73
FEMALE	7	280	.91	.90	.95	-

[1] differences are nonsignificant by a "Student" t-test (Kempthorne, 1957).

[2] total number of NO-chromosomes scored for VERY SHORT class is 140.

Table 3. Relative size distribution of NOR's in long, medium, short, and very short chromosomes in seven female and seven male dogs.[1]

DOG	CHROMOSOME SIZE											
	LONG			MEDIUM			SHORT			VERY SHORT		
	SIZE OF NOR[2]			SIZE OF NOR			SIZE OF NOR			SIZE OF NOR		
	●	●	•	●	●	•	●	●	•	●	●	•
D14♂	7	29	4	7	28	3	6	19	11	9	6	1
D13♀	0	14	18	3	11	3	13	26	1			
D1-1♂	10	20	8	22	12	3	15	23	0	11	8	1
D38♀	14	7	16	18	19	4	23	14	1			
D35♀	26	8	2	36	2	0	20	14	3			
D24♀	12	16	12	20	16	1	8	16	15			
D3-1♂	30	8	2	33	6	1	18	13	14		1	4
D33♂	31	6	3	37	3	0	7	28	2	4	6	5
D2-2♂	24	11	4	20	13	6	28	7	3	11	3	5
D40♀	29	4	3	19	5	11	28	9	0			
D41♂	23	9	5	28	6	0	32	4	3	18	2	0
D42♀	16	7	12	21	10	3	31	5	0			
D45♂	18	9	2	20	6	1	34	2	0	17	0	0
D43♀	16	15	7	17	12	5	39	1	0			

[1] 20 metaphase spreads/individual were observed.

[2] ● = Size 3
● = Size 2
• = Size 1

Table 4. Frequency of telomeric associations in chromosomes bearing NOR's.

CODE	ASSN./20 CELLS	NO. OF CHROMOSOMES INVOLVED IN ASSN.	f OF CHROMOSOMES IN ALL ASSN.: LONG	MEDIUM	SHORT	VERY SHORT
♀D43	7	18	.38	.33	.27	
♀D42	4	10	.30	.50	.20	
♀D40	6	17	.41	.41	.18	
♀D13	11	22	.23	.27	.50	
♀D38	6	12	.17	.03	.58	
♀D24	14	36	.39	.33	.28	
♀D35	12	29	.24	.41	.41	
Mean f	8.65[1]		.295	.37	.35	
♂D45	6	17	.18	.29	.29	.23
♂D41	16	40	.18	.38	.20	.28
♂D2-2	9	24	.25	.16	.40	.25
♂D1-1	7	14	.21	.43	.14	.14
♂D3-1	15	34	.35	.32	.21	.03
♂D33	8	18	.22	.39	.33	.05
Mean f	10.1 [1]		.23	.33	.26	.16

[1] t value = .647, no significant difference between mean frequencies.

Table 5. Frequency of nucleolar associations involving different number of chromosomes.

SEX	NUMBER OF CHROMOSOMES ASSOCIATING:					
	2	3	4	5	6	7
MALE	.92	.08	0	0	0	0
FEMALE	.90	.06	.01	.01	.01	.01

Table 6. Relative size distribution of NOR's in long, medium, short, and very short chromosomes in a family of dogs.[1]

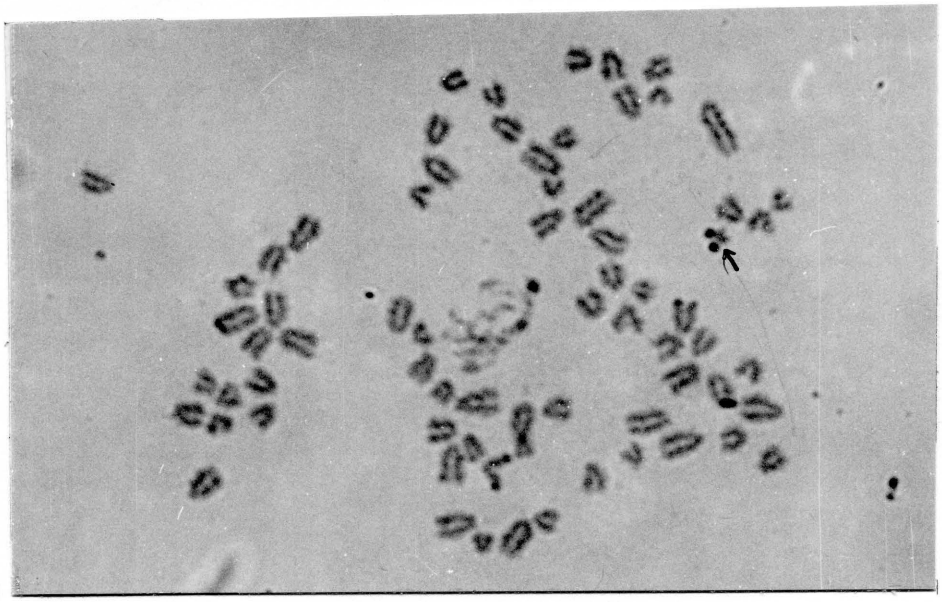
DOG NUMBER	CHROMOSOME SIZE											
	LONG[2]			MEDIUM			SHORT			VERY SHORT		
	●	●	•	●	●	•	●	●	•	●	●	•
D40	29	4	3	19	5	11	28	9	0			
D41	23	9	5	28	6	0	32	4	3	18	2	0
D45	18	9	2	20	6	1	34	2	0	17	0	0
D44	9	6	3	9	3	3	12	0	0	7	1	0
D43	16	5	7	17	12	5	39	1	0			
D42	16	17	12	21	10	3	31	5	0			

[1] 20 cells/individual were scored for all animals except D44 in which only nine cells were scored.

[2] ● = size 3, ● = size 2, • = size 1 of NOR's.

Figure 1. Silver stained metaphase of a male dog. Arrow indicates NOR on the biarmed Y chromosome.

Figure 2. Silver stained metaphase of a female dog. Six NOR's are present on the telomeric ends (black regions).



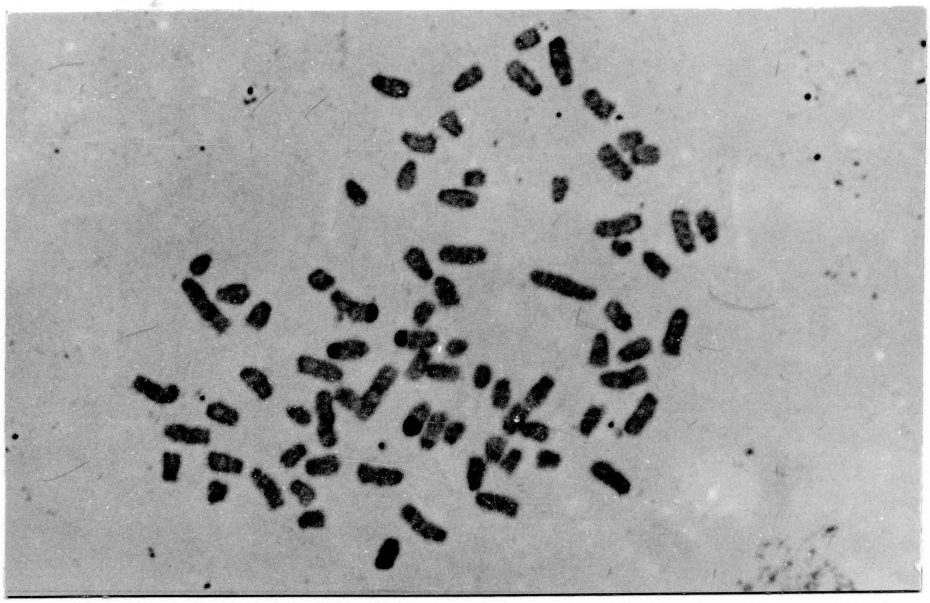
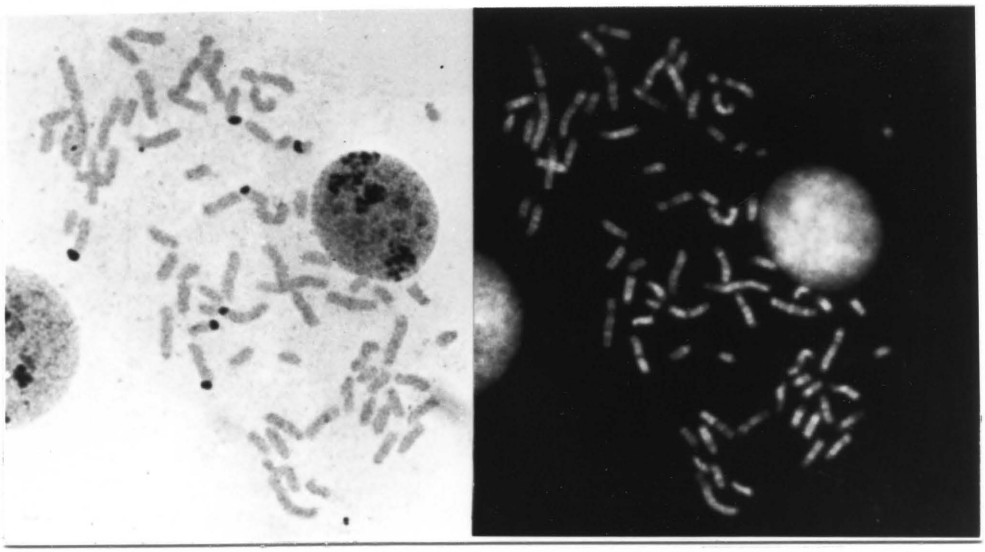


Figure 3. Sequentially stained metaphase in a male dog. The silver stained preparation is on the left, QM on the right. Seven NO-chromosomes are present.

Figure 5. Q- and G-band comparisons of NOR-bearing chromosomes. From left to right, chromosome numbers 7, 17, 31, and the Y.



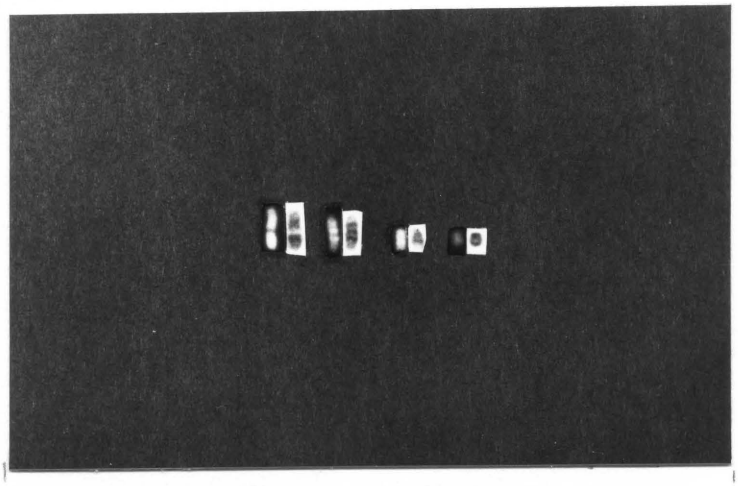
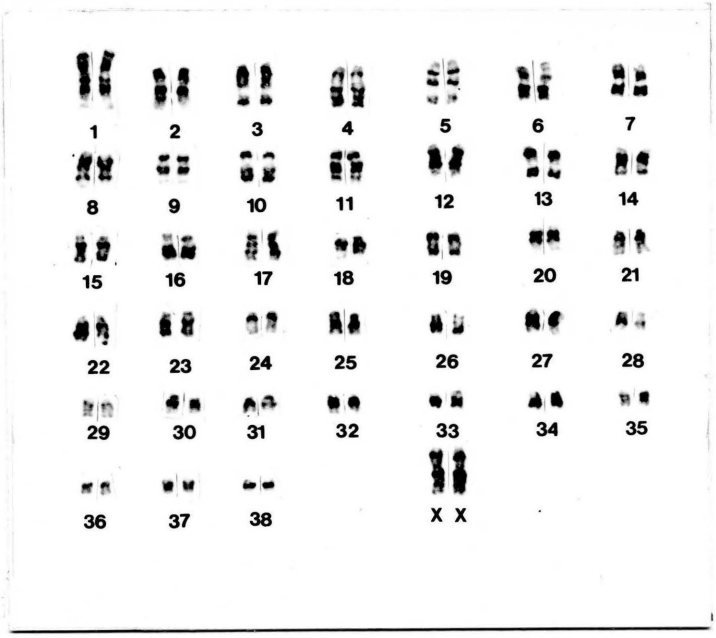


Figure 4. The G-banded karyotype of Canis familiaris.

Figure 6. Telomeric associations in a female dog. Six NOR's are present, five of which are in association.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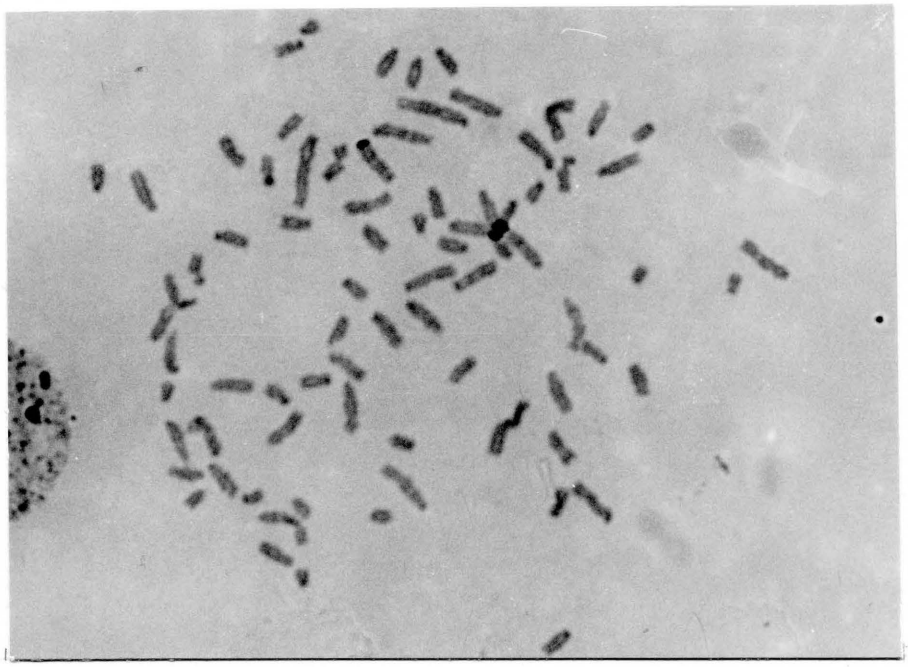

DISCUSSION

The NOR's in the chromosomes of the domestic dog occur terminally and do not correspond to visible secondary constructions, therefore, silver stained metaphase preparations are needed to identify the NOR-bearing chromosomes. In the six breeds studied, no difference has been found in position of NOR's on the chromosomes or in identification of the NO-chromosomes. The maximum number of Ag-NOR's per metaphase is six in females and seven in males. Pathak et al. (1982), studied a male dog and found the modal number of NOR's to be seven with a range of three to eight. Centromeric regions of some autosomes stain darkly with silver possibly accounting for the count of more than seven NOR's in some cells (Pathak et al., 1982). In this study, female dogs had a modal value of six NOR's and a range of three to six (Table 1). Furthermore, the modal number of NOR's observed in an individual is usually the same as the highest value in the range (Table 1) which suggests that most often all of the possible NOR's are detected. It appears that the NO-chromosomes in dogs are conserved and that number and location of NOR's is highly stable. The stability of the NO-chromosomes might be preserved by the following mechanisms: 1) little or no unequal sister chromatid exchanges in premeiotic cells, which would reduce the chance that all of the NOR material could be transferred to one chromatid with the resulting loss of the NOR from the sister chromatid, 2) low frequency of unequal meiotic crossover in the terminal regions of the

NO-chromosomes, resulting in the same consequences as (1) above, and/or 3) infrequency of chromosomal rearrangements (inversions, translocations, fusions, etc.) which would otherwise change the location of the NOR's in the complement.

In the present investigation, no chromosomal rearrangements were observed in the dogs studied. The NOR-bearing chromosomes in the genus Canis appear to be highly conserved. Preliminary observations from this laboratory on the Mexican wolf (C. lupus baileyi) and the coyote (C. latrans) suggest that the number and chromosomal location of NOR's are the same in dogs, wolves, and coyotes; if this is true, the degree of chromosomal similarity between the species would be remarkable. Such similarity does occur in other groups. For example, in four members of Bovidae (sheep, goat, cattle, and audad) the NOR's are all located terminally on chromosomes with homologous G-banding patterns (Henderson and Bruère, 1979). NO-chromosomes are not always highly conserved. Change in the location of NOR's occurs in primates, and Tantravahi et al. (1976) account for this change as a result of chromosomal rearrangements which have occurred by a variety of mechanisms. Inbred strains of laboratory mice also exhibit changes in location of NOR's on the chromosomes and changes in the number of NOR's as well (Henderson et al., 1974).

The long, medium, short, and very short chromosomes bearing NOR's in the domestic dog were identified as numbers 7, 17, 30 or 31, and the Y based on comparison of Q-bands with the G-bands of the karyotype assembled for this purpose (Figure 4). Manolache et al. (1976), found

G- and Q-banding patterns of dog chromosomes to be identical. G- and Q-banding patterns of the NO-chromosomes in this study were also found to be identical (Figure 5) in four individuals studied in detail and lead to the following conclusions. I believe the long NOR-bearer is chromosome number 7 by virtue of its banding pattern. This chromosome has a distinctive non-staining (G negative) gap in its distal third. The medium-sized NO-chromosome is distinctly shorter than the number 7 and exhibits three equally spaced G positive bands along its length. I believe the medium-sized NOR-bearing chromosome is best identified as number 17 (Figures 3, 4, and 5). The small NOR-bearing chromosome is difficult to identify because only spreads with very elongated chromosomes permit distinction of banding patterns among the small chromosomes of the karyotype. In my preparations, the small NO-chromosome had two bands, one at the shoulder and one halfway down its length. I feel these observations best describe those chromosomes numbered 30 and 31 in both my karyotype and that of Selden et al. (1975). Extensive studies with extended chromosomes and high resolution banding techniques would be necessary to unequivocally identify this small NO-chromosome. Finally, data from this study support the conclusion that the smallest chromosome carrying an NOR is the Y chromosome because: 1) the small unpaired NOR-bearing chromosome is found only in males (Tables 2, 3, and 4); 2) the chromosome is G-band positive and does not fluoresce brightly, properties which are characteristic of the Y chromosome in domestic dogs (Selden et al., 1975; Manolache et al., 1976; Figures 3 and 5);

and finally, 3) the chromosome is the only small biarmed chromosome in the complement (Figure 1).

In a similar study, Kopp et al. (1982), have recently identified the NO-chromosomes in the domestic dog as numbers 7, 8, 27, and 38. They used an Ag-I/Giemsa sequential staining technique and compared the Giemsa band patterns to those of the karyotype of Selden et al. (1975). The data in the present study do not support the chromosomal assignment of NOR's proposed by Kopp et al. (1982). First, I feel the medium-sized NOR-bearing chromosome is misassigned as number 8, because the number 8 chromosome is roughly the same size as the number 7, while the medium-sized NO-chromosome is distinctly shorter. The G-bands of chromosome number 8 do not match those of the medium NOR-bearer in either the Selden et al. (1975) karyotype or my karyotype (Figure 4). Secondly, although the small NO-chromosome is difficult to identify by banding, my preparations show a distinctive G positive centromeric band. Chromosome number 27 observed in the present study and as proposed by Kopp et al. (1982), is G negative in the region of the centromere (Selden et al., 1975; Figure 4). Therefore, I feel chromosome 27 is not this NOR-bearer. Lastly, the small NO-chromosome is not number 38 as Kopp et al. (1982), suggest. I object to their conclusion because: 1) neither their data nor photographs allow unequivocal identification of chromosome 38, and 2) number 38 is the smallest acrocentric chromosome in the complement and has a bright fluorescent band (G+ band) in the middle of the arm, neither of which are properties of the smallest NO-chromosome. The data in this study support the conclusion that the Y

chromosome, rather than number 38, is the small NOR-bearing chromosome, a finding confirmed by data from Pathak et al. (1982). These workers, in a study of meiosis in male dogs, found the smallest NOR-bearer to pair with the X chromosome, thus identifying it as the Y.

It is of interest that the Y-chromosome in domestic dogs bears an NOR as mammalian Y chromosomes generally carry little genetic information. NOR's have been reported on both the X and Y chromosome in the fruit bat, Carollia castanea (Hsu et al., 1975). This observation supports a dosage compensation hypothesis, and it is observed that males and females of this species have the same number of NOR's. Three other mammals have been reported to exhibit NOR's on the Y chromosome: the primate, Hylobates (symphalangus) syndactylus (Ledbetter, 1981), the antelope squirrel, Ammospermophilus harrisii (Elder et al., 1980), and the raccoon dog, Nyctereutes procyonoides (Mäkinen and Fredga, 1980); however, none of these species express an NOR on their X chromosome. Pathak et al. (1982), suggest that the presence of the NOR on the Y and its absence on the X probably has little effect, if any, on the phenotype. In other mammals, differences in number of active NOR's occur without any apparent effect on phenotype (humans: Bloom and Goodpasture, 1976, and Miller et al., 1977; sheep: Henderson and Bruère, 1979; deer mice and Chinese hamster: Hsu et al., 1975).

In the current study, 73 percent of the Y chromosomes in somatic cells exhibited silver stained NOR's in the seven males studied (Table 2). The range for Y expression was as high as 100 percent and as low as 25 percent indicating considerable variability of expression in this NO-chromosome. Pathak et al. (1982), using data from one individual,

suggest the Y chromosome is more active in somatic cells (61.6 percent) than in pachytene stages of meiotic cells (32.7 percent). It would be of interest to carry out similar studies in meiotic cells of the other mammals with Y bearing NOR's. The NOR on the Y may be a useful tool for the characterization of sex chromosome function in mammals.

Unpublished data from this laboratory on observations from silver stained metaphase spreads of C. latrans (coyote) and C. lupus baileyi (Mexican wolf) show that the Y chromosome in these species also exhibits an NOR. In the phylogenetic tree of Canidae proposed by Todd (1970), the genera Canis, Nyctereutes, and Vulpes all arose from a common ancestor. Silver staining studies in addition to the current study, have revealed Canis and Nyctereutes as well to have Y bearing NOR's (Mäkinen and Fredga, 1980; Pathak et al., 1982) while Vulpes (Ellenton and Basrur, 1980) does not. Further karyological studies on other Canidae should provide useful information on phylogenetic relationships and positive assignment of other members to the Canid group.

Telomeric associations among NO-chromosomes are common in the domestic dog. Miller et al. (1977), have proposed that the frequency with which a chromosome is involved in an association is based on the number of genes it carries which code for 18S and 28S ribosomal subunits. Jacobs, Mayer, and Morton (1976), in testing the Miller et al. (1977), hypothesis, found no significant preference for associations between satellite chromosomes in humans. Henderson, Warburton, and Atwood (1972), and Evans, Buckland, and Pardue (1974), suggest that the probability of a chromosome being in an association is not a function

of the number of genes it carries which code for rRNA but is instead a result of chromosomes participating in nucleolus formation. In this study, males were found with associations in 50.5 percent of all metaphases investigated, while females showed 43.25 percent associations. No significant differences were found in the frequency of associations between NOR-bearing chromosomes in males and females (Table 4). Kopp et al. (1982), found associations in 60 percent of all investigated metaphases. The chromosomes involved in associations appear to be at random (Table 4), and no preference was found for associations based on size (i.e. short chromosomes will associate equally with long, medium, or very short chromosomes). Randomness in association has also been reported in a study of humans (Curtis, 1974). I have found the most frequent types of NO-chromosome associations involve two chromosomes (about 90 percent), while those involving three or more, as in Figure 6, occur less frequently (about 10 percent of the total)(Table 5). Similar results were also reported by DiBerardino et al. (1981), in the water buffalo. Polymorphic markers, other than the NOR itself, for NO-chromosomes would be required to adequately study these chromosomal associations in dogs. Markers would permit the identification of chromosomes involved in associations and the distinction of maternal and paternal chromosomes in NOR heritability studies.

Quinacrine fluorescence polymorphisms in humans (Milkelsaar et al., 1977; Markovic et al., 1978; Taylor and Martin-DeLeon, 1981), and translocation polymorphisms in domestic sheep (Henderson and Bruère, 1980), have been used to trace the inheritance of Ag-NOR's. In both

cases, evidence was found to support the heritable nature of these regions. In the current study, although no polymorphic markers were available with which to trace chromosomes of paternal and maternal origin, the following evidence was obtained to suggest that NOR size in domestic dogs is a heritable property. The relative size of Ag-NOR's in a family of mixed breed dogs is shown in Table 6. D40 is the female parent, D41 is the presumed male parent, and D42, D43, D44, and D45 the female and male progeny. The long and medium chromosome did not provide any definitive data on inheritance. The short NO-chromosome, however, was noteworthy. Both parents had large NOR's on this chromosome (Figure 7), and male and female pups consistently exhibited this character. This may suggest that the large NOR's were inherited as a constant morphological feature of chromosome number 30 or 31 and not merely of large size as a result of activity. The Y chromosome of the male parent displayed an unusually large silver deposit in 18 of the 20 cells observed. The male pups, D45 and D44, had similarly large NOR's in 17 of 20 and seven of nine cells, respectively. Since the pups inherited the Y chromosome from their father, they probably inherited this large NOR.

SUMMARY

The chromosomes in male and female domestic dogs, Canis familiaris, exhibit three pairs of autosomes with telomeric NOR's In addition, males of this species display a telomeric NOR on a small unpaired biarmed chromosome. These chromosomes have been identified as numbers 7, 17, 30 or 31, and the Y using sequential staining with QM followed by Ag-NO_3. For identification, Q-banding patterns of the NO-chromosomes were compared to a G-banded standard karyotype assembled for this purpose. The NO-chromosomes appear to be conserved and location and number of NOR's is highly stable in the six breeds studied. Associations between NOR-bearing chromosomes are common, those involving two chromosomes being the most frequent type. No preference was found for associations based on NOR size and chromosomes involved in associations appear to be at random. The present study suggests the size of the silver deposit on an NO-chromosome is inherited as a constant morphological feature of that chromosome.

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