NUCLEOLUS ORGANIZERS IN *MUS MUSCULUS* SUBSPECIES AND IN THE RAG MOUSE CELL LINE[1]

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ABSTRACT

Silver staining has been used to detect active nucleolus organizer regions (NOR's). By this criterion six mouse chromosomes, numbers *12*, *15*, *16*, *17*, *18* and *19*, can have an NOR. The number and distribution of chromosomes with NOR's vary among inbred strains of *Mus musculus musculus* (C57BL/6J, BALB/cJ, C3H/HeJ and C3H/StCr1BR) and in *M. musculus molossinus*. In a *musculus* × *molossinus* F_1 hybrid, nucleolus organizers from each parent are silver stained.—Chromosomes which have NOR's in diploid cells also show them in tetraploid cells and in established cell lines. The BALB/cJ strain shows Ag-staining of NOR's on chromosomes *12*, *15*, *18* and occasionally *16*. In the RAG cell line, which was derived from BALB/c, active NOR's are seen on *12*, *15* and *18*, even after these chromosomes have undergone structural rearrangements in the cell line. Some correlation exists between the amount of Ag-stain and the size of a secondary construction region, with a large amount of Ag-stain present on a chromosome which has a prominent secondary constriction. There is no correlation between the amount of Ag-stain and the presence or absence of C-band material.

THE banding patterns along the length of the chromosomes of various species Mus (including *M. musculus musculus*, *M. musculus molossinus* and *M. cervicolor*) are so similar that a single mouse karyotype can be prepared. However, differences in the appearance of the centromeric region of certain chromosomes make it possible to distinguish the chromosome complements of these mice, as well as those of various inbred strains of the laboratory mouse. These differences include the brightness of quinacrine staining, the size of the C-band, the distribution of chromosomes which have a secondary constriction distal to the C-band region, and the size of the secondary constriction (DEV *et al.* 1971, 1973, 1975; DEV, MILLER and MILLER 1973; FOREJT 1973; and MILLER *et al.* 1976b). The secondary constriction is, in many species, the site of the DNA coding for 18S and 28S ribosomal RNA (rRNA) (HSU, SPIRITO and PARDUE 1975), suggesting that the distribution of chromosomes which carry rDNA may also vary among inbred strains of mice. This appears to be the case. HENDERSON *et al.* (1974), using *in situ* hybridization with I^{125}-labeled rRNA, showed in a mouse heterozygous for

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three translocations that rDNA was located in the secondary constriction regions of chromosomes *15* and *19* and on a chromosome tentatively identified as *18*. ELSEVIER and RUDDLE (1975) studied the SEC/1ReJ mouse and demonstrated that in this inbred strain there are rDNA sites on chromosomes *12*, *16* and *18*.

The nucleolus organizer regions (NOR's), which are the sites of rRNA synthesis and processing, have been visualized in a variety of species by staining metaphase chromosomes with ammoniacal silver by the Ag-AS method (GOODPASTURE and BLOOM 1975; TANTRAVAHI *et al.* 1976). GOODPASTURE and BLOOM reported that in what they refer to as the Swiss strain of the laboratory mouse four to five chromosomes are stained by the Ag-AS method. We have now used this method to locate the NOR's in chromosomes from *M. musculus musculus* and *M. musculus molossinus* and from the RAG cell line.

MATERIALS AND METHODS

Pregnant mice of the C57BL/6J, C3H/HeJ and BALB/cJ stains were purchased from The Jackson Laboratory. A pregnant C3H/StCr1BR mouse was purchased from Charles River Laboratories. Twelve–fourteen day mixed embryo cultures were set up and metaphase chromosome preparations made as described previously (MILLER *et al.* 1971). Preparation of the *M. musculus molossinus* chromosomes has been reported previously (DEV *et al.* 1975); most of these preparations were derived from adult lung cultures. All slides were stored at 4° until use, *i.e.*, for periods of up to three years.

The slides were first stained with quinacrine and then by either the C-banding method of DEV *et al.* (1972) or the Ag-AS method of GOODPASTURE and BLOOM (1975). In addition to staining the nucleolus organizer regions, the Ag-AS method stains the C-band regions of mouse chromosomes. To minimize the C-banding in most cases, a modification of the Ag-AS method was used in which the slides were incubated with 50% silver nitrate solution for 6–18 hours at 50° and developed in 3% formalin, pH 4.5. The omission of ammoniacal silver in this modification considerably reduces the amount of C-banding, and permits the NOR's to be scored more reliably. The staining obtained by either the Ag-AS method or its modification will be referred to as Ag-staining.

Quinacrine-stained slides were viewed with a fluorescence microscope, and well spread metaphases were photographed on H & W control film, which was developed in D-19 developer for 6 minutes at 20°. Prints were made on Ilford paper, grade 3 or 4. C-banded or Ag-stained metaphases were relocated and photographed on high contrast copy film, which was developed in Acufine for 5½ minutes at 20° and printed on Ilford paper, grade 2.

Double karyotypes (1–5) were prepared for each animal by simultaneously cutting out the corresponding chromosomes from prints of an Ag-stained cell to locate the NOR's and prints of the same cell stained with quinacrine to identify each chromosome. Additional cells were scored by marking on a print each chromosome which had an Ag-stained NOR and then identifying the chromosome on a print of the same cell stained with quinacrine.

RESULTS

The distribution of chromosomes which have an Ag-stained NOR is different in each of the three inbred strains of mice (Table 1). In the C57BL/6J strain, pairs, *12*, *15*, *18* and *19* are stained (Figure 1), in the BALB/cJ strain pairs *12*, *15* and *18*, and occasionally *16*, and in the C3H/HeJ and C3H/StCr1BR strains pairs *15*, *16* and *18* (Figure 2). Mouse cultures develop tetraploid cells rather rapidly; in these cells approximately twice as many copies of NOR's are stained as in diploid cells (Figure 2 and Table 2).

TABLE 1

Distribution of chromosomes which have an NOR stained by Ag-AS

Mouse strain	Chromosomes stained by Ag-AS					
	12	*15*	*16*	*17*	*18*	*19*
C57BL/6J	+	+			+	+
BALB/cJ	+	+	+*		+	
C3H/HeJ		+	+		+	
C3H/StCr1BR		+	+		+	
MMH-2	+		+	+	+	+
MLV			+	+	+†	+

* In only a few cells.
† Only one member of the pair.

The size of the Ag-stained region also varies among strains. For example, in C57BL/6J it is largest on pairs *12* and *19* (Figure 1), whereas in C3H/StCr1BR it is largest on pair *15* (Figure 2). In another C3H strain, C3H/HeJ, pair *15* has about the same amount of stain as pairs *12* and *18*. Chromosomes with smaller

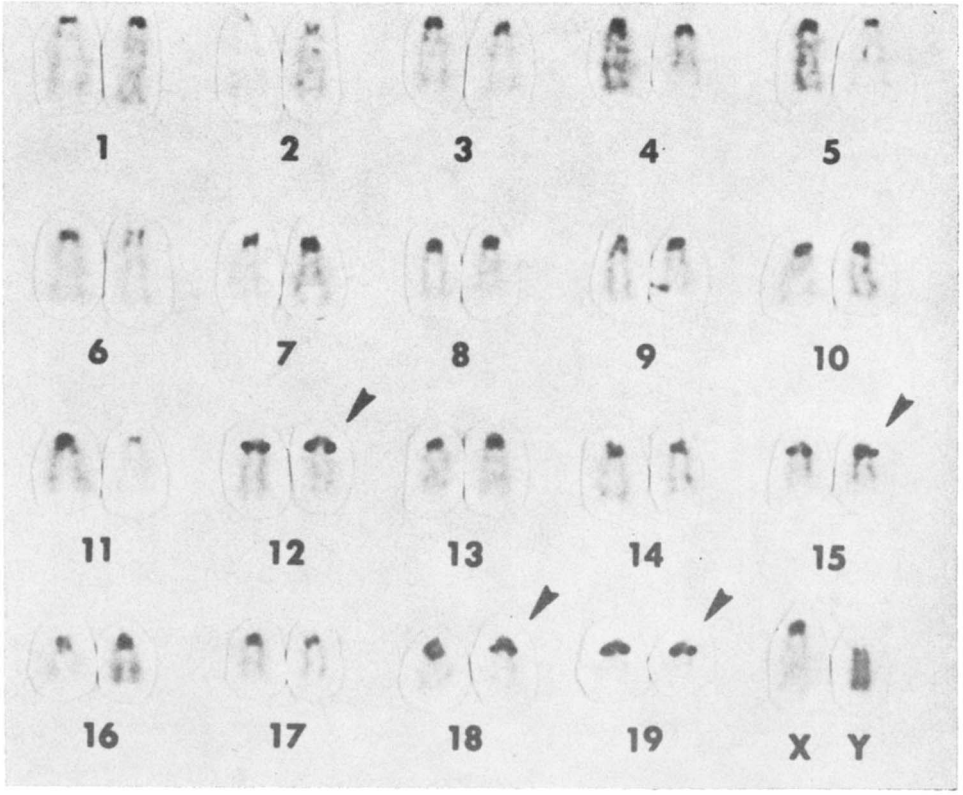

FIGURE 1.—Karyotype of a cell from a strain C57BL/6J male mouse showing Ag-stain of numbers *12*, *15*, *18* and *19*. The chromosomes have been identified by quinacrine staining of the same cell (not shown).

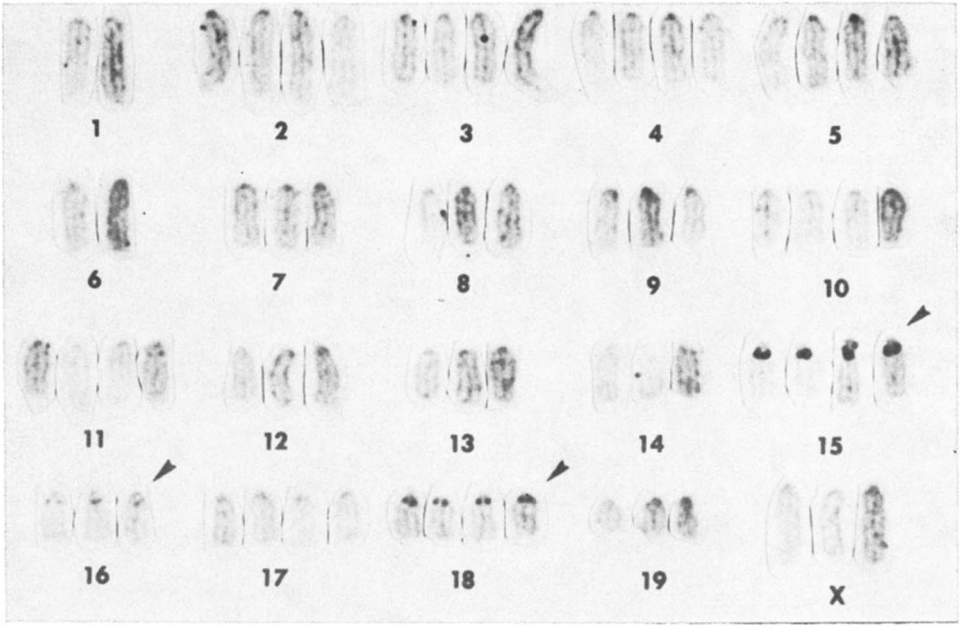

FIGURE 2.—Karyotype of a near-tetraploid cell from a strain C3H/StCr1BR female mouse embryo showing Ag-stain of numbers *15*, *16* and *18*. The chromosomes have been identified by quinacrine staining (not shown).

Ag-stained regions are not always detected, with the result that often only one member of a pair is stained, even in cells from animals known to be homozygous. In the diploid BALB/c cells, for example, using the Ag-AS method as described by GOODPASTURE and BLOOM (1975), only about one copy per cell of numbers *12* or *18* is found to be stained (Table 2). Using the modified procedure, in which the C-banding does not obscure the NOR's, a much greater percentage of the NOR's is detected.

The *M. musculus molossinus* mice used in this study are not completely inbred. Mouse MMH-2 has Ag-stained material on numbers *12*, *16*, *17*, *18* and *19*, with about the same amount on both members of each pair (Figure 3a). Mouse MLV has Ag-stained material on numbers *16*, *17*, *18* and *19* (Figure 3b). The absence of stain from one *18* and the different amounts on pair *19* are indications of persistent heterozygosity (Figure 3b).

TABLE 2

*Number of BALB/cJ chromosomes which have an NOR stained by Ag-AS**

Cell type	No. of cells	Mean number of copies per cell of chromosome *12*	*15*	*16*	*18*
Diploid	17	0.8	1.9	0.3	1.2
Tetraploid	4	1.0	3.8	0	2.2

* Ag-AS method described by GOODPASTURE and BLOOM (1975). Using the modified method, a mean of six chromosomes per diploid cell were stained.

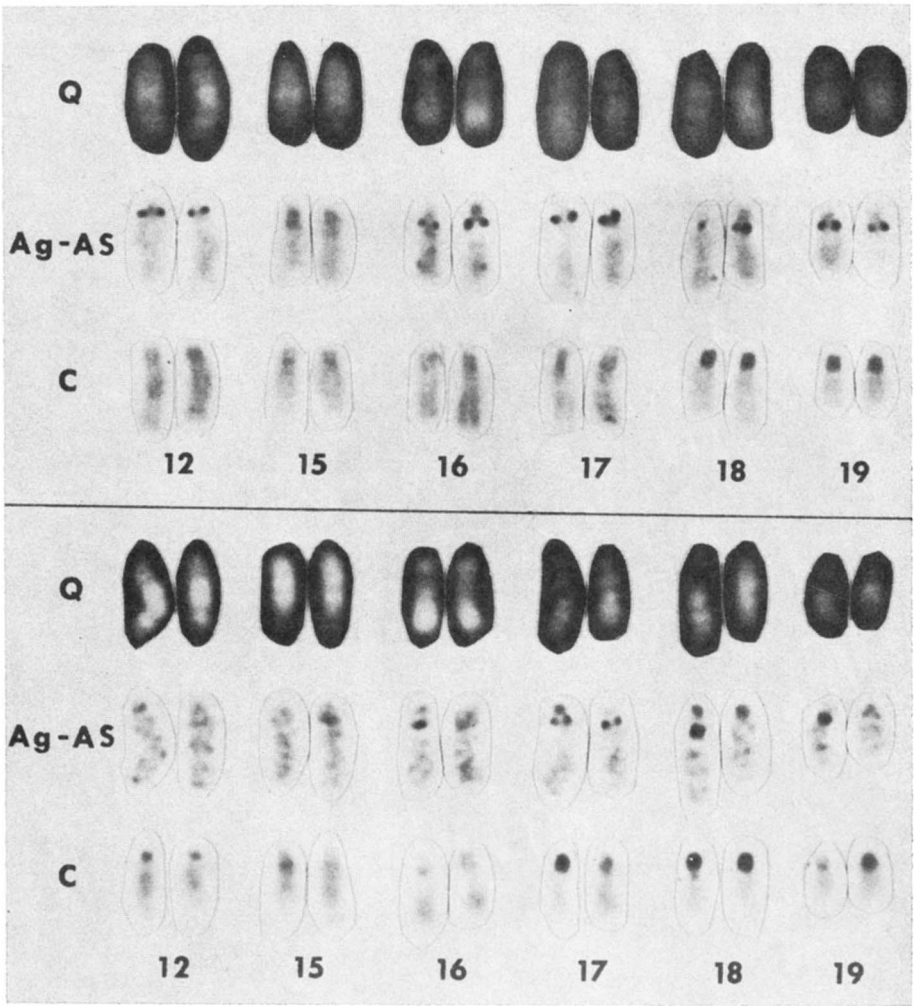

FIGURE 3.—(a, top) Partial karyotypes showing chromosomes from the MMH-2 *molossinus* mouse stained to show quinacrine (Q), Ag-AS and centromeric heterochromatin (C) band patterns. Q and Ag-AS were done sequentially on the same cell. Q (not shown) and C were done sequentially on a different cell. Homologues which could be distinguished by the appearance of the secondary constriction are placed in the same relative position within each pair.

(b, bottom) Partial karyotypes showing chromosomes from the MLV *molossinus* mouse stained to show Q, Ag-AS and C-band patterns, as described in (a).

Differences in the distribution of Ag-stained chromosomes can be used to show that NOR's from both subspecies are active in a *musculus* × *molossinus* F_1 hybrid. Chromosome *15* is Ag-stained in C57BL/6J but not in MMH-2, and chromosomes *16* and *17* are stained in MMH-2 but not in C57BL/6J. Copies of chromosomes *15*, *16* and*17*, as well as numbers *12*, *18* and *19*, have Ag-stain in the few cells which were available from an F_1 hybrid and from a transformed line derived from it.

The size of the secondary constriction can be related to some extent to the size of the Ag-stained region. Chromosomes which have a very prominent secondary constriction, such as number *15* in C3H/StCr1BR (Figure 2) and number *18* in MLV (Figure 3b), have a large amount of Ag-stain. Chromosomes which have no visible secondary constriction, such as number *15* in MMH-2 and MLV, have no Ag-stain (Figure 3a,b). Between these extremes is difficult to correlate the size of the secondary constriction and the amount of Ag-stain.

The Ag-stained NOR of mouse chromosomes is always located a short distance from the centromere, very close to the C-band region. Ag-staining is not related to the presence or absence of material which is stained by C-banding, as shown by comparison of chromosomes in *molossinus*. In the MMH-2 mouse, three pairs of chromosomes that are Ag-stained (*12*, *16* and *17*) have little, if any, C-band but the other two pairs which are Ag-stained (*18* and *19*) have a large C-band (Figure 3a). MLV shows even greater diversity. Both chromosomes *18* have C-bands, but only one has an Ag-stained region. One member each of pairs *17* and *19* has a C-band and one does not, but both members of each pair are Ag-stained, though to a different extent in the case of *19*. Finally, neither number *16* has a C-band, but each has an Ag-stained region (only one of which appears in Figure 3b).

Chromosomes that have nucleolus organizers in diploid cells, as indicated by Ag-staining, carry out the same function in cell lines derived from them. For example, in BALB/cJ chromosomes *12*, *15* and *18* are usually stained (and number *16* only occasionally). In the RAG cell line, which was derived from BALB/c, and in a RAG × human hybrid the same chromosomes (mouse numbers *12*, *15* and *18*) are stained (Figure 4). Normal copies of *18* and sometimes normal copies

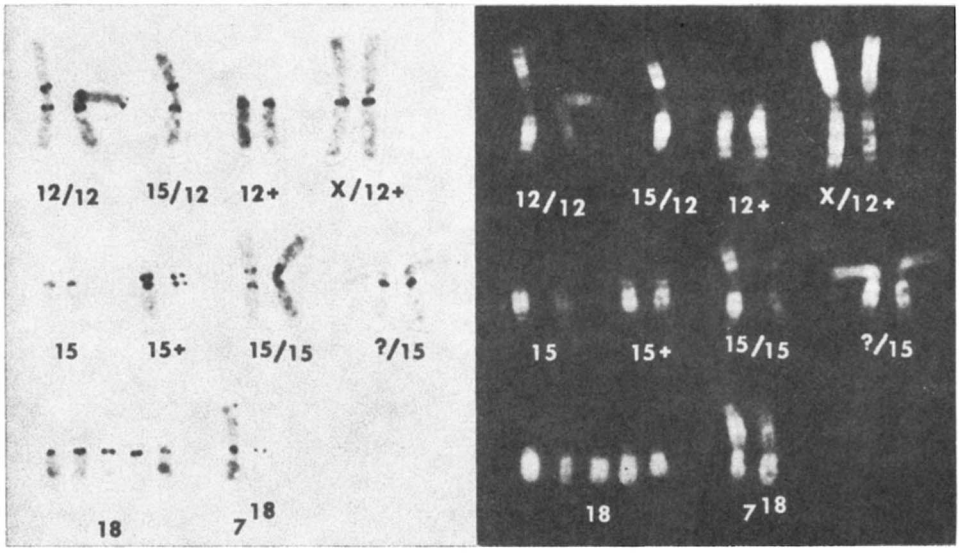

FIGURE 4.—Partial karyotype of a RAG × human somatic hybrid cell; (a, left) all the chromosomes which show Ag-AS staining, (b, right) the quinacrine patterns of these chromosomes. All of them are derived from mouse numbers *12*, *15* and *18*.

of *12* and *15* are found, but more often these chromosomes are present in a variety of structurally altered forms. These include centric fusion of various kinds, addition of material to the distal end of *12*, insertion of material into a number *12* to form an abnormal chromosome, which has undergone centric fusion with an *X*, and translocation of the centromeric end of *18* to the distal end of *7* (Figure 4).

DISCUSSION

Ag-staining has shown that an NOR can be present on six different mouse chromosomes: *12*, *15*, *16*, *17*, *18* and *19*. The distribution is different in various mouse strains, making this a potentially useful method for distinguishing between strains, as well as between certain homologous chromosomes in F_1 hybrids or in intraspecific somatic cell hybrids. Mice of the various strains examined have three, four or five pairs of chromosomes with Ag-stained NOR's. This is a greater number than might have been expected from the maximum of six nucleoli found in diploid cells from a laboratory mouse (SHEA and LEBLOND 1966). On the other hand, the number of chromosome pairs with secondary constrictions can be greater than three (BENNETT 1965; MILLER 1976b) and secondary constrictions are the sites of the 18S and 28S ribosomal RNA (rDNA) (HSU, SPIRITO and PARDUE 1975).

In the present study, the presence of a prominent secondary constriction is an indication of a large amount of material which can be Ag-stained, but as the size of the secondary constriction decreases, so too does its reliability as an indicator of the presence of Ag-staining material. This can be seen by comparing the results obtained using quinacrine to detect secondary constrictions with the results obtained by Ag-staining. In the C57BL/6J strain, no chromosome has a prominent secondary constriction, although in some cells chromosomes *12*, *16* and more often *19* have a small secondary constriction (MILLER *et al.* 1976b). In this strain, Ag-stained NOR's are present on numbers *12*, *15*, *18* and *19*. In the BALB/cJ strain a prominent constriction is found on chromosome *15* and less prominent constrictions on numbers *12*, *18* and *19* (MILLER *et al.* 1976b); Ag-stained NOR's are found on numbers *12*, *15*, *16* and *18*. Thus in many, but not all, cases secondary constrictions are an indication of rDNA sites, but not all rDNA sites are associated with a secondary constriction. ELSEVIER and RUDDLE (1975) reached similar conclusions using *in situ* hybridization in the SEC/1ReJ strain of mouse: rDNA was localized to two chromosomes (*12* and *18*) which had a secondary constriction and to one (*16*) which did not. The most extensive hybridization of radioactively labeled 18S and 28S rRNA was to the larger secondary constriction. HENDERSON *et al.* (1974) found more extensive hybridization of 18S and 28S rRNA to the 15^{14} chromosome of the T6 translocation, which had a prominent secondary constriction, than to the number *15* in the Rb1 translocation, whose constriction was extremely difficult to detect.

In the *in situ* hybridization studies of both HENDERSON *et al.* (1974) and ELSEVIER and RUDDLE (1975), only three pairs of chromosomes per mouse had rDNA. This is probably characteristic of the particular mice examined. However, ELSEVIER and RUDDLE pointed out that some rDNA sites may not be detected by

in situ hybridization because there are too few copies of the rDNA genes present. It is possible that such sites might be detected by Ag-staining.

Five of the chromosomes that have Ag-stained NOR's have been shown by *in situ* hybridization to have genes coding for 18S and 28S rRNA: numbers *12*, *15*, *16*, *18* and *19* (HENDERSON *et al.* 1974; ELSEVIER and RUDDLE 1975). ATWOOD *et al.* (1976) did not find any rDNA on chromosome *17* in mice of the HTG/Go strain or its outcross progeny with mice heterozygous for the T/t^{12} locus However, finding rDNA on number *17* is not unexpected because the appearance of the secondary constriction region of this chromosome, as well as those of the other five chromosomes, has been found to vary from strain to strain (DEV *et al.* 1971, 1175). The list may be expanded even further when additional strains of mice are studied.

It has been possible to equate the presence of Ag-staining of an NOR with the presence of rRNA genes (GOODPASTURE and BLOOM 1975; HSU, SPIRITO and PARDUE 1975). However, because Ag-staining is based on a reaction with protein rather than with DNA (GOODPASTURE and BLOOM 1975), the absence of Ag-stain could indicate either that rRNA genes are not present or that they are inactive. Study of somatic cell hybrids has provided evidence that not all NOR's are active and that inactive NOR's are not stained by silver-staining methods. In RAG-human hybrids that lost some or most of the human chromosomes, mouse NOR's were stained but human NOR's were unstained (MILLER *et al.* 1976a). In such hybrids only mouse 28S rRNA was produced (ELICEIRI and GREEN 1969; MARSHALL, HANDMAKER and BRAMWELL 1975). Conversely, in BALB/c-human hybrids that lost some or most of the mouse chromosomes, only human NOR's were stained and mouse NOR's were unstained (MILLER *et al.* 1976c). In these hybrids only human 28S rRNA was produced (CROCE *et al.* 1977). That is, in mouse-human somatic cell hybrids, there was species-specific regulation of 28S rRNA synthesis and only active NOR's were Ag-stained.

Regulation of rRNA function has also been observed in hybrid animals. In interspecific hybrids of *Xenopus laevis* × *X. mulleri*, suppression of *mulleri* NOR activity (CASSIDY and BLACKLER 1974) and suppression of production of *mulleri* 28S rRNA (HONJO and REEDER 1973) was observed. In F_1 hybrids of the more closely related *musculus* subspecies studied here, suppression of these functions does not occur, as shown by the fact that chromosomes of both parental types are Ag-stained.

The present studies provide no evidence of regulation of NOR's in either tetraploid cells or in cells from established lines. The same chromosomes are Ag-stained to the same relative extent in tetraploid as in diploid cells. In addition, chromosomes which have Ag-stained NOR's in diploid cells also have Ag-stained NOR's in cell lines, even if the chromosomes have undergone structural changes. In the A9 cell line, as in the C3H mouse strain from which it was derived, chromosomes *15*, *16* and *18* have Ag-stained NOR's (MILLER *et al.* 1976a), even though these chromosomes are present in centric fusion form in the A9 cells. In RAG cells, and in cells of the parental BALB/c strain, the NOR of every recognizable copy of numbers *12*, *15* and *18* is Ag-stained and the relative amount of

stain is constant from cell to cell for each kind of normal or altered chromosome. In BALB/c, an occasional number *16*, as well as *12*, *15* and *18*, has an Ag-stained NOR (CROCE *et al.* 1977).

There is some evidence that changes can take place in the amount of Ag-stain on a particular chromosome. The number *15* chromosome in C3H/StCr1BR consistently has a very large block of Ag-stained material, whereas the *15*'s in C3H/HeJ and in A9 do not, even though all these chromosomes presumably had the same origin. Similarly, the normal *15* in the RAG cell line has a noticeably smaller amount of Ag-stained material than do the *15*'s in some of the abnormal chromosomes, though again these presumably had the same origin.

We do not know if the differences in size and distribution of NOR's seen among inbred strains of mice represent differences in the number of copies of rRNA genes or regulation of gene expression. A comparison of the results of *in situ* hybridization of rRNA with that of Ag-staining of NOR's on metaphase spreads in which individual chromosomes have been identified might resolve this problem and thus provide information on the extent to which there is regulation of rRNA genes in diploid cells.

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