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# Visualization of Nucleolar Organizer Regions in Mammalian Chromosomes Using Silver Staining

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*Abstract.* A simple ammoniacal silver staining procedure, designated Ag-AS, differentially stains the chromosomal locations of ribosomal DNA in certain mammalian species. This was critically demonstrated by Ag-AS staining of the nucleolus organizer regions in karyotypes of the same species and cell lines used for locating the ribosomal cistrons by DNA/RNA *in situ* hybridization. With Ag-AS, silver stained NORs (Ag-NORs) are visualized as black spherical bodies on yellow-brown chromosome arms. Ag-NORs were visualized throughout mitosis at the secondary constrictions in the rat kangaroo, Seba's fruit bat, Indian muntjac, and Rhesus monkey. The Chinese hamster and cattle have telomeric Ag-NORs, the mouse subcentromeric Ag-NORs, and the field vole Ag-NORs as minute short arms or chromosomal satellites. Ag-NORs occur at both secondary constrictions and at telomeres in the cotton rat. Variability in Ag-NOR pattern included differences in the number of Ag-NORs per cell within a cell population, size of Ag-NORs among chromosomes of a complement, and presence of Ag-NOR on particular chromosomes in two cell lines of the Chinese hamster. The available cytochemical data suggest that the Ag-AS reaction stains chromosomal proteins at the NOR rather than the rDNA itself.

## Introduction

Studies employing *in situ* DNA/RNA or DNA/DNA hybridization and chromosome banding have shown that somatic metaphase chromosomes of vertebrates can be resolved at a level far beyond the traditionally recognized features of size, position of centromere, presence of secondary constriction, and degree of heteropycnosis. These techniques have allowed biochemical and morphological dissections of metaphase chromosomes. For example, various satellite, ribosomal and other fractions of DNA have been localized at particular chromosomal sites (reviews by Hsu, 1973; Wimber and Steffensen, 1973).

*In situ* hybridization experiments have positively demonstrated that the familiar achromatic secondary constrictions (nucleolus organizer regions or NORs) represent the chromosomal locations of genes coding for 18S+28S ribosomal RNA (Henderson *et al.*, 1972; Henderson *et al.*, 1974a,b; Pardue and Hsu, 1975). Hsu *et al.* (1975) further showed that in karyotypes where no obvious NOR can be detected with conventional staining, the ribosomal gene clusters may be located in the centromeric or telomeric regions.

Although *in situ* hybridization experiments identify the locations of ribosomal cistrons on a molecular basis, these experiments are tedious and cannot be easily performed in laboratories without resources and technical expertise. Thus, a simple cytochemical method which can produce results corresponding to those

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obtained from *in situ* experiments should be more desirable as a general procedure. Matsui and Sasaki (1973) and Funaki *et al.* (1975) advocated the N-band technique using Giemsa to stain the NOR. Unfortunately, we were not able to obtain satisfactory results with this technique. Other laboratories encountered a similar problem.

Ammoniacal silver (A-S) has been used to differentially stain chromosomes of *Drosophila* (Black and Ansley, 1974), chick embryos (Bloom and Buss, 1969), *Trillium* (Utsumi and Takehisa, 1974), and humans (Bartalos and Rainer, 1972; Howell and Denton, 1974). Specific regions and structures visualized with A-S include the classical bands of polytene chromosomes, coils (chromonemata) along somatic metaphase chromosomes, constitutive heterochromatin, and human satellite III DNA regions. Recently, the satellites of human D and G group chromosomes were selectively stained with an A-S technique (Howell *et al.*, 1975). This finding suggests that under certain conditions silver staining is specific for nucleolus organizer regions (NOR) in the human genome.

In the present work we tested the feasibility of using A-S as a specific NOR stain in mammalian karyotypes. An improved A-S technique was applied to metaphase chromosomes of several mammalian cell lines representing 6 mammalian species. The same cell lines were analyzed for the cytological locations of 18S and 28S ribosomal cistrons by *in situ* DNA/RNA hybridization (see Pardue and Hsu, 1975; Hsu *et al.*, 1975). We show conclusively that the new technique, designated Ag-AS, selectively stains the same chromosome areas (NORs) displaying heavy labeling by the *in situ* technique. In addition, our results using Ag-AS are in accord with previous reports on the locations of rDNA in man Henderson *et al.*, 1972), mouse (Henderson *et al.*, 1974a), and Rhesus monkey Henderson *et al.*, 1974b).

## Materials and Methods

*Cell Source and Cytological Preparations.* For the present study, we employed the following cell lines, all of which were studied by Pardue and Hsu (1975) and Hsu *et al.* (1975) using the DNA/RNA *in situ* hybridization technique.

1. *Potorous tridactylis* (rat kangaroo): This female cell line, designated Pt-$K_1$, has 11 chromosomes, monosomic for the smallest autosome.

2. *Carollia perspicillata* (Seba's fruit bat): Male and female fibroblast lines, TCH-151 and TCH-674, were used.

3. *Carollia castanea*: This male cell line has a piece of an autosome translocated to the short arm of the submetacentric X chromosome. The short arm of this new X chromosome represents the long arm of the original X.

4. *Microtus agrestis* (European field vole): Female cell line Ma 504.

5. *Muntiacus muntjak* (Indian muntjac): Male cell line 1626.

6. *Cricetulus griseus* (Chinese hamster): We used the diploid male cell line Don, the diploid female cell line Dede and bone marrow cells aspirated from animals.

In addition to the aforementioned cell lines, we used primary cultures or early subcultures of the following materials:

7. *Mus musculus* (Swiss strain of the laboratory mouse): Cultures were initiated from embryonic cells.

8. *Sigmodon arizonae* (Arizona cotton rat): Female fibroblast culture.

9. *Macaca mulatta* (Rhesus macaque): Male fibroblast culture.

All cells received a short-term Colcemid treatment prior to harvest. Conventional air-dried preparations were used for silver staining.

*Silver Staining.* To stain the NORs of chromosomes, a modification of the AG-SAT procedure of Howell *et al.* (1975); Denton *et al.* (in press) was employed. Three solutions were prepared: 1) A pretreatment (Ag) solution of 50% aqueous silver nitrate. 2) An ammoniacal silver (As) solution prepared by dissolving 4 gm silver nitrate in a solution of 5 cc distilled water and 5 cc concentrated ammonium hydroxide (pH 12–13). 3) A developing solution of 3% formalin first neutralized with sodium acetate crystals and then adjusted to a pH of 5–6 with formic acid. It was usually necessary to alter the developer by adding additional sodium acetate and/or formic acid to achieve optimal chromosomal staining.

The Ag solution was pipetted onto a slide, covered with a coverglass and the slide placed about 25 cm below a photo flood (2800° K bulb) for 10 minutes. The coverglass was rinsed off in distilled water and the slide developed by adding 4 drops of As solution followed by 4 drops of developing solution. The slide was immediately covered with a coverglass and staining progress was monitored under the microscope. When the chromosomes reached a golden yellow color, the slide was rinsed in distilled water, dehydrated in an ethanol series, soaked in xylene and mounted in permount.

We term this modified silver technique Ag-AS which should not be confused with the original A-S histone staining technique of Black and Ansley (1964) or the AG-SAT technique of Howell *et al.* (1975).

## Results

As in all cytochemical procedures, the Ag-AS method for staining the NOR requires monitoring for optimal results. As the staining reaction proceeds, the NORs rapidly assume a black coloration while the rest of the chromosome slowly assumes color. Over-staining by prolonged treatment will result in undesirable preparations. Generally, the chromosomes should exhibit a deep yellow to a light brown color with NORs appearing as conspicuous black spherical bodies (Ag-NORs). If desired, staining can be stopped just after the NORs become conspicuous (Fig. 1 a) and the chromosomes can be lightly counterstained with Giemsa. It should be noted that contrastingly dark centromeric staining is frequently evident in over-stained slides. For example, in the Indian muntjac, centromere areas usually stain dark brown and NORs deep black. In the mouse, the centromeric areas as well as the NORs take up silver after prolonged staining (Fig. 1 b). Gross over-staining causes chromosomes to become dark brown to black throughout their lengths (Fig. 1 c).

Several hundred metaphase plates of each species were examined for the presence of differentially stained regions. Diploid cells with conspicuous black bodies on dark golden yellow to light orange-brown chromosomes were critically examined and photographed. Although a low level of background silver precipitation was present in many of our preparations, no consistent precipitation over chromosomal areas other than nucleolus organizer regions was observed. In all species studied, a black coloration of the NORs was seen. Secondary constrictions appeared heavily stained or as a series of black dots across the chromatids. Telomeric NORs were visualized as paired dots. Unlike achromatic regions at secondary constrictions, Ag-NORs were consistently visualized from mid-prophase through telophase. The silver stained NORs were usually most conspicuous in highly contracted C-metaphase chromosomes.

Table 1 is a summary comparing our Ag-NOR data with the *in situ* data of previous investigations. It is obvious that the correlation between the two sets of results is extremely high. However, for direct correlation between the achromatic NOR and silver staining, we applied the duplicate photography technique on

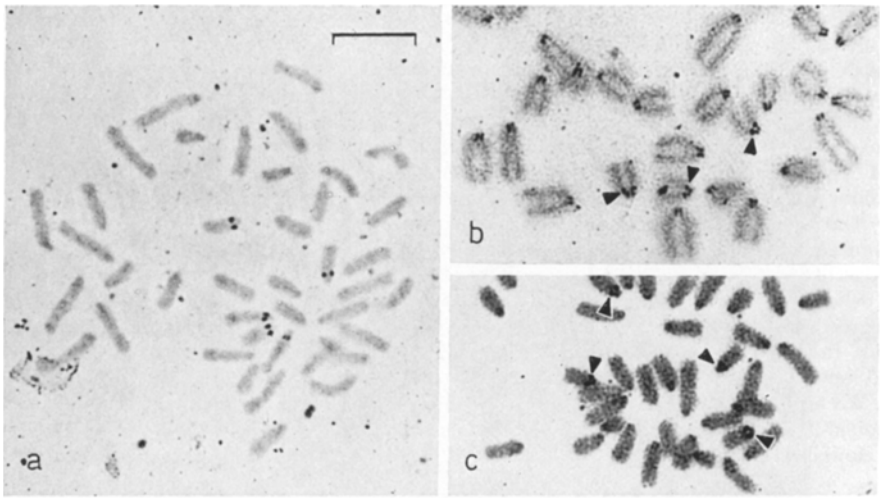


Fig. 1a—c. Ag-AS preparations of *Mus musculus* (2n=40). (a) Metaphase plate showing 5 chromosomes with Ag-NORs. Staining time 1 minute. Phase contrast. Chromosome arms are faintly stained. (b) Partial metaphase plate. Staining time 5 minutes. Brightfield. The centromeric areas of all chromosomes are darkly stained. Chromosome arms are stained bright yellow. Three subcentromeric Ag-NORs are visible (arrows). (c) Partial metaphase plate. Staining time 10 minutes. Brightfield. Chromosome arms are stained dark brown. Arrows indicate Ag-NORs. Bar indicates 10 μm

cytological preparations of the Rhesus macaque, Indian muntjac, fruit bat and rat kangaroo. Slides were first stained with Giemsa for positive identification of the marker chromosomes which contained conspicuous achromatic regions. The same slides, after removal of Giemsa, were then stained with Ag-AS. Fig. 2a and b show this correlation between achromatic region and Ag-NOR for the Rhesus macaque.

*Species with Ag-NOR at Secondary Constrictions*

*Macaca mulatta*: Silver staining of the secondary constriction on the marker chromosomes (Rothfels and Siminovitch, 1958) was unequivocal. A perfect correspondence between secondary constriction and Ag-NOR was found in a sample of 10 diploid cells first stained with Giemsa and then with Ag-AS (Fig. 2). A sample of 25 additional diploid cells, stained with the Ag-AS technique, showed at least one of the marker chromosomes silver stained; 21 cells showed both marker homologues stained.

*Protorous tridactylis*: Silver (Ag) staining was restricted to the NORs of the X chromosomes in this species (Fig. 3a). In a random sample of 100 metaphases, Ag spots were observed on only the X chromosomes. In 18 of these cells, the secondary constriction of at least one X chromosome was clearly represented by 4 dots (Fig. 3a). Two dots per chromatid were resolved in favorable anaphase cells.

*Carollia perspicillata*: As in the rat kangaroo, silver staining was restricted to the secondary constrictions of the X chromosomes (Fig. 3b). The Ag-NOR of the

Table 1. Correspondence between *in situ* hybridization data and silver stained nucleolus organizer regions (Ag-NORs) on the chromosomes of seven mammals

| Species | *In situ* hybridization | | Silver staining | | | |
|---|---|---|---|---|---|---|
| | Chromosomes bearing heavy grain cluster | Reference | Chromosomes bearing Ag-NOR | No. of Ag-NOR chromosomes per cell | | No. of cells examined |
| | | | | Mode | Range | |
| *Macaca mulatta* | marker | Henderson *et al.*, 1974a | marker | 2 | 1–2 | 35 |
| *Potorous tridactylis* | X | Hsu *et al.*, 1975 | X | 2 (♀) | 1–2 | 100 |
| *Carollia perspicillata* | X | Hsu *et al.*, 1975 | X | 2 (♀), 1 (♂)[a] | — | 100 |
| *Muntiacus muntjak* | 1, X, $Y_1$ | Pardue and Hsu, 1975 | 1, X, $Y_1$ | 4 | 2–4 | 20 |
| *Mus musculus* | 15, 18[b], 19 | Henderson *et al.*, 1974b | 3 pairs | 4 | 4–5 | 40 |
| *Cricetulus griseus* | 4, 5, 6, 7, 9 10, X[c] | Hsu *et al.*, 1975 | 4, 5, 6, 7, 9, 10, X | 7 | 4–10 | 188 |
| *Microtus agrestis* | several autosomes | Hsu *et al.*, 1975 | 8 autosomes | 8 | 5–10 | 31 |

[a] No variation observed in sample.
[b] Tentative assignment.
[c] Low grain counts but probably above background level.

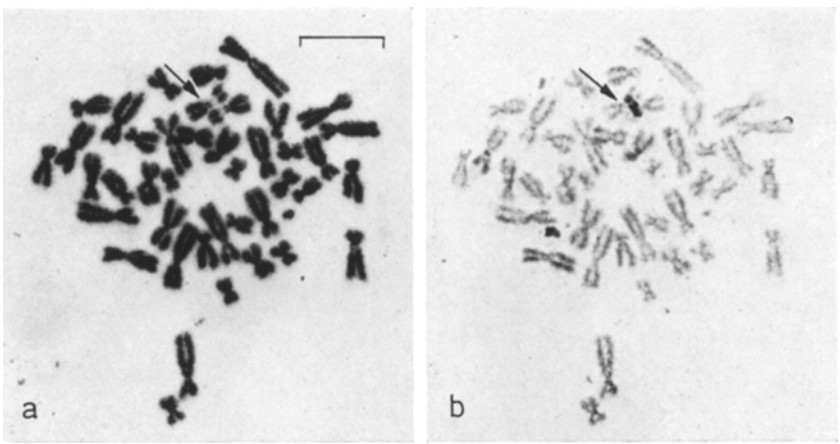


Fig. 2a and b. Metaphase preparation of *Macaca mulatta* (2n=42). (a) Stained with 2% Giemsa solution. (b) Destained with alcohol and restained with Ag-AS. Arrows show the secondary constrictions (a) stained by Ag-AS (b). Brightfield illumination. The bar indicates 10 μm

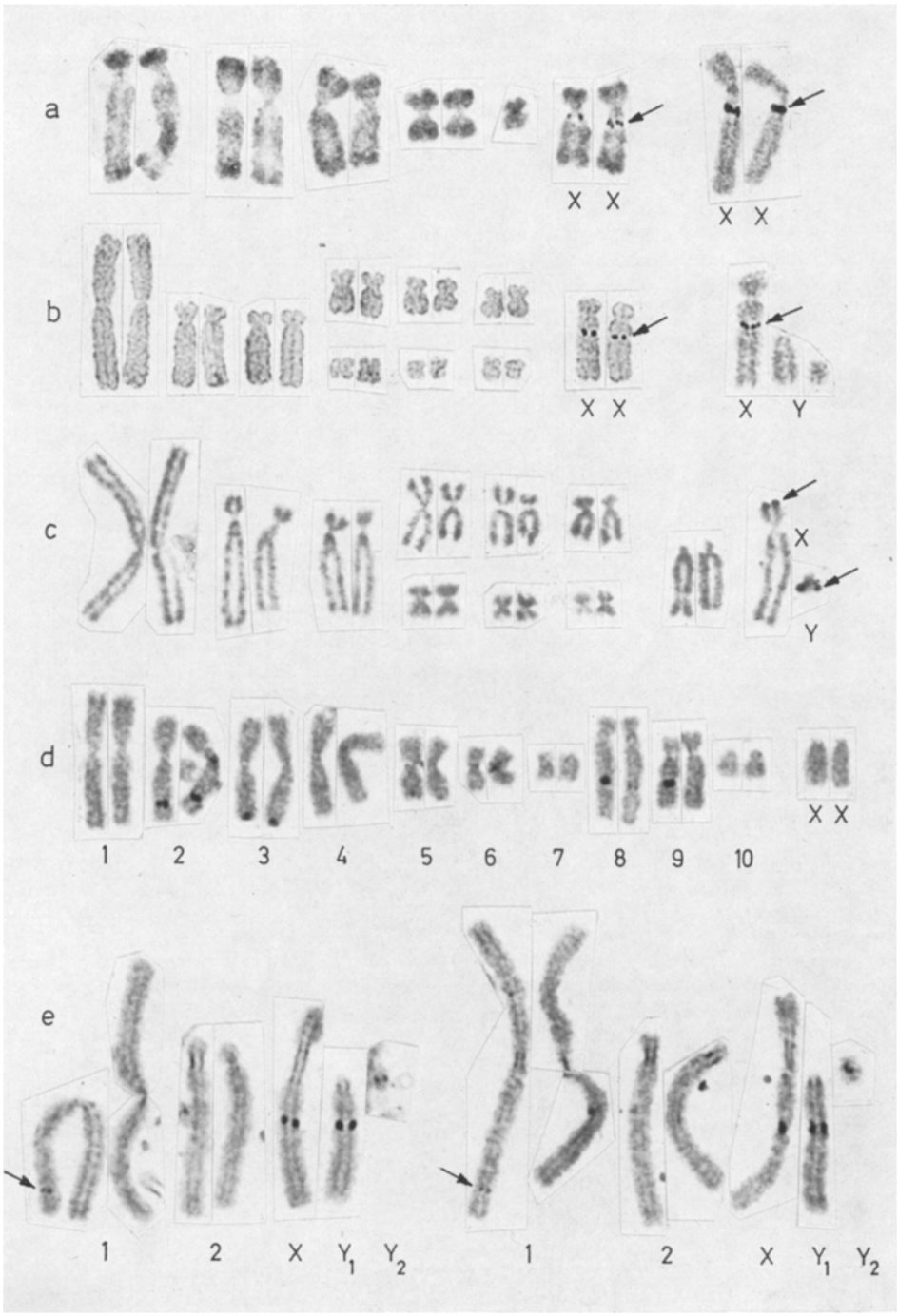


Fig. 3a–e

X in the male and both Xs of the female appeared equal in size. The silver stained region occasionally appeared quadripartite.

*Muntiacus muntjak*: Four of the seven chromosomes of the male muntjac bear secondary constrictions (Kato *et al.*, 1974). Conspicuous achromatic regions are located in the autosomal arm of the compound X chromosome and in the homolog of the translocated autosome (customarily termed the $Y_1$ chromosome). A smaller achromatic region is present near the distal end of one arm of the large metacentric no. 1 pair.

Ag staining of secondary constrictions was scored in 20 metaphases. Ag-NORs were evident in the X and $Y_1$ in all 20 cells; 3 cells showed one no. 1+X and $Y_1$ Ag stained, and 13 cells showed both no. 1 homologues plus X and $Y_1$ stained. The Ag-NOR in the $Y_1$ chromosome is the largest of the complement. Ag stained regions on both the X and $Y_1$ were considerably larger than Ag regions on the no. 1 pair (Fig. 3e). This staining pattern appears to correspond with the relative sizes of grain clusters over these chromosomes on *in situ* autoradiographs (Pardue and Hsu, 1975).

*Sigmodon arizonae*: The diploid number of the cotton rat is 22 and each chromosome pair can be identified in silver-stained karyotypes. The presence of secondary constrictions in the longest arms of several chromosomes is variable among individuals and also among cells from the same animal (Zimmerman and Lee, 1968). In our material, secondary constrictions were observed in the long arms of chromosomes 2, 8, and 9 (Zimmerman and Sihronen, 1973). Conspicuous Ag-stained areas were seen at the secondary constrictions in chromosomes 2, 8, and 9 and at the telomere regions of the long arm of chromosome 3. Ag-NORs of chromosomes 2 and 3 were intensely stained in most cells examined. In contrast, one of the homologues of chromosomes 8 and 9 invariably showed greatly reduced Ag staining or complete absence of staining. A representative karyotype is illustrated in Fig. 3d.

### *Species with Terminal NORs*

*Cricetulus griseus*: In the Chinese hamster silver staining was localized at the telomeres of several autosomes and occasionally at the secondary constriction of the X chromosome. The NOR was represented by a black dot at the extreme tip of the chromatid or by a pair of minute dots on the X. In most cells, Ag spots were graded in size, smaller autosomes bearing smaller spots (Fig. 4). Associations of 2 to 3 NOR-bearing chromosomes were visualized in occasional cells (Fig. 4c). Also, dark staining connections between Ag-NORs were seen (Fig. 4d).

The distribution of Ag-NORs in the chromosomes of two cell lines of the Chinese hamster is plotted in Fig. 5. The data were obtained from a sample of 85 metapha-

Fig. 3a—e. Karyotypes from Ag-AS stained metaphases. (a) *Potorous tridactylis* with Ag-NORs (arrows) on the X chromosomes. Two sets of X chromosomes are shown, the larger pair from a prometaphase. (b) *Carollia perspicillata* with Ag-NORs (arrows) on the X chromosomes. Sex chromosomes of male on right. (c) *Carollia castanea* with Ag-NORs on the X and Y. (d) *Sigmodon arizonae* with multiple Ag-NORs. (e) *Muntiacus muntjak* with Ag-NORs on no. 1 (arrows) and X and $Y_1$ chromosomes. Two cells are shown. All figures × ca. 2000

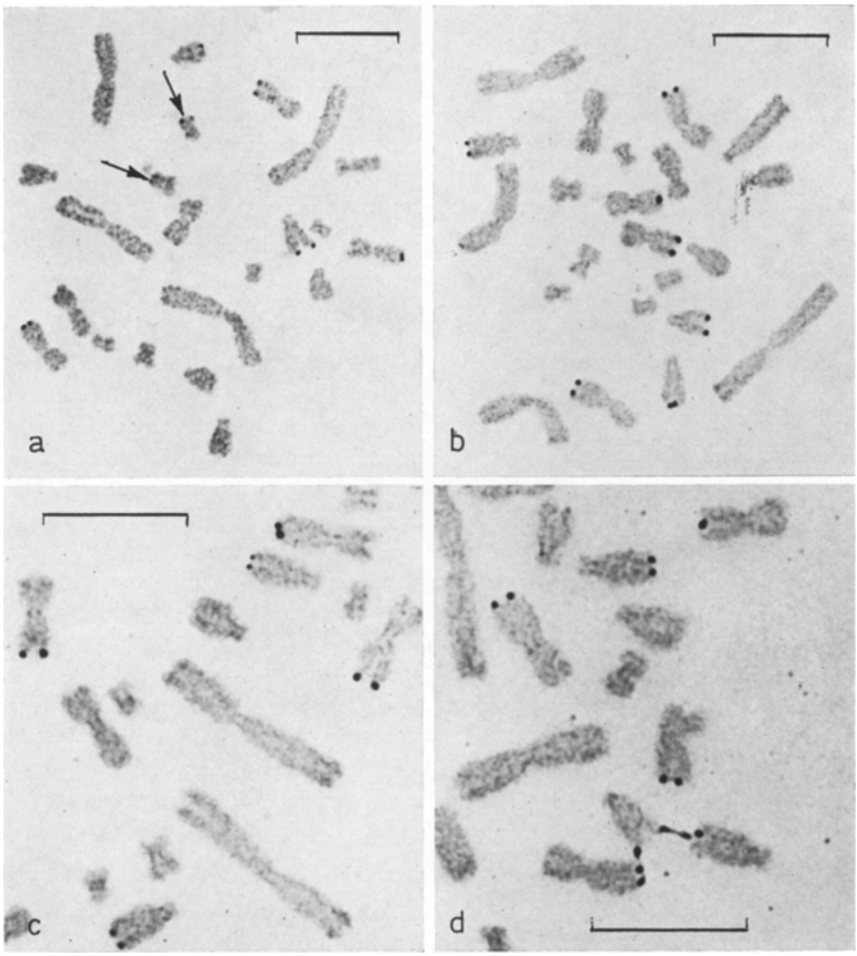


Fig. 4a—d. Metaphase chromosomes of the Chinese hamster (2n=22) from Ag-AS preparations. (a) Cell line Don, with 7 Ag-NOR chromosomes visible, including both chromosomes 9 (arrows). (b) Cell line Dede. Seven chromosomes bear telomeric Ag-NORs. (c) Cell line Dede. Five Ag-NOR bearing chromosomes are included to illustrate variation in sizes of Ag-NORs. Bar indicates 10 μm. (d) Bone marrow cell. Partial metaphase plate showing an association involving three Ag-NOR chromosomes. Seven chromosomes show distinct telomeric Ag-NORs in this cell

ses showing distinct Ag stained NORs. In the male cell line Don, chromosomes 4, 5, 6, 9, and 10 (or 11) bear Ag spots in most cells. Chromosome 7 (or 8) was stained in 39% and the X in 6% of the cells examined. The frequency of occurrence of Ag-NORs in this cell line corresponds closely with the distribution of cumulative grain counts from *in situ* autoradiographs (see Hsu *et al.*, 1975, Fig. 5). In the female cell line Dede, chromosomes 5, 6, and 7 were stained more frequently than in Don, and 9, 10 and X were usually unstained (Fig. 4a and b). Ag staining in male and female bone marrow metaphase cells appeared the same as that of Don

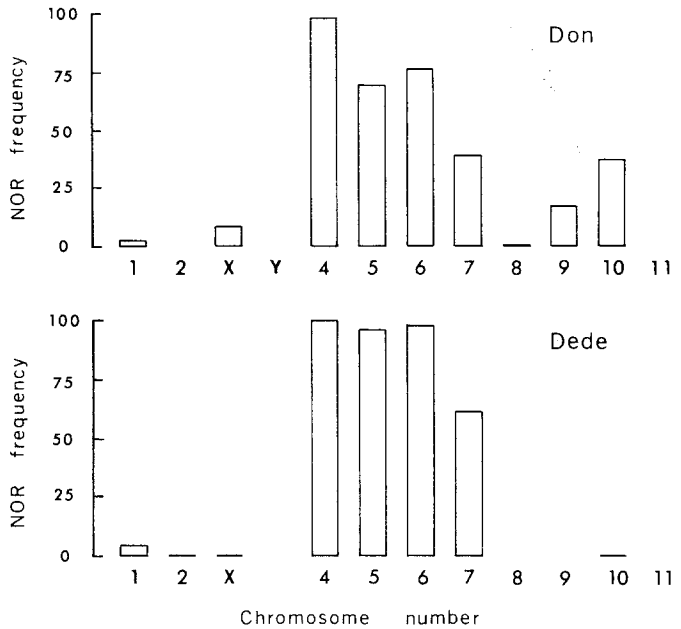


Fig. 5. Distribution of Ag-NORs in chromosomes of two cell lines of the Chinese hamster. A sample of 85 metaphases showing distinct Ag stained NORs was selected for counts from each cell line. Abscissa: Chromosome number. Ordinate: Frequency of occurrence of Ag-NOR expressed as a percentage of the total number metaphases scored (*i.e.* 100% or 170 no. 4 chromosomes observed showed distinct Ag-NORs in cell line Dede). Chromosomes were identified by size and position of centromere (Hsu and Zenzes, 1964)

cells in that a modal number of 7 Ag-NORs per cell was observed aud chromosomes 9 and/or 10 usually showed Ag-NORs

*Carollia castanea*: Ag-AS staining revealed a deeply stained dot at the end of the acrocentric Y chromosome and at the tip of the short arm of the modified X (Fig. 3c). These data confirm a telomeric location of the NOR in this cell line and lend support to the interpretation of Hsu *et al.* (1975) that the original NOR was at the telomeric end of the long arm of the X. In *Carollia perspicillata* the NOR region was involved in a translocation with an autosome.

### *Species with Centromeric NORs*

*Mus musculus*: *In situ* hybridization experiments of Henderson *et al.* (1974a) suggest that the ribosomal cistrons of the laboratory mouse are located in the paracentromeric areas of chromosomes 15, 18, and 19. In our material, Ag-NORs were identified in 5 of the 40 acrocentric chromosomes. Out of 40 diploid cells critically examined, 22 cells showed 4 chromosomes with Ag-NOR, and 18 cells showed one additional chromosome with a much smaller Ag-NOR (Fig. 1a). Ag spots were located just below the centromeric heterochromatin, which can be stained with silver if the staining time is prolonged or the silver nitrate and formalin concentrations are higher than optimum (Fig. 1b and c). The relative lengths of the chromosomes carrying an NOR as well as the locations of their NORs suggest

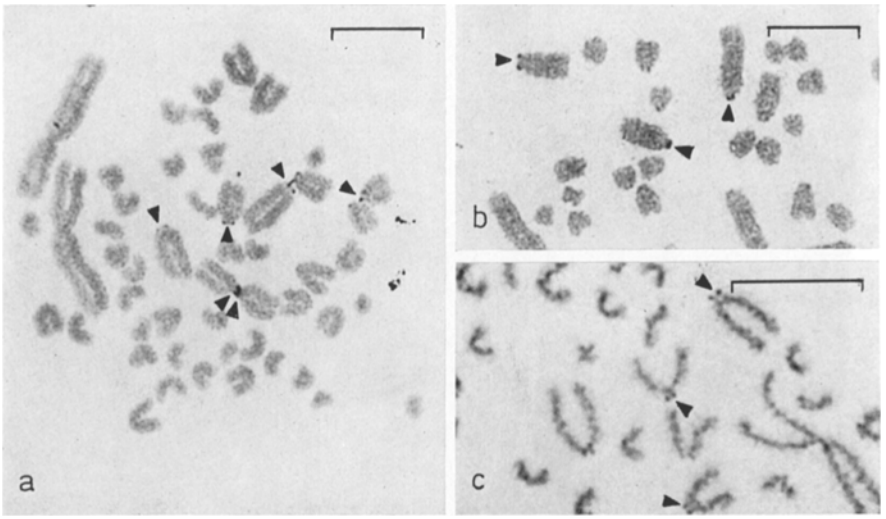


Fig. 6a—c. Ag-AS preparations of *Microtus agrestis* (2n=50). (a) Metaphase plate showing 8 chromosomes with Ag-NORs (arrows). Two large acrocentrics appear fused at the Ag-NOR (double arrow). (b) Partial metaphase plate showing Ag-NORs as short second arms (arrows). (c) Partial metaphase plate showing satellites or short arms on some of the acrocentric chromosomes (arrows). Bar indicates 10 μm

that our data correspond to those determined by the *in situ* method.

*Microtus agrestis*: Minute Ag spots were detected at the centromeric ends of 4 or 5 of the larger pairs of acrocentric chromosomes (Fig. 6a). In favorable cells the Ag-NOR was visualized as a minute "second arm" (Fig. 6b–c). These short arms were not detected with Giemsa staining in our material. With Giemsa staining, chromosomes involved in NOR associations showed a conspicuous achromatic gap between their adherent centromeric ends (Fig. 6a). This achromatic area was darkly stained in Ag-AS preparations. A maximum number of 4 chromosome pairs per diploid cell showed end-to-end Ag-NOR associations.

## Discussion

### *Specificity of Ag-AS for rDNA Regions in Chromosomes*

Our experiments using Ag-AS staining indicate that in properly controlled preparations, the nucleolus organizer regions can be revealed with certainty and accuracy. We have selectively stained the known locations of 18s and 28s ribosomal cistrons (NORs) in several mammals. These data include silver staining of the same cell lines used by Pardue and Hsu (1975) and Hsu *et al.*, (1975) for locating the NOR by DNA/RNA *in situ* hybridization. In all species studied (including human), silver stained nucleolus organizer regions (Ag-NORs) were observed only at chromosomal locations that demonstrated heavy labeling with the *in situ* technique (Table 1). Thus, the hypothesis that ammoniacal silver stains NORs is confirmed for 9 mammalian species.

It is conceivable that both the *in situ* technique and the silver technique require a certain degree of DNA sequence redundancy for effective demonstration of the NOR. If the ribosomal genes are dispersed as single copies or very small clusters, only minute amounts of stainable material may occur at some NORs. Thus, in species with numerous rDNA sites, Ag-NORs might be difficult to detect due to minute size, reduced staining intensity and proximity to darkly staining centromeres.

*Distribution of Ag-NORs on Chromosomes*

In their report on location of rDNA in mammalian genomes, Hsu *et al.* (1975) show that karyotypes can be classified according to patterns of distribution of rDNA along chromosomes. Our data support this classification and extend the *in situ* work by revealing more precisely the location of the NORs. The Ag-AS technique shows a single dark area on the chromatid rather than a cluster of grains over a chromosome region. This precision is appreciated where NORs are terminal or near centromeres. Thus, we show that the Chinese hamster has telomeric NORs, the mouse subcentromeric NORs, and the field vole NORs as minute short arms or satellites. The Ag-AS technique can now be extended to study NORs in other mammals for which *in situ* data are not yet available. Using Ag-AS we have found Ag-NORs at both telomeres and secondary constrictions in the cotton rat (Fig. 3d) and exclusively at telomeres in cattle (unpublished data). These data suggest that the ribosomal genes are located at multiple sites in the genomes of the cotton rat and cattle.

*Variation in Ag-NORs in Mammalian Genomes*

We found that the number of Ag-NOR chromosomes per cell varies in a given cell population (Table 1). For example, in the Chinese hamster cell line Don, most cells showed 7 chromosomes with Ag-NORs, while other cells showed from 4 to 10. Although a similar pattern of variation in number of Ag-NORs per cell was found in the female Chinese hamster cell line Dede, the distribution of Ag-NORs in the Dede karyotype was somewhat more restricted than that of the Don line (Figs. 4a, b and 5).

Variation in size of Ag-NOR was also observed. One of the marker chromosomes of the squirrel monkey, *Saimiri sciureus*, consistantly shows a small area of Ag staining while its homologue is extensively stained (unpublished data). In the cotton rat, the large Ag-NOR of one homologue of chromosome 9 and the complete absence of Ag staining of the other homologue is particularly striking (Fig. 3d). Since the relative sizes of Ag-NORs in the Chinese hamster and Indian muntjac appear to correspond to grain cluster sizes on *in situ* autoradiographs, differences in Ag-NOR staining may reflect the amounts of rDNA present at the NORs.

In general, our observations with Ag staining support the idea of a characteristic number and pattern of distribution of NORs within a cell line as well as within an individual. The silver data are also in agreement with observations of chromosomal heteromorphism in rDNA (Evans *et al.*, 1974). It is presently unknown whether the variation in Ag-NOR pattern among cells is real or represents variability in the technique. However, the marked differences in Ag-NOR patterns within and between cell lines of the Chinese hamster suggests that there is a real

variation in number, location, or stainability of NOR in these cell lines. Analyses of Ag-NORs in clonal derivatives may throw more light on the problem of variability.

### *Cytochemical Basis for Ag-NOR Staining*

Although it is clear that Ag-AS stains the chromosomal locations of rDNA, silver probably does not stain rDNA itself. Several pieces of indirect evidence point to this conclusion: 1) Our preliminary experiments using DNase treatments followed by Ag-AS did not eliminate the characteristic staining of Ag-NORs in our preparations. 2) Brief pretreatments with trypsin or pronase eliminated Ag-NOR staining. 3) Unlike grain cluster sizes on *in situ* autoradiographs, the nucleoli of interphase cells showed much heavier silver staining than the metaphase chromosomes. If Ag-AS indeed stains rDNA, the amount of silver stained material in interphase and in metaphase should be more or less equal unless an amplification process is operative in interphase nuclei of mammalian cells. 4) Since ribosomal genes usually contain GC-rich sequences (Sinclair and Brown, 1971), other GC-rich areas of chromosomes should demonstrate heavy staining with Ag-AS. However, DNA sequences with high GC-content, such as those in the centromeric areas of cattle chromosomes (Krunit *et al.*, 1973), showed no Ag staining. Thus, staining specificity with Ag-AS is probably not related to base composition.

Speculations have been made by other investigators that ammoniacal silver (A-S) stains nucleoproteins. A variety of cytochemical procedures yielded results compatible with the idea that A-S stains histones (Black and Ansley, 1964; Black *et al.*, 1960; Black and Ansley, 1966; Utsumi and Takehisa, 1974). Howell *et al.* (1975) have used an A-S technique involving a series of treatments with silver solutions to stain the chromosomal satellite (SAT) regions of human D and G group chromosomes, and present evidence that silver stains acid proteins associated with the SATs. Similarly, it was suggested that differentiation of the NOR with Giemsa (N-banding) involves staining of acid or residual proteins (Matusi and Sasaki, 1973; Matsui, 1974).

The various ammoniacal silver procedures, including the present modification (Ag-AS), seem to stain chromosomal proteins rather than nucleic acids. Further studies are required to determine the nature of NOR specific proteins (if they exist) and how they might be bound to the ribosomal cistrons.

### *Action of Staining Solutions Used in the Ag-AS Technique*

A probable basis for the reaction of silver solutions with nucleolar material was discussed by Das (1961), who suggested that submicroscopic silver grains or nuclei impregnate the nucleolus selectively and that additional silver is then deposited at sites where silver nuclei are present due to the action of a physical developer. In a similar manner, silver may impregnate the NOR selectively during pretreatment of chromosomes with aqueous silver nitrate solution. After the formalin developer is applied additional silver is apparently deposited at the sites where silver is present and the characteristic black color of the NOR is formed.

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