The Ribosomal RNA Gene Cluster in Aneuploid Chickens: Evidence for Increased Gene Dosage and Regulation of Gene Expression

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ABSTRACT   In the chicken, the nucleolus organizer regions, or sites of the genes encoding 18S, 5.8S, and 28S ribosomal RNA (rRNA), map to one pair of microchromosomes that can be identified by silver nitrate cytochemistry. This nucleolar organizer chromosome also contains the major histocompatibility complex. Chickens aneuploid for this chromosome have been identified and reproduced for over seven generations. Crossing two trisomic parents results in the production of viable disomic, trisomic, and tetrasomic progeny, showing two, three, and four nucleoli and nucleolar organizers per cell, respectively. A molecular analysis of rRNA genes was undertaken to establish the gene copy numbers in the aneuploid genotypes, and to determine if elevated numbers of rRNA genes are stably maintained and inherited over multiple generations. Gene copy numbers were determined using hybridization analysis of erythrocyte DNA obtained from individuals comprising a family which segregated disomic, trisomic, and tetrasomic genotypes. The values obtained were 290, 420, and 570 rDNA repeats per cell for disomic, trisomic, and tetrasomic animals, respectively. These results provide molecular confirmation of the two aneuploid states and show that elevated gene copy numbers have been maintained over multiple generations. Fibroblasts derived from disomic and tetrasomic embryos were found to grow at similar rates in culture, and mature rRNA levels in chicken embryo fibroblasts from disomic, trisomic and tetrasomic embryos were also found to have similar levels of mature rRNA. Therefore, despite the increase in rDNA content, the level of rRNA is regulated to diploid amounts in aneuploid fibroblasts.

In higher eukaryotic organisms, the genes encoding 18S, 5.8S, and 28S ribosomal RNAs (rRNAs)[1] are tandemly repeated up to several hundred times at specific chromosomal locations known as nucleolus organizer regions (NORs). Each NOR directs the formation of a single nucleolus, the site of synthesis of the large and small ribosomal subunits. The extensive reiteration of rRNA genes within the NOR allows for the synthesis of the large amounts of rRNA required for ribosome biogenesis. Ribosomal RNA synthesis and ribosome production are closely coordinated events in eukaryotic and procaryotic cells (10, 25). Together they are related directly to cell

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[1] *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; MHC, major histocompatability complex; NOR, nucleolus organizer region; rDNA/rRNA, ribosomal DNA/RNA; SSC, 0.01 M sodium citrate + 0.15 M NaCl.

growth rate and are modulated in response to a variety of environmental and nutritional factors. In *Escherichia coli*, regulation of ribosome production occurs primarily at the level of rRNA transcription: the amount of rRNA present in the cell determines the amount of ribosomal protein that is translated (9, 25). Exactly how regulation of ribosome production is achieved in eukaryotic cells is unclear, although regulation of initiation of rRNA transcription has been inferred to be involved under nutritional shift-down conditions in cell cultures (13, 14), and regulation of translation of ribosomal proteins has been demonstrated in yeast, *Xenopus* oocytes, and mouse 3T6 cells (11, 26, 28).

Mutants possessing alterations in rRNA gene copy number have been described in several organisms including *E. coli, Neurospora,* and *Drosophila.* In all of these cases regulation

leads to normal levels of rRNA when additional rRNA genes are present.

Transcriptionally active NORs can be identified in metaphase chromosomes by silver nitrate staining (12, 23). In general, the chromosomal assignments of NORs (as well as the redundancy of rDNA repeats within an NOR) are similar for all individuals of a species, but vary among different species. Most mammals, including the mouse and humans, contain several NORs per haploid genome (19). The domestic chicken is a simpler system in that it has only one NOR per haploid genome, which is located on a microchromosome, and is linked to the major histocompatability complex (MHC) (3, 4).

Because in higher eukaryotes rRNA genes typically occur in clusters dispersed over more than one chromosome, and because the presence of extra genetic material in cells most often has detrimental consequences, no model system for studying rRNA gene dosage in higher vertebrate organisms has been described. Chromosomal trisomies in mice are lethal in the embryonic period (42). Mice that are chimeric for normal and trisomic cells have been made (6, 8), but must be experimentally produced for each study, cannot be generated in large numbers, and have variable numbers of trisomic cells in tissues. In addition, only a small proportional increase in rRNA gene dosage is obtained in trisomic mouse cells.

Here we describe a line of domestic chicken that is trisomic for the chromosome bearing the rRNA genes. An aneuploid series of progeny which are disomic, trisomic, or tetrasomic for the nucleolar organizer chromosome is obtained in an F_1 generation by crossing two trisomic parents. All three genotypes survive the embryonic period, and most individuals can be reared to adults. Since there is only one nucleolar organizer chromosome per haploid chicken genome, cells from disomic, trisomic, and tetrasomic chickens show two, three, and four NORs and nucleoli, respectively (20).

The presence of additional NORs and nucleoli cannot be taken as direct evidence for the presence of more than diploid amounts of rRNA genes in cells. In other organisms reduction of gene copy number can occur over multiple generations. We have analyzed, by molecular hybridization, the rDNA level in this line of chickens. We show that the increase in rRNA gene copy number directly correlates with the number of NORs, and that additional rRNA genes are stably maintained and inherited over multiple generations. Fibroblasts from disomic and tetrasomic embryos grow at similar rates in cell culture, and fibroblasts from all three genotypes contain the same levels of mature rRNA.

MATERIALS AND METHODS

Experimental Chickens: The chickens used in this work were obtained from the Trisomic line maintained in the Department of Poultry and Avian Sciences, Cornell University. Blood samples for DNA were obtained from an F_1 generation of chickens produced from a trisomic male × trisomic female cross. The parents used in this cross were proven breeders. Their genotypes were established by classification of nucleoli from feather pulp cells, blood typing for MHC haplotypes and by progeny testing. Random segregation of this chromosome was previously demonstrated using the latter two methods. All three genotypes (disomic, trisomic, and tetrasomic) were represented in the F_1. Genotypes of the F_1 experimental chickens were established by classification of nucleoli at 1 wk and verified later at 20 wk before bleeding. Acridine orange cytochemistry was used to determine the genotypes of parental and F_1 chickens.

Cytochemistry to Detect Nucleoli: The genotypes of the chickens used were established by determining the maximum number of nucleoli present in somatic cells. This was done nondestructively by plucking developing pin feathers (which are rich in pulp tissue) from the body of growing or mature

chickens. Nucleoli are very prominent in this growing tissue. Feather pulp cells were prepared for cytological analysis as follows. Pulp tissue was squeezed from the shaft of freshly plucked pin feathers, washed in Hanks' balanced salt solution for about 1 h, and then fixed (and softened) in 50% acetic acid for not more than 24 h. Small pieces of the fixed tissue were dissected apart in a well slide containing 50% acetic acid. A cell suspension was produced by teasing the tissue apart with steel needles and repeated pipetting into a microhematocrit tube. The cell suspension was air dried on a slide heated to 45–48°C. For diagnosis of number and morphology of nucleoli, slides were stained in 0.01% acridine orange and examined by fluorescence microscopy (29). Low pH McIlvaine's buffer (0.1 M citric acid, 0.2 M disodium phosphate, pH 4.5) was used to reduce the rate of fading under UV exposure without altering the 4-way color differentiation. Several cells were analyzed for the determination of the maximum number of nucleoli, since two or more nucleoli will often be fused in a single nucleus.

Silver Nitrate Cytochemistry: A modification of the silver nitrate staining procedure of Goodpasture and Bloom (12) was used to detect the presence of actively transcribing rRNA genes in nucleoli and in chromosome preparations. In the latter, the precise location of NORs was also achieved. The modifications were as follows. Before staining, slide preparations were stored in a dry oven at 40°C for 1–2 d. Slides were then treated in 50% silver nitrate prepared in Walpole's buffer (0.1% sodium acetate) at pH 6.0 for 15–20 min at 60°C. After washing off the coverslip, the slides were then developed in 4 drops of 3% formalin (pH 4.5) plus 4 drops of ammoniacal silver solution. Development under a coverslip was monitored using a phase-contrast/bright field system using a Heine condensor. The development times varied from 15 s to 15 min and development was terminated by a rinse in distilled water. After several water rinses, the slides were dehydrated in an ethanol series and then dried. Slides were observed unmounted to avoid any further changes in staining. Cells were observed with a Leitz Ortholux II microscope system equipped with an Orthomat automatic camera. Cells were photographed under 100x oil immersion using Kodak High-Contrast Copy film. Color slides were taken with Ektachrome slide film (ASA setting at 16) and were later printed using a Cibachrome processing kit.

Isolation of Nuclei and DNA: Chickens were bled through the wing vein into a 5-ml syringe containing 0.5 ml of 0.07 M sodium citrate and 0.07 M NaCl (pH 7.5). The blood was mixed with an additional 2–3 vol of the same solution. (This and all subsequent steps were performed at 4°C or on ice.) Cells were collected by centrifugation at 1,000 g for 5 min, and washed a second time in the citrate solution. Cells were then lysed by resuspending and vortexing the pellet in 2–3 vol (relative to the initial volume of blood) of 0.1% Triton X-100, 10 mM Tris-Cl (pH 7.5), and 0.1 mM MgCl. Nuclei were pelleted at 1,000 g for 10 min. This was repeated several times until a white nuclear pellet, which was visibly free of hemoglobin, was obtained.

Nuclei were lysed by the addition of 3 vol of 0.2 M NaCl, 10 mM EDTA, 10 mM Tris-Cl (pH 7.8), 2% SDS. Proteinase K (0.5 mg/ml) was added and the lysate was continuously mixed at 37°C for at least 1 h, then extracted three times with water-saturated phenol and precipitated with ethanol. The DNA was dissolved in 10 mM Tris-Cl (pH 7.5), 1 mM EDTA (TE buffer) and dialyzed against 10 vol of the same buffer with a minimum of six changes of buffer over several days at 4°C. After dialysis, the DNA was treated with RNase A (10 μg/ml) at 37°C for 1 h, extracted with phenol, precipitated with ethanol, and dialyzed as above. DNA concentration was determined by absorbance at 260 nm (assuming $A_{260} = 1$ for a 50 μg/ml solution), and by the diphenylamine procedure (5). All $A_{260/280}$ ratios were between 1.75 and 1.85, indicating that the DNA preparations were free from protein and RNA.

Hybridization Analysis: The chicken rDNA clone, which contains part of the 18S and 28S coding regions plus all of the internal transcribed spacer and the 5.8S gene in bacteriophage lambda, was provided by A. Skalka (22) and grown in E. coli DP 50 SupF. The probe used in the hybridization was obtained by digesting the recombinant lambda DNA with Eco R1 and isolating the 8-kb rDNA insert, from a 0.8% low melting agarose gel. The fragment was labeled by nick translation using α-^{32}P-labeled deoxyribonucleoside triphosphates (30). The hybridization analysis was performed as follows: 5 or 10 μg of Eco R1-digested cellular DNA was electrophoresed into a 0.8% agarose gel in Tris-borate buffer (0.09 M Tris base, 0.09 M boric acid, 25 mM EDTA). Known amounts of cloned rDNA were also run on each gel as standards. The DNA was transferred to nitrocellulose and hybridized to the labeled probe (39). To obtain a linear standard curve, a minimum of a fivefold excess of probe was required. In addition, we found that an excess of E. coli DNA in the prehybridization and hybridization mix was necessary to minimize nonspecific hybridization. (Use of either salmon sperm or calf thymus DNA resulted in an excessive amount of background, presumably due to homology to the 18S and 28S chicken rDNA probe.) After hybridization the filter was washed successively with 2 ×, 1 ×, and 0.5 × SSC (1 × SSC = 0.015 M sodium citrate, 0.15 M NaCl) at 60°C for a total of 3 h. The DNA fragments were

visualized by fluorography using a Dupont Cronex intensifying screen and Kodak XAR-5 film. The amount of hybridization was quantitated either by densitometry, or by cutting the appropriate regions out of the nitrocellulose filter and determining the amount of ^{32}P by scintillation counting. The amount of rDNA clone hybridized to the genomic DNA sample relative to the standards was determined and rDNA gene copy number was calculated based on a molecular mass for the 8-kb Eco R1 fragment of 5×10^6 daltons and assuming 2.4 pg of DNA per diploid chicken cell (24a; for review see 7).

Cell Culture and Analysis of RNA: Primary cultures of chicken embryo fibroblasts (CEFs) from 9-d embryos were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY; 4,500 mg glucose/liter) supplemented with 10% fetal calf serum, 2% chicken serum, sodium pyruvate (1 mg/ml), glutamine 0.58 mg/ml), penicillin, streptomycin, and Fungizone. Cell cultures were passed every other day until approximately the 10th passage. At that time cell growth slowed and cells were passed every 3 d. To obtain a growth curve, plates (Falcon Labwares, Oxnard, CA, 60 mm) were seeded with 5×10^5 cells and duplicate plates were inoculated at each passage. Cell numbers were determined using a hemocytometer. Doubling times were calculated based on the increase in cell number per plate over the original inoculum.

Relative amounts of rRNA in CEFs were determined by slot blot analysis of formaldehyde-denatured cytoplasmic RNA (41) that hybridized to the lambda-rDNA clone. Briefly, 5×10^6 cells were collected by centrifugation after trypsinization and resuspended in 180 μl of 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, and lysed by the addition of 20 μl of 5% Nonidet P-40. Nuclei were removed by centrifugation for 2.5 min in an Eppendorf centrifuge. The supernatant solution was transferred to a new tube, and SDS was added to a final concentration of 1%. An equal volume of a formaldehyde solution (3 parts 20 × SSC to 2 parts 37% formaldehyde), was then added, and the mixture was heated at 60°C for 15 min. Dilutions of the denatured cytoplasm in 10 × SSC were loaded onto zeta-probe blotting membrane (Bio-Rad Laboratories, Richmond, CA) using an S&S Slot Blot apparatus (Minifold II, Schleicher & Schuell, Inc., Keene, NH). The total sample volume was 300 μl for all dilutions, and triplicate samples were loaded for each dilution. Dilutions in the range of 1:1,000 gave a linear increase in hybridization for various amounts of input rRNA. The filters were baked, prehybridized, hybridized, and washed in the same manner as previously described for gene copy number determination, except that the prehybridization mixture also contained 10% dextran sulfate, and prehybridization time was increased to 12 h. Blots were quantitated by densitometry and by scintillation counting.

RESULTS

Cytochemical Analysis of Aneuploids

The observation of three MHC haplotypes and three nucleoli in an individual chicken led to the discovery of the linkage between the MHC and rDNA in chickens, and to the identification of a trisomy for this chromosome (4). Animals in this trisomic line have since been bred for over eight generations. By crossing two trisomic animals, disomic, trisomic, and tetrasomic offspring are produced in an F_1 generation. We have used two methods to analyze the genotypes of the F_1 progeny from such a cross: (a) determination of the maximum number of nucleoli per cell by phase-contrast microscopy and staining with acridine orange, and (b) silver nitrate staining of metaphase chromosomes to detect nucleolar organizer regions.

The maximum number of nucleoli was determined in feather pulp cells. Since this procedure inflicts no harm on the animal, it is possible to analyze a single animal at different stages of development. Nucleoli were visualized in unstained cells by phase-contrast and dark field microscopy, and were further differentiated by acridine orange staining/fluorescence. The nucleoli appeared bright orange against a background of green of the euchromatin (Fig. 1). Heterochromatin

FIGURE 1   Nuclei from feather pulp cells showing differential fluorescence of nucleoli (orange color) after staining with acridine orange. (a) Disomic nucleus with two nucleoli. (b) Trisomic nucleus with three nucleoli. (c) Tetrasomic nucleus with four nucleoli. × 2,060.
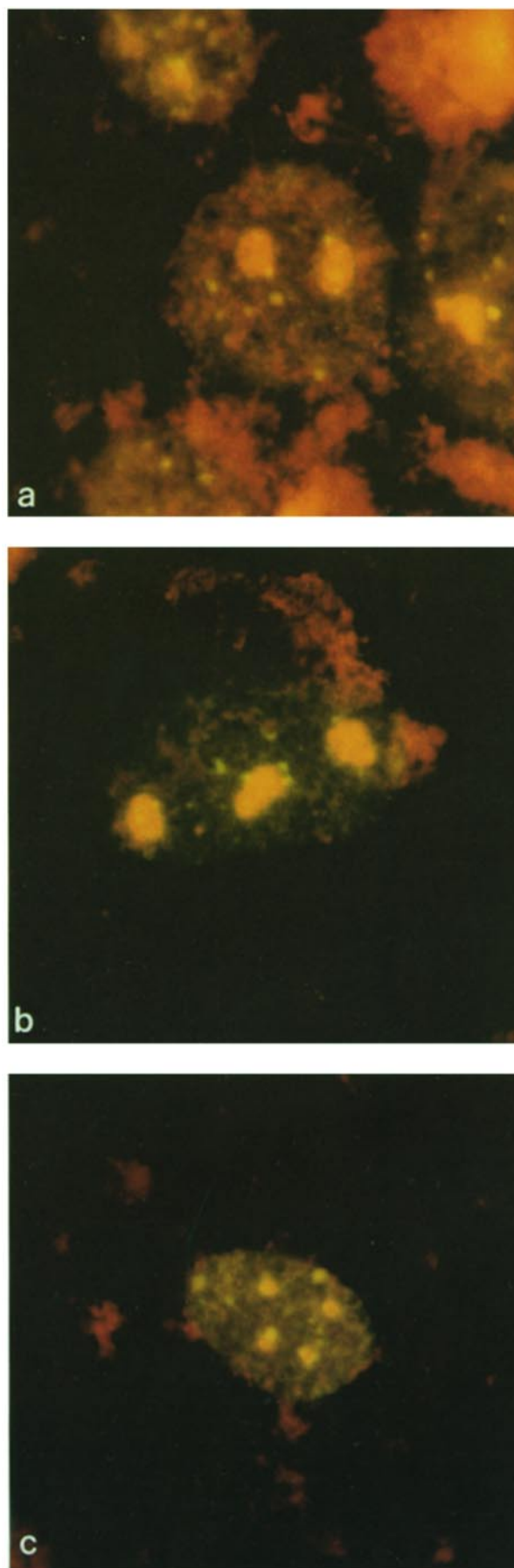
appeared yellow and cytoplasm was red-orange. Acridine orange fluorescence was particularly useful in detecting the multiple nucleoli in trisomic and tetrasomic cells and in cases where nucleoli were small and could not be easily distinguished from larger spots of heterochromatin by phase-contrast microscopy.

Silver nitrate cytochemistry was also used for the analysis of nucleoli. Silver nitrate stains nucleoli very strongly and with a dark brown color. The same maximum number of nucleoli was detected in aneuploid cells with both staining techniques. These results, which were obtained using interphase cells, coincided with data obtained from silver nitrate cytochemistry of chromosomal preparations, namely, that all nucleolar organizer chromosomes in the three genotypes studied were actively transcribing rRNA and enough gene product was generated to be detected at the cytochemical level.

Silver nitrate staining of chromosome preparations from normal chickens revealed two differentially stained microchromosomes that were previously assigned to the size range of pairs 15–18 (Fig. 2a) (3). Three and four NORs were seen in cells from chickens showing three and four nucleoli, respectively (Fig. 2, b and c). The characteristic association of nucleolar organizer chromosomes was also seen in some metaphases from aneuploid individuals. The maximum number of NORs and nucleoli (i.e., 2, 3, and 4 for disomic, trisomic, and tetrasomic cells, respectively) were seen in embryonic cells and feather pulp samples taken from male and female chickens at various ages to adult and in CEF cell cultures.

Molecular Analysis of rDNA

To determine if the number of NORs detected by silver staining corresponds to the amount of rDNA in the cell, and if the additional amounts of rDNA are stably maintained and inherited over several generations, gene copy numbers were determined for individual animals. Genomic DNA was isolated from erythrocytes, digested with the restriction enzyme Eco R1, and subjected to analysis by blotting hybridization (Fig. 3a). This enzyme cuts twice within the rDNA coding region, generating an 8-kb fragment that contains the 3' half of the 18S gene, the internal transcribed spacer and 5.8S gene, and most of the gene for 28S rRNA. This same fragment, which was cloned in bacteriophage lambda by McClements and Skalka (22), was used as the probe in these experiments. A standard curve consisting of varying amounts of the 8-kb fragment was generated to insure that the amount of hybridization was linear over the range of values in the genomic samples. Visual inspection of the fluorograph revealed that the amount of hybridization to equivalent amounts of DNA from trisomic (lane B) and tetrasomic animals (lane C and D) was greater than that for the disomic chicken (lane A). Quantification of this fluorograph by densitometry (Fig. 3b) indicated that the ratio of hybridization among the four samples shown was close to the expected ratio of 1.0:1.5: 2.0:2.0. That is, the relative increase in the amount of rDNA for the trisomic and tetrasomic individuals was 50% and 100%, respectively, over the amount for disomics.

Subsequent hybridizations were quantitated by cutting the appropriate regions from the nitrocellulose filters, counting the amount of ^{32}P, and comparing counts obtained from the bands representing genomic DNA with those for known amounts of rDNA in the standard curve. Gene copy numbers

were determined for animals in a pedigree consisting of a trisomic male and female, and their disomic, trisomic, and tetrasomic progeny. A summary of the number of rDNA repeats per diploid cell for a total of 11 individuals constituting this family is shown in histogram form (Fig. 4). Two additional tetrasomics (numbers 267 and 416) were also included since proportionally fewer animals of this genotype survive. Although there is some variation of gene copy numbers within each genotype, this variation is within 10–15% of the average copy numbers for that group, which is within the experimental error obtained for replicate samples. A gene copy number of 290 ± 30 per cell obtained for the disomic animals is within the range of published values for rDNA content for the chicken (21, 22, 34, 35). The rDNA values clearly fall into three separate categories (Table I) corresponding to the number of NORs present, with the trisomic animals having ~420 rDNA repeats and tetrasomics having ~570 rDNA repeats per diploid cell. The F_1 animals in this pedigree represent the seventh generation of the trisomic line. Similar copy numbers were also found for unrelated animals spanning three generations (data not shown), indicating that no reduction of rDNA content had occurred over successive generations. In addition, copy numbers for a second cell type, the CEF, were also determined (data not shown). Gene copy numbers for the CEFs harvested after the fourth passage in culture were found to be similar to those of the erythrocytes.

Characterization of Fibroblast Cultures

A preliminary characterization of CEFs was performed in order to assess the effects of rDNA gene dosage on cell growth. The growth of disomic and tetrasomic CEFs over 18 d in culture was compared (Fig. 5). At this point, a marked decline in growth rate was observed for both genotypes. This limited lifespan in culture is typical for chick cells, and was consistently observed in this study. No statistical difference was apparent in the growth rate of cells of the two genotypes, using an analysis of variance of the cell doubling times from 2–10 d in culture.

rRNA in Fibroblast Cultures

The relative amount of mature rRNA in fibroblasts from disomic, trisomic, and tetrasomic embryos was determined by slot-blot analysis. For two separate trials (Table II), no consistent increase in the amount of hybridization of the rRNA probe to cytoplasmic extract from equivalent numbers of cells could be detected. Similar amounts of total RNA were also detected when cells were labeled with [^{14}C]thymidine and [^{3}H]uridine, and the amount of ^{3}H incorporated into RNA was normalized to a constant amount of ^{14}C in DNA (data not shown). Thus, the formation of mature rRNA in the cultured CEFs does not appear to reflect the number of rRNA genes in these cells, but rather, is regulated to diploid levels. Whether this regulation results from transcriptional control, increased degradation, or regulation of RNA processing is currently being investigated in more detail.

DISCUSSION

We have shown that the additional rDNA genes in a line of chickens aneuploid for the nucleolar organizer chromosome are stably maintained and inherited over several generations. In addition, all of the NORs are transcriptionally active in
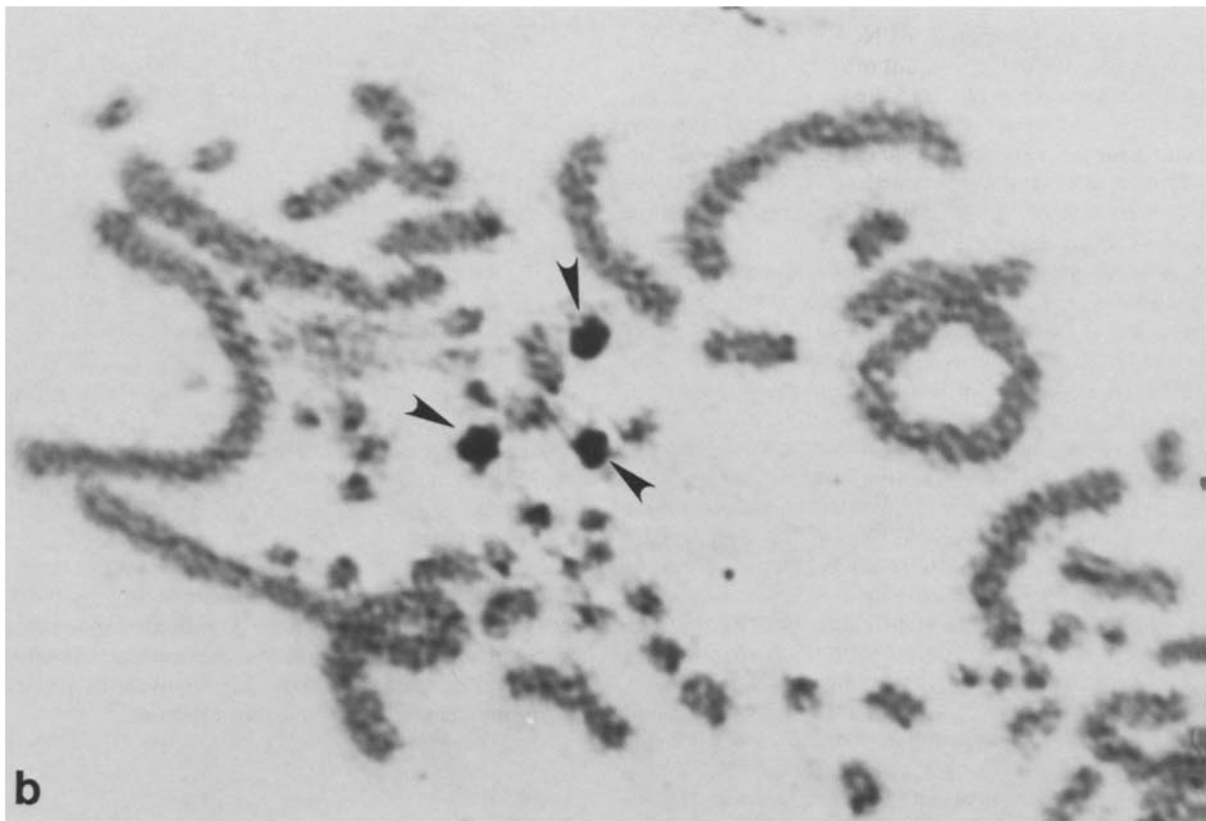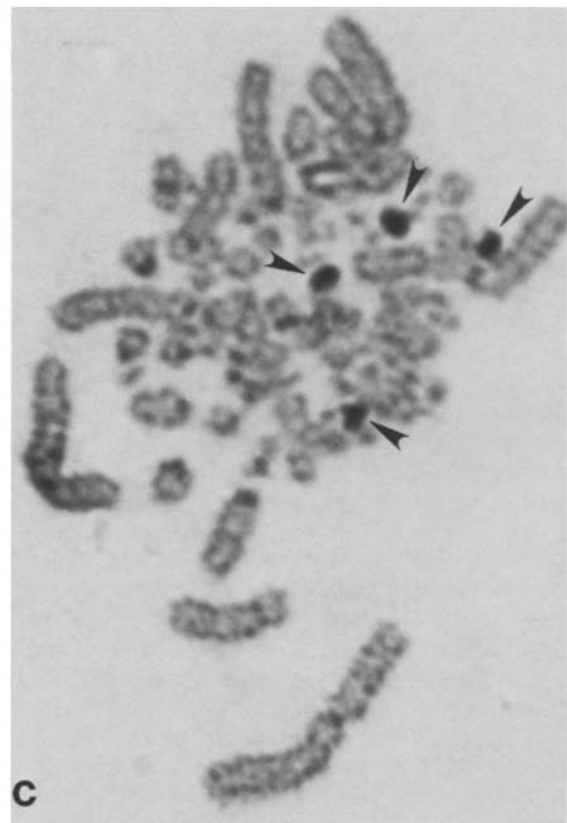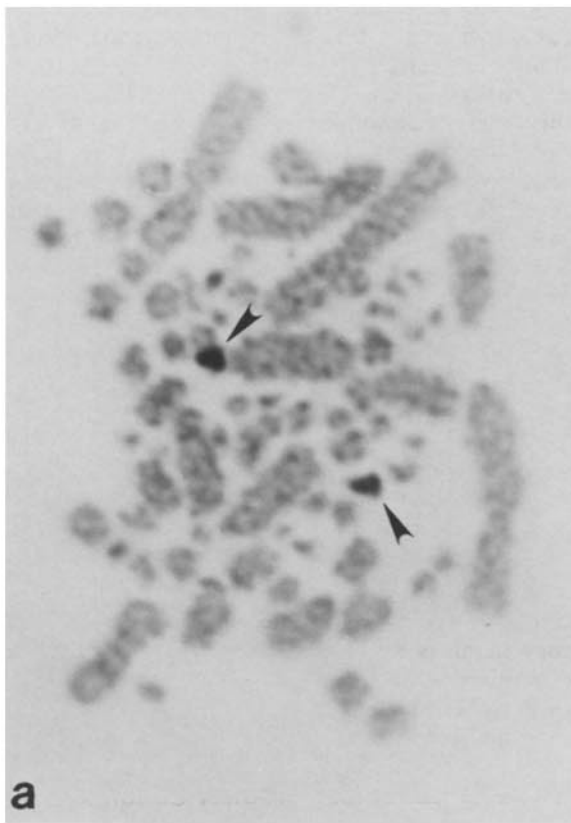
FIGURE 2   Metaphase chromosomes stained with silver nitrate to detect the NORs (arrowheads). a, disomic; b, trisomic; c, tetrasomic. (a and b) × 3,560; (c) 2,720.
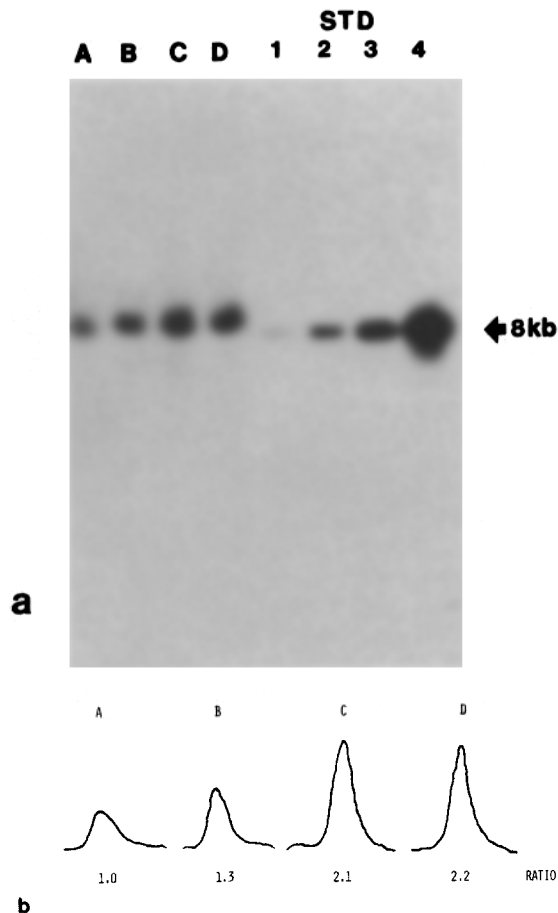
FIGURE 3 (a) Fluorograph of blot of DNA from disomic (lane a), trisomic (lane b), and tetrasomic (lanes c and d) chickens. 10 μg of Eco R1-digested DNA isolated from erythrocytes was loaded in each lane. The 8-kb Eco R1-rDNA fragment was used as standards of 15 (STD) (1), 50 (2), 150 (3), and 500 ng (4). (b) Densitometer tracings of genomic samples from same fluorograph showing increasing amount of hybridization to trisomic (B) and tetrasomic (C and D) samples as compared to disomic sample (A).
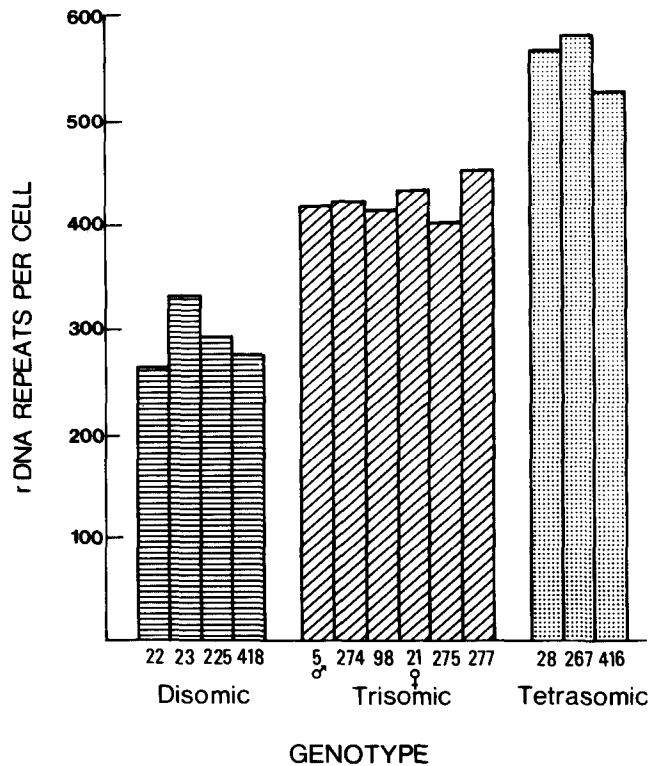

FIGURE 4 Histogram of rRNA gene copy numbers determined in a pedigree of a male and female trisomic chicken (numbers 5 and 21, respectively) and their disomic, trisomic, and tetrasomic offspring. Genotypes for all animals were established using acridine orange cytochemistry. Each bar represents the average of data from four determinations (i.e., replicate samples from two separate hybridizations of erythrocyte DNA). Standard deviations among the individual determinations for each animal were within ±15%. Parental male and female chickens are indicated (♂, ♀). Tetrasomic numbers 267 and 416 were not from same family.

TABLE I. rDNA Determinations in Diploid and Aneuploid Chickens

Genotype	Repeats per cell*	rDNA‡	Ratio
		%	
Disomic	290 ± 30	0.5	1.0
Trisomic	424 ± 17	0.73	1.5
Tetrasomic	568 ± 8	0.98	2.0

* Values represent the means and SDs for the three groups of individuals shown in Fig. 4. Copy numbers were determined from the amount of rDNA probe that hybridized to the genomic DNA samples relative to standards, and were calculated based on a molecular weight for the 8-kb Eco R1 fragment of 5×10^6 daltons and a DNA content of 2.4 pg per diploid chicken cell.

‡ The percent of rDNA per cell represents the amount of total rDNA (i.e., coding and spacer regions) and was calculated based on an rDNA repeat size of 40 kb.

normal and aneuploid cells. Thus, the trisomic line of chickens provides unique material to study the effects of rRNA gene dosage at the molecular, cellular, and organismal level.

The aneuploid animals that we have studied are viable presumably because only a minimal genetic imbalance is created by the addition of one or two microchromosomes. Approximately half of the genomic DNA in the chicken is packaged into 68 microchromosomes, each comprising ~0.5–1.5% of the genome. Trisomic chickens do show a small, but statistically significant, decrease in body weight as compared to disomics. The size and viability of tetrasomics is reduced even further (unpublished data), thus indicating that a gene dosage effect on one or more developmental pathways is occurring in these animals. By contrast, the growth of embryonic fibroblasts in culture is the same for cells from disomic and tetrasomic chickens. The effects of aneuploidy observed in the animals may be due to rDNA, or to other genes linked to the nucleolar organizer, such as those of the MHC.

Mammalian chromosomes are large compared to the chicken microchromosomes, so chromosomal trisomy is usually lethal. Trisomy for any of the macrochromosomes is also lethal in ovo for chickens (1, 2). Mice that are chimeric for normal and trisomic cells have been used to study the effect

of aneuploidy on growth and development (8). In this case, trisomic cells appear to have a proliferative disadvantage in vivo, since they were found to comprise less than half of the cells in most tissues studied. Cell cultures of skin fibroblasts from human trisomy 21 (Down's syndrome) have been used to study the effect of aneuploidy on levels of mRNA specific to that chromosome (17). The level of poly-A mRNA transcribed from chromosome 21 was found to be directly proportional to the dosage of the chromosome in cells. Although this chromosome does possess an NOR, rRNA levels were not determined. However, since NORs in many animals are located on several chromosomes (there are up to six NORs in
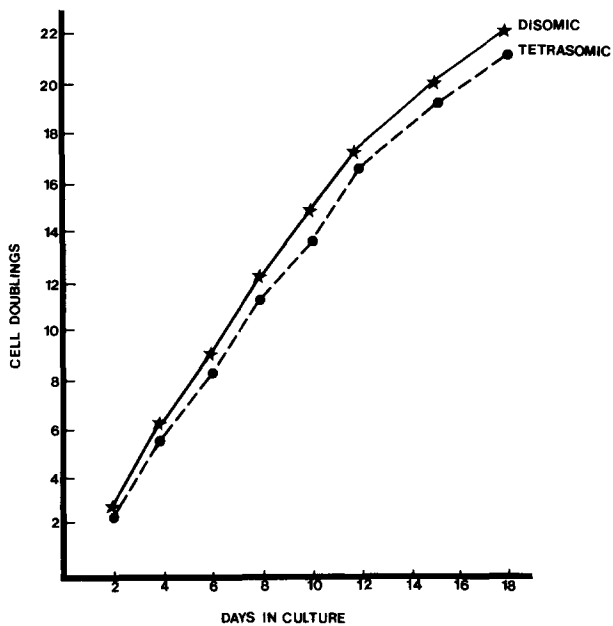
FIGURE 5 Growth curve for CEFs isolated from disomic and tetrasomic embryos showing cumulative cell growth over 18 d in culture. Each point represents a day at which two plates for each cell type were passed and counted.

TABLE II. Relative Amounts of Mature rRNA in CEFs*

| | Relative amounts of rRNA per cell | |
Genotype	Trial 1	Trial 2
Disomic	4.9 (1.0)	4.2 (1.0)
Trisomic	5.9 (1.2)	3.9 (0.9)
Tetrasomic	5.6 (1.1)	4.5 (1.1)

* Values represent average cpm of ^{32}P-labeled rDNA probe (see Materials and Methods) hybridized per cell to filter-bound cytoplasmic RNA from samples containing 1 × 10³ disomic, trisomic, or tetrasomic CEFs. (Ratio of values with respect to disomic cells is indicated in parentheses.)

mice and 10 in humans) (19), only a relatively slight increase in rRNA gene copy number can be expected in trisomic cells. Thus, it is difficult to study rRNA gene dosage effects using aneuploid cells from these organisms. Since the NORs of chicken are located on a single chromosome pair, animals that are trisomic and tetrasomic for this chromosome are expected to have a much larger proportional increase in rRNA gene copy number, and thus provide a useful model for studying rDNA gene dosage effects.

The data presented here indicate that the rRNA gene copy number corresponds to the number of NORs present in cells from normal and aneuploid chickens. Therefore, the NORs detected by silver staining appear to represent all of the rDNA present in the disomic chicken. In addition, the 1.0:1.5:2.0 ratio of rRNA gene copy numbers obtained for disomic, trisomic, and tetrasomic individuals, which were from the seventh generation that this strain has been reproduced, is consistent with the hypothesis that regulation (i.e., reduction) at the level of gene copy number is not occurring in this line. This resembles the situation found in maize, where rRNA gene copy numbers in monosomic and trisomic plants were found to be proportional to the number of nucleolar organizers present, with no changes occurring over time (27). A different situation has been reported in some other eukaryotic systems. A double-NOR strain of *Neurospora*, having a trans-

location involving the nucleolar organizer chromosome, possesses a twofold increase in rRNA gene copy number, and a reduced growth rate (31, 32). But within ten generations of culture, the rRNA gene copy number is reduced to diploid levels and a normal growth rate of cells is restored. Some plants also possess mechanisms to adjust rDNA content. For example, in wheat, only some aneuploidies for nucleolar organizer chromosomes result in stably increased rRNA gene copy numbers, whereas others show a reduction in copy number (18). These findings suggest that at least some chromosomes may have a regulatory function with respect to rDNA redundancy. Increases and decreases in rRNA gene copy number also occur in the bobbed mutant of *Drosophila*, where unequal sister chromatid exchanges (38) and mitotic crossing-over (33) have been invoked as possible mechanisms for such changes. Sister chromatid exchanges within the rRNA gene cluster have also been reported to occur in yeast (36) and mammalian tissue culture cells (40), resulting in alterations in gene copy numbers. It is possible that the slight variation in rRNA gene copy number that we observed among different animals within each of the three genotypes reflects real genetic differences. However, it does not appear that large changes in rDNA redundancy are occurring in the trisomic line.

Regulation of rRNA levels in the presence of extra rRNA genes may also be achieved by means other than reduction of gene copy number. Transcriptional regulation occurs in *E. coli* that have additional rRNA gene sequences on a multi-copy plasmid (15). In this case, there is a reduction of rRNA transcription per gene, so that overall a normal amount of rRNA is produced. A rat hepatoma cell line that has a 10-fold increase in the number of rRNA genes also has been reported to contain diploid levels of rRNA (24, 37). These amplified gene sequences were shown to be heavily methylated, and did not stain with silver nitrate, which has been shown to stain transcriptionally active NORs. In strains of *Drosophila* that have additional nucleolar organizer chromosomes, only two of the chromosomes per cell were active (16). We found that all of the NORs are transcriptionally active in aneuploid chickens. This is supported by several lines of evidence. First, all of the NORs stain with silver nitrate. Second, all of the nucleoli are RNA rich, as indicated by orange fluorescence with acridine orange. And third, the nucleoli are morphologically complete at the ultrastructural level (20). Despite the fact that all of the additional NORs are transcriptionally active in all tissues studied, mature rRNA levels appear to be the same for all three genotypes in at least one cell type, the chicken embryo fibroblast. However, we cannot exclude that extra rRNA is produced in other tissues for only brief periods in the growing chicken. Such an effect would be missed in studying CEFs obtained from aneuploid embryos whose growth is known to be normal.

These findings raise several questions concerning the nature of regulation of the additional rRNA genes in CEFs and other tissues from aneuploid embryos. Specifically, at what level, such as transcription initiation or rRNA processing, does regulation of rRNA synthesis occur, and how does regulation in the cultured fibroblasts compare to that in other tissues in vivo? Answers to these questions may provide insight into the key factors involved in the regulation of ribosome biogenesis in animal cells.

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