Characterization of Ribosomal RNA Synthesis in a Gene Dosage Mutant: The Relationship of Topoisomerase I and Chromatin Structure to Transcriptional Activity

Donna E. Muscarella,* Volker M. Vogt,* and Stephen E. Bloom‡

*Section of Biochemistry, Molecular and Cell Biology, and ‡Department of Poultry and Avian Sciences, and Division of Biology, Cornell University, Ithaca, New York 14853

Abstract. The genes encoding 18S, 5.8S, and 28S ribosomal RNA (rRNA) are tandemly repeated at the nucleolus organizer region (NOR). The NORs in the chicken map to one pair of microchromosomes. A line of chickens that contains individuals that are either disomic, trisomic, or tetrasomic for this chromosome, and have two, three, or four nucleoli and NORs, per cell, respectively, has been described previously. Aneuploid animals display a proportional increase in the rRNA gene copy number per cell. But, despite an increase in rDNA dosage, the levels of mature rRNA are regulated to normal levels in cells from aneuploid chickens (Muscarella, D. E., V. M. Vogt, and S. E. Bloom, 1985, *J. Cell Biol.*, 101:1749–1756). This paper addresses the question of how regulation of mature rRNA synthesis occurs in cells with elevated levels of rDNA.

An analysis of rRNA transcription in chicken embryo fibroblasts (CEFs) revealed that the relative rates of rRNA synthesis and processing and the amounts of precursor rRNA per cell are similar for all three genotypes. A comparison of chromatin structure, as determined by sensitivity of rDNA in nuclei from CEFs to digestion by DNase I, revealed that some of the rRNA genes from aneuploid cells are more resistant to digestion than corresponding sequences in the disomic cells. A determination of the distribution of topoisomerase I on rDNA has also been performed using the compound camptothecin, which introduces single- and double-strand breaks in topoisomerase–DNA complexes. Quantitation of camptothecin-induced cleavages revealed that a larger proportion of the rRNA genes in aneuploid cells was resistant to cleavage than in disomic cells, and therefore have no detectable amounts of topoisomerase I. These results suggest that the regulation of rRNA synthesis in CEFs with elevated levels of rDNA is achieved by the use of a subset of the rRNA genes.

Ribosomal RNA (rRNA) synthesis and ribosome biogenesis are closely coordinated events in eukaryotic and prokaryotic cells. Together they are related directly to cell growth rate and are modulated in response to a variety of environmental and nutritional factors (1, 13, 25). Although the mechanisms involved in the regulation of these processes in prokaryotes have been well characterized, far less is known about the regulation of ribosome biogenesis in eukaryotes. The initiation of rRNA transcription has been inferred to be the primary point of regulation in cultured cells under a variety of conditions, including inhibition of cell growth by serum or amino acid deprivation (21, 48) and stimulation of cell growth by the addition of hormones or mitogens (2). Most of the ultrastructural studies of transcription complexes on rRNA genes suggest that these genes are either fully loaded with molecules of RNA polymerase, each carrying a nascent transcript, or devoid of actively transcribing polymerase (36). The observation that specific transcription factors, which have recently been identified for several different species (18, 28, 31, 38, 42, 50), must first interact at the rRNA promoter before transcription by RNA polymerase I can occur, provides a probable mechanism by which the selective activation of rRNA gene in vivo may occur. Thus, the principles controlling activation of the rRNA genes in vivo may be similar to those of the 5S RNA genes, in that a particular gene is either "turned on" or "turned off." Once a particular 5S RNA gene is activated, it is transcribed at a maximal rate (3, 12, 49). According to this hypothesis, cells may respond to differences in their requirement for ribosome biogenesis by the transcriptional activation of a defined number of rRNA genes.

Relatively few techniques have been developed to allow transcriptionally active genes to be distinguished from inactive genes in vivo. One well established method is to digest DNA in isolated nuclei with nucleases (11, 30, 53, 56). Transcriptionally active genes display a chromatin structure that is more accessible to digestion by nucleases than that of inactive genes. This has been demonstrated for the rRNA genes in several species. Differences in the chromatin structure of the rRNA genes in rapidly dividing and nondividing mouse

cells have been observed (7). An increased sensitivity to digestion by nuclease has also been described for the rRNA genes of *Xenopus laevis* in hybrids of *X. laevis* × *X. borealis* (32). In such hybrids, only the rDNA sequences derived from *X. laevis* are transcribed.

Another potentially useful probe for actively transcribed sequences is the enzyme topoisomerase I. Recently, topoisomerase I has been shown to be specifically associated with the transcribed regions of several genes in *Drosophila*, and absent from, or present in extremely low amounts in, nontranscribed regions (14, 15). Presumably, this enzyme functions to relieve torsional stress associated with transcription. Although it is likely that topoisomerase I is a component of many, if not all, transcribed genes, this enzyme has also been shown to be particularly abundant in nucleoli of a variety of cell types (10, 43). Camptothecin is a compound that specifically inhibits topoisomerase I in vitro (24). Exposure of cells to camptothecin in vivo results in the introduction of single- and double-strand breaks in DNA at the sites of topoisomerase I–DNA interaction. Since topoisomerase I appears to interact preferentially with transcribed genes, we reasoned that it should be possible to use this compound to distinguish between active and inactive rRNA genes, and thus, to gain insight into the way in which these genes are used in vivo.

We previously described a line of domestic chicken that contains individuals that are aneuploid for the chromosome containing the rRNA genes. In the chicken, the nucleolar organizer regions (NORs),[1] or site of the genes encoding the 18S, 5.8S, and 28S rRNAs, are located on a single pair of microchromosomes (4, 5). The mutant strain contains individuals that are either disomic (i.e., normal), trisomic, or tetrasomic for this chromosome, and show two, three, or four NORs and nucleoli per cell, respectively. The increase in the number of NORs correlates directly with an increase in copy number for the rRNA genes: disomic, trisomic, and tetrasomic cells have ~290, 420, and 570 rRNA repeats per cell, respectively (4, 5, 44). But despite an increase in rRNA gene copy number, the amount of mature rRNA per cell is the same for all three genotypes (44).

In this study, we attempted to determine how regulation of the amount of mature rRNA is achieved in cells with increased numbers of rRNA genes. An analysis of the rates of pre-rRNA transcription and processing revealed that transcriptional regulation of rRNA synthesis is operative in these cells. We have further characterized the nature of the regulation by comparing the chromatin structure, the degree of methylation, and the distribution of topoisomerase I in the rDNA of normal and aneuploid cells. Our results indicate that the same number of rRNA genes are active in disomic, trisomic, and tetrasomic cells, suggesting that the regulation of rRNA synthesis in cells with increased numbers of rRNA genes is achieved by the use of a subset of the rRNA genes on each chromosome.

Materials and Methods

Description of Clones

The plasmid pETS was derived from a chicken rDNA clone, pVCT 135, which was provided by I. Mattaj (34). A 3.2-kb Bam HI–Sal I fragment, that contained the site for initiation of transcription was isolated from pVCT 135 and subcloned into pBR 322. The plasmid pITS was derived from a clone of chicken rDNA, which was provided by A. Skalka (35). It consists of a 4.3-kb Bam HI-Eco RI fragment that contains a portion of the coding regions for the 18S rRNA and the internal transcribed spacer. Both clones are shown in Fig. 4 *a*.

Experimental Chickens and Culture of Chicken Embryo Fibroblasts

Eggs were obtained from matings of trisomic × trisomic chickens. Such crosses yield a 1:2:1 ratio of disomic/trisomic/tetrasomic progeny. After 9 d of incubation, embryos were collected. A sample of tissue, usually from the wing or thigh, was processed using the acridine orange staining technique for nucleoli, as previously described (44). Genotypes of the embryos were established by determining the maximum number of nucleoli per cell; disomic, trisomic, and tetrasomic embryos possessed two, three, and four transcriptionally active nucleoli per cell, respectively. Individual cultures of chicken embryo fibroblasts (CEFs) were established from each embryo, and were grown in DME (4,500 mg glucose/liter; Gibco, Grand Island, NY) supplemented with 10% FCS, 2% chicken serum, sodium pyruvate (1 mg/ml), glutamine (0.58 mg/ml), penicillin, streptomycin, and Fungizone. To ensure that no changes in nucleolar number occurred in the CEFs, the nucleolar number was monitored in all cultures throughout the experimental period. In addition, cultures from each genotype were used at the same passage number for each experiment, and no cultures were used beyond the tenth passage.

Isolation of Total Cellular RNA by Guanidine Isothiocyanate/Cesium Chloride Method

Total cellular RNA was isolated by a modification of the method described in Maniatis et al. (33). Monolayers of CEFs were washed with PBS. All excess buffer was removed, and 2.5 ml of a guanidine isothiocyanate solution (6 M guanidine isothiocyante, 5 mM sodium citrate pH 7.0, 0.1 M β-mercaptoethanol, and 0.5% Sarkosyl) that was pre-warmed to 37°C was pipetted into the plate. The cells lysed immediately, and the viscous solution was scraped to the edge of the plate and transferred into a polypropylene tube. 1 g of cesium chloride was added per 2.5 ml of lysate, which was then transferred into an SW-60 centrifuge tube. Approximately 1 ml of a cushion (5.7 M CsCl in 0.1 M EDTA) was layered under the homogenate, and the tubes were centrifuged at 32,000 rpm for 24 h at 20°C in an SW-60 rotor. The RNA pellet was dissolved in 10 mM Tris pH 7.5, 5 mM EDTA, 1% SDS, and extracted with 4:1 chloroform/butanol. The organic phase was re-extracted with the above solution, and the aqueous phases were pooled and precipitated by the addition of 0.5 vol of 3 M sodium acetate plus 2 vol of absolute ethanol.

Formaldehyde Gels for RNA Analysis

A denaturing gel of agarose-formaldehyde in a phosphate buffer was found to give the best resolution of the large (~10–12 kb) rRNA precursors. For a typical 1% gel (150 ml), 1.5 g of agarose was added to 124.5 ml of water, boiled, and cooled to 50°C, followed by the addition of 24 ml of 37% formaldehyde solution and 1.5 ml of 1 M $NaPO_4$ pH 7.0. The running buffer consisted of 160 ml of formaldehyde solution (37%) and 10 ml of 1 M $NaPO_4$ pH 7.0 per liter. RNA that was dissolved in water containing 0.1% diethylpyrocarbonate was added to sample mix (75% formamide, 3.3 M formaldehyde, 13 mM $NaPO_4$) at a ratio of 1:2 (RNA/mix). The samples were heated to 50°C for 15 min, and the loading buffer (5× = 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol) was added to each sample. Samples were subjected to electrophoresis at 15 mA overnight, then at 40 mA for 4–5 h. The running buffer in the two chambers was mixed every 1–2 h during the last 5 h of electrophoresis to prevent electroendoosmosis of the gel (continuous pumping of the buffer resulted in smearing of the RNA bands).

Blotting of Formaldehyde-RNA Gels

Formaldehyde gels were soaked in 50 mM NaOH (containing 5 μg/ml of ethidium bromide) for 60 min, then in 10 mM $NaPO_4$ pH 7.0 for an additional 60 min, with at least five changes of buffer. The gel was photographed using a short wave UV light box. (Background staining of formaldehyde gels was typically very high, even after extensive de-staining.) The RNA was transferred to Zeta-probe (Bio-Rad Laboratories, Richmond, CA) blotting

1. *Abbreviations used in this paper*: CEF, chicken embryo fibroblast; ETS, external transcribed spacer; ITS, internal transcribed spacer; NOR, nucleolus organizer region.

membrane (that was pre-wetted with 4× SSC) overnight in 20× SSC using the method described by Maniatis et al. (33). After the transfer was complete, the blotting membrane was air dried and baked in vacuo at 80°C for 3 h. The filter was prehybridized overnight at 42°C in the following solution: 5× SSC, 1% Denhardt's solution (1× = 0.02% each of BSA, Ficoll, and polyvinylpyrrolidone), 20 mM NaPO pH 7.0, 10% dextran sulfate, 5 mg/ml *E. coli* DNA (sonicated), and 50% formamide. The solution was heated to 90°C and cooled before use. The prehybridization solution was discarded, and the filter was hybridized overnight at 42°C in a hybridization mix consisting of the same solution to which the nick-translated probe had been added. To ensure a linear increase in the amount of hybridization to increasing amounts of RNA on the filter, a minimum of a fivefold excess of nick-translated probe was used. This was estimated based on the expected amount of rRNA on the filter that was complementary to the probe. Hybridization was complete within 24 h, and the filter was washed in 2×, 1×, and 0.5× SSC at 70°C for several hours. Filters could be hybridized sequentially with different probes simply by repeating the prehybridization/hybridization procedure described above. Alternatively, the first probe could be removed by washing the filter in 0.02 mM EDTA at 80°C for 1 h.

Pulse-Chase Labeling for rRNA Processing

CEFs were plated at a concentration of 2–3 × 10^5 per 60-mm plate and incubated for ~2 d, or until the cultures were ~90–100% confluent. (At this point cells are growing optimally.) All solutions were pre-warmed to 39°C and cell cultures were maintained at this temperature through the pulse-chase experiment. Cultures were pulse-labeled with 50 μCi of [^{3}H]uridine (specific activity, 40 Ci/mmol) in 1.0 ml of DME per plate for 10 min. Cultures were chased by removing the medium, washing the monolayer with 3 ml of PBS, and adding 3 ml of DME containing 10% FCS and 0.05 μg/ml actinomycin D. After the chase period, the monolayers were washed with 3 ml of PBS, and RNA was isolated from nuclei.

All solutions for RNA contained 0.1% diethylpyrocarbonate, which was added immediately before use. Nuclear RNA from CEFs was isolated as follows: Monolayers were washed with 3 ml of PBS, followed by 1.5 ml of hypotonic solution (10 mM Tris pH 7.4, 1 mM $MgCl_2$). An additional 1.5 ml of hypotonic solution was added, and then cells were scraped off the plate and transferred into a centrifuge tube. Triton X-100 was added to a final concentration of 0.1%, and the cell suspension was vigorously vortexed for 30 s. The tubes were placed in an ice bath for 10 min, and the vortexing was repeated. The liberation of the nuclei was monitored by clearing of the solution and by phase microscopy. After lysis of the cells, 0.5 ml of a sucrose cushion (0.2 M sucrose in the hypotonic solution) was layered into the bottom of the tube. Nuclei were pelleted by centrifugation at 2,000 rpm at 4°C in an HB4 rotor. The nuclear pellet was resuspended in 100 μl of hypotonic solution containing 0.1% Triton X-100. Sodium acetate (pH 6) and $MgCl_2$ were added to a final concentration of 0.1 and 0.01 M, respectively. DNase I solution (0.1 mg/ml DNase I in 0.1 M sodium acetate, treated with 0.5% diethylpyrocarbonate overnight at room temperature) was added to give a final concentration of DNase I of 20 μg/ml. The nuclei were incubated at room temperature for 10 min, and were then lysed by the addition of SDS to 1%. The lysate was extracted two times with Leder phenol (50 ml of phenol/50 ml chloroform/0.5 ml isoamyl alcohol) and precipitated with 0.5 vol of 3 M sodium acetate plus 2 vol of absolute ethanol at −20°C for several hours. RNA was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C and dissolved in water. RNA was subjected to electrophoresis through agarose-formaldehyde gels as described above.

Fluorography of Formaldehyde Gels

Formaldehyde gels containing ^{3}H-labeled RNA were fluorographed as follows: Gels were rinsed in several changes of water for 1 h, then soaked in 4 M sodium salicylate for 30 min. The gels were then briefly rinsed in water and dried onto 3-mm paper (Whatman Inc., Clifton, NJ) using a Bio-Rad slab gel-dryer. Gels were dried at room temperature for ~1 h, then at 60°C for 5–10 min. (Heating the hydrated gels resulted in excessive crystallization of the salicylate.) The gel was exposed to Kodak XAR-5 film overnight.

In Vitro Transcription Using Isolated Nuclei

Cell cultures were established with ~3 × 10^6 cells in a 100-mm plate, and incubated for ~2 d, or until the cultures were confluent. The cultures were fed several hours before isolation of the nuclei. Cells were collected by trypsinization, and cell counts were done using a hemacytometer. An equivalent number of cells for each genotype were transferred into 15-ml centrifuge tubes, with a maximum of 5 × 10^7 cells per tube. (Larger numbers of cells resulted in extensive clumping of nuclei and cell debris.) The cells were washed with ice-cold PBS and resuspended in 10 ml of hypotonic solution (10 mM Tris pH 8.0, 5 mM KCl, 1 mM dithiothreitol [DTT], 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 10% glycerol), and allowed to swell in this solution, on ice, for 10 min. NP-40 was then added to a concentration of 0.5%, and the cell suspension was vigorously vortexed for 20 s, allowed to sit on ice for an additional 10 min, and vortexed again. Disruption of the cells and release of intact nuclei were monitored by phase-contrast microscopy. After the cells were lysed, 2 ml of cushion (hypotonic solution plus 0.2 M sucrose) was pipetted into the bottom of the tube using a pasteur pipette. The nuclei were pelleted by centrifugation at 2,000 rpm for 10 min at 4°C in an HB-4 rotor. The nuclei were then resuspended in nuclei buffer (100 mM Tris pH 8.0, 0.2 mM EDTA, 1 mM DTT, 25% glycerol, 0.125 mM phenylmethylsulfonyl fluoride [PMSF]). Approximately 100 μl of buffer was added for every 10^7 nuclei. Nuclei were immediately frozen at −70°C in 500-μl aliquots. (Freezing of the nuclei did not interfere with their ability to transcribe in vitro.)

For the transcription assay, nuclei were allowed to thaw on ice, and α-amanitin (Sigma Chemical Co., St. Louis, MO) and RNasin (Promega Biotech, Madison, WI) were added to a final concentration of 100 μg/ml and 1 U/ml, respectively. The nuclei were preincubated for 10 min on ice. Tubes containing 250 μl of 2× transcription buffer (1× = 100 mM Tris pH 8.0, 5 mM magnesium acetate, 2 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.6 mM NaF, 0.1 mM PMSF, 1 mM ATP, GTP, CTP, and 2 μm UTP plus 25 μCi of α-[^{32}P]UTP [specific activity, 410 Ci/mmol]) were set up. The transcription reaction was initiated by the addition of 250 μl of nuclei into the reaction tube, and transferring the tube to 37°C. Reactions were stopped by the addition of EDTA to 5 mM and diethylpyrocarbonate to 0.2%. Sodium acetate (pH 6) and $MgCl_2$ were added to final concentrations of 0.1 and 0.01 M, respectively, and the reaction was treated with DNase I (1 μg/reaction) that had been treated with diethylpyrocarbonate for 5 min at room temperature. The nuclei were then lysed by the addition of SDS to a concentration of 0.1%. Duplicate samples, each comprising one-tenth of the total reaction, were aliquoted for determination of incorporated counts by TCA precipitation.

For hybridization experiments, the nuclear lysates were extracted twice with Leder phenol and precipitated with ethanol. Intact RNA was separated from free counts by chromatography on a 3-ml P-60 column. The RNA collected from the column was used as a probe for filter-bound plasmid DNA by the hybridization procedure previously described, with the exception that the filter-bound DNA was present in excess in the hybridization. Typically, 5 μg of plasmid DNA on a single strip of filter (from one lane of a gel) was hybridized to RNA that was synthesized from a reaction containing 2 × 10^7 nuclei (~200,000–400,000 cpm of ^{32}P) in a total volume of 10 ml.

DNase I Sensitivity of rDNA

Nuclei were isolated from CEFs or erythrocytes as previously described, with the exception that the hypotonic solution used consisted of 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 10% glycerol. Nuclei were also resuspended in this same buffer, except that 25% glycerol was added. Nuclei from ~10^7 cells were resuspended in 1 ml and aliquoted into 100-μl samples. 5 μg of DNase I, in a volume of 50 μl, was added to the nuclei, and the reactions were stopped by the addition of EDTA to 25 mM and SDS to 0.2%. 1 μg of lambda DNA was added to each reaction, which contained ~5 μg of genomic DNA. (The lambda DNA was used to ensure that no DNA was lost during the purification steps, and to ensure that all of the DNA was digested to completion.) The lysates were treated with Proteinase K (25 μg/reaction) for 1 h at 37°C, then extracted twice with Leder phenol, three times with water-saturated ether, and precipitated at least twice with 0.5 vol of 7.5 M ammonium acetate and 2 vol of absolute ethanol. A portion of each reaction (1/20th of the total reaction) was subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide to determine the extent of digestion by DNase I. The remainder of the DNA was digested with restriction enzyme and hybridized to nick-translated probes as described above.

Camptothecin–Topoisomerase I Analysis

Camptothecin (lactone form; NSC 94600) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. It was stored as a 10 mM stock in DMSO at −70°C. For topoisomerase I cleavage experiments, exponentially growing cultures of CEFs were exposed to 20 μM camptothecin in PBS for 5 min at 37°C (2.5 ml total for 100-mm plate). The camptothecin solution was removed, and the monolayers were immediately flooded with 2.5 ml of 10 mM Tris pH 7.5, 50 mM NaCl, 25 mM EDTA,

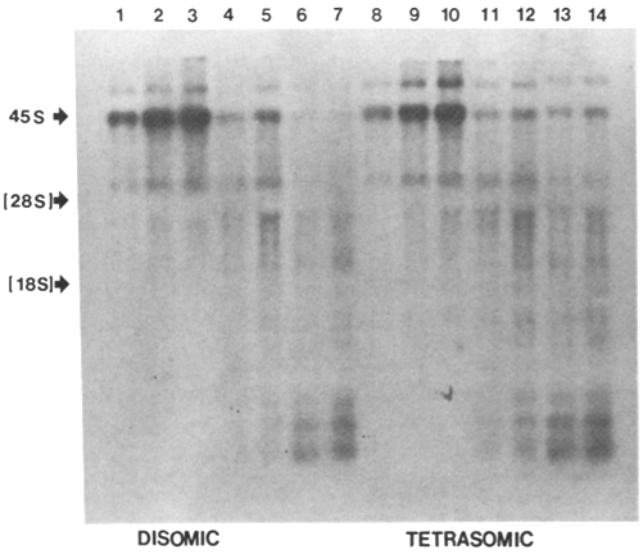

Figure 1. Quantitation of pre-rRNA levels in CEFs. Autoradiograph of blot of total cellular RNA from CEFs hybridized to pETS. Lanes *1–3* and lanes *8–10* represent increasing amounts (2.5, 5, and 10 μg) of total RNA from disomic and tetrasomic cells, respectively. Lanes *4* and *5* and lanes *11* and *12* are 5 and 10 μg of RNA after 15-min exposure to actinomycin D (50 ng/ml). Lanes *6* and *7* and lanes *13* and *14* are 5 and 10 μg of RNA after a 30-min exposure of cells to actinomycin D. This filter was then probed with a fragment corresponding to the 28S rRNA (not shown) for determination of ratio of precursor to mature rRNA (Table I). (A band above the 45S precursor is seen occasionally in these blots. Although its identity is unknown, it may arise from transcripts that extend beyond the termination site for the 45S rRNA.)

2% SDS. The lysates were treated with Proteinase K (100 μg/ml) for 4 h at 55°C, extracted twice with phenol, once with Leder phenol and once with 24:1 chloroform/isoamyl alcohol. The samples were precipitated with ethanol twice, then treated with RNase A (10 μg/ml) for 1 h at 37°C. The organic extractions were then repeated. The DNA was dissolved in 10 mM Tris pH 7.5, 1 mM EDTA. DNA concentrations were determined by absorbance at 260 nm (assuming A_{260} = 1 for a 50 μg/ml solution. The DNA was then digested with the appropriate restriction enzyme.

To detect single-strand cleavages, samples were treated with S1-nuclease as follows: 5 μg of DNA in 25 μl 10 mM Tris pH 7.5, 1 mM EDTA was mixed with an equal volume of 2× S1 buffer (1× = 50 mM sodium acetate pH 4.5, 150 mM NaCl, 2 mM $ZnSO_4$) containing ~10 U of S1 nuclease. Samples were incubated at 37°C for 30 min. The reaction was stopped by addition of EDTA to 25 mM and NaCl to 300 mM. Samples were extracted with phenol and precipitated with ethanol. The DNA was subjected to electrophoresis into a 1% agarose gel in Tris-borate buffer (0.09 MM Tris-base, 0.09 M boric acid, 25 mM EDTA), transferred to Zeta-probe blotting membrane, and hybridized to nick-translated probe as described above.

Mapping of Initiation Site with S1-Nuclease

Total genomic RNA was isolated by the guanidine-isothiocyanate method described above. For each reaction 500 ng of Sma I-digested pETS was mixed with 10 μg of RNA (or an equivalent amount of salmon sperm DNA in control samples). The nucleic acids were precipitated with ethanol and dissolved in 20 μl hybridization buffer (40 mM Pipes pH 6.4, 1 mM EDTA pH 8.0, 0.4 M NaCl, 80% formamide). Samples were denatured by heating to 80°C for 15 min. Reannealling was allowed to proceed at 60°C for 4 h. (Conditions for the preferential formation of DNA–RNA hybrids over DNA–DNA hybrids were empirically determined in previous experiments.) After hybridization, 300 μl of ice-cold S1 buffer (described above) containing 10, 100, or 1,000 U of S1 nuclease was added to each reaction. Samples were incubated at 37°C for 1 h. Reactions were terminated with the addition of 50 μl of 4 M ammonium acetate plus 100 mM EDTA. Nucleic acid was precipitated with an equal volume of isopropyl alcohol. Samples were subjected to electrophoresis in an alkaline-agarose gel (33), transferred to Zeta-probe membrane, and hybridized to nick-translated probe.

Densitometry

All autoradiographs were scanned on a Bio-Rad model 620 video densitometer. Different exposures of each autoradiograph were scanned to ensure that the intensities of the bands were within a linear range for the x-ray film.

Results

Precursor rRNA Transcription and Processing

We reported previously that the amount of mature 18S and 28S rRNA per cell is the same for normal and aneuploid CEFs. In the present study, we attempted to determine how regulation is achieved in aneuploid cells. rRNA is transcribed as a large primary transcript that is processed by a series of cleavages to yield the mature 18S, 5.8S, and 28S rRNAs. Since transcription and processing of the primary transcript are possible control points for rRNA production we compared both of these aspects of mature rRNA synthesis in normal and aneuploid CEFs.

The relative levels of pre-rRNA for disomic and aneuploid CEFs were determined in two ways: (*a*) by blotting hybridization and (*b*) by nuclear run-on transcription. In the first experiment (Fig. 1), 2.5, 5, and 10 μg of total cellular RNA from disomic (lanes *1*, *2*, and *3*) and tetrasomic (lanes *8*, *9*, and *10*) cells were subjected to electrophoresis in an agarose-

*Table I. Ratio of Precursor to 28S rRNA**

Genotype	− Actinomycin D	+ Actinomycin D	Inhibition
			%
Trial No. 1			
Disomic	0.35	0.07	80
Tetrasomic	0.40	0.10	75
Trial No. 2			
Disomic	0.73	–	–
Trisomic	0.76	–	–
Tetrasomic	0.74	–	–

* The ratio of 45S pre-RNA to 28S rRNA was determined from the relative areas of the peaks in densitometer scans. The autoradiograph for trial No. 1 is shown in Fig. 1. The blot shown was re-probed with a fragment corresponding to the coding region for the 28S rRNA to obtain the 45S/28S rRNA ratios. Trial No. 2 represents a similar experiment that included trisomic samples. The percent inhibition by actinomycin D was determined in trial No. 1 after exposure of CEFs to the compound for 15 min at a concentration of 50 ng/ml. (The differences in the absolute value of the ratios between the two experiments represents differences in the specific activities of the probes used for the precursor and mature rRNAs.)

formaldehyde gel, transferred to blotting membrane, and hybridized to a nick-translated probe derived from part of the external transcribed spacer (pETS). The filter was probed a second time with a probe derived from the 28S rRNA coding region (not shown). The intensities of the bands corresponding to the 45S pre-rRNA as well as that of the 28S rRNA was quantitated by densitometry, and the ratios of RNA precursor to mature RNA were calculated based on the relative areas of the peaks in the densitometer scans (Table I). In trial No. 1 (shown in Fig. 1), similar ratios of precursor RNA to mature RNA were obtained for the disomic and tetrasomic samples. Trial No. 2 also included a trisomic sample. Once again, the ratio of precursor to mature rRNA was found to be similar in all of the genotypes. (The different absolute values of the numbers between the two trials represent differences in the specific activities of the probes used.) Since the level of 28S rRNA in normal and aneuploid CEFs is the same, these results indicate that the steady-state amount of pre-rRNA per cell is also the same.

Actinomycin D was included in some disomic and tetrasomic cultures (Fig. 1, lanes *4–7* and lanes *11–14*, respectively). Because rRNA synthesis is particularly sensitive to inhibition by actinomycin D, a low concentration of this compound (50 ng/ml) is sufficient to inhibit rRNA synthesis without affecting pre-rRNA processing or synthesis of mRNA (45, 52). The extent of reduction in the amount of 45S precursor by actinomycin D was determined (Table I). For both genotypes, the amount of 45S rRNA rapidly decreased after exposure of cells to the compound for 15 min, indicating that disomic and tetrasomic cells are equally sensitive to the inhibition of rRNA synthesis by this compound. Three bands, corresponding to small RNAs ~0.5–1-kb long, accumulated with time in the cells exposed to actinomycin D. Similar small RNA species have been observed in actinomycin D–treated mouse cells, and may be due to aborted initiation by RNA polymerase I (9). But it is also possible that these bands arise from the transcription of sequences upstream of the pre-rRNA start site (pETS; Fig. 4 *a*). Transcripts that are derived from the "non-transcribed spacer" and terminate immediately upstream of the pre-rRNA start site have been reported in mouse and in *Xenopus* (20, 23, 37, 50). The spacer transcripts are highly unstable. However, it is possible that their stability is enhanced when pre-rRNA synthesis is inhibited by actinomycin D, leading to the accumulation of these small RNAs.

This rapid inhibition of pre-rRNA synthesis by actinomycin D was used to compare processing of rRNA in normal and aneuploid cells. CEFs were pulse-labeled with [^{3}H]uridine for 10 min, then re-fed with fresh medium containing actinomycin D for chase periods of 10, 20, and 30 min. A profile of the major processing intermediates can be seen using this labeling regime (Fig. 2 *a*). The size of these processing intermediates was assigned by analogy with mammalian processing intermediates (54, 55). Although other, alternative processing pathways also occur in most mammalian cells, the RNA species visible in this experiment coincide with the most abundant mammalian species. The 45S rRNA, which is the largest stable pre-rRNA that can be identified, is rapidly cleaved at the 5′ end to form a 43S intermediate, then a 41S intermediate. The 41S rRNA is then cleaved to produce a 20S species, from which the 18S rRNA is derived, and a 32S species, which is cleaved to

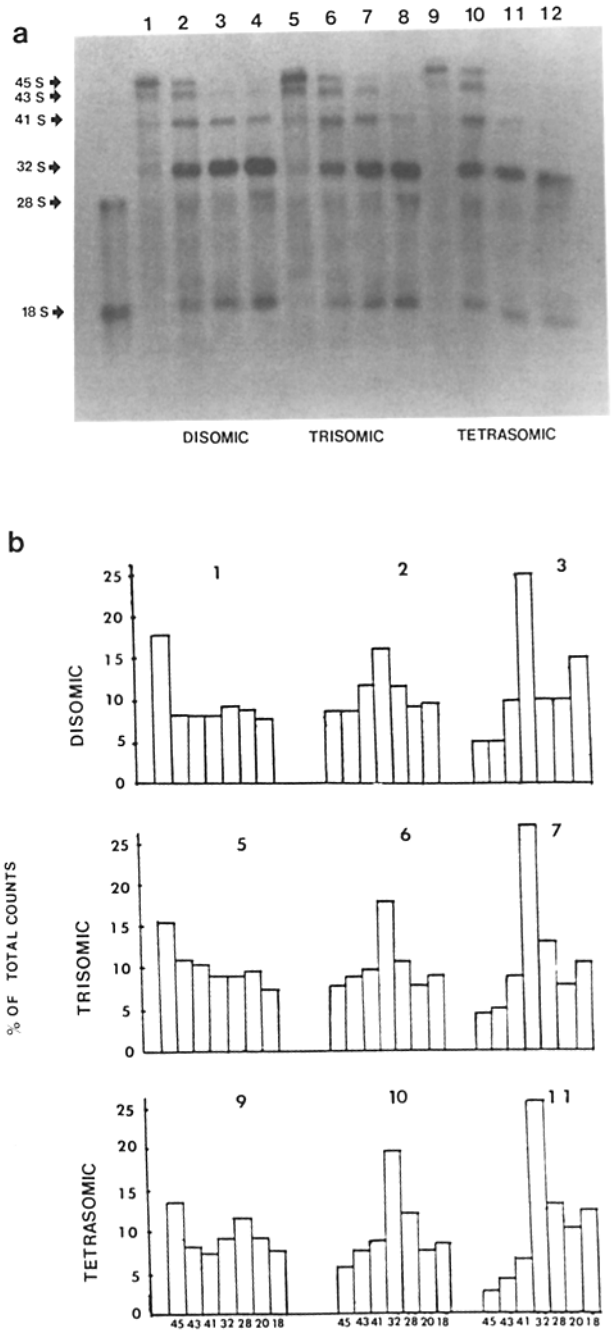

Figure 2. Processing of pre-rRNA in CEFs. (*a*) Autoradiograph of nuclear RNA after 10-min pulse labeling with [^{3}H]uridine (lanes *1*, *5*, and *9*) followed by 10-min chase (lanes *2*, *7*, and *10*), 20-min chase (lanes *3*, *8*, and *11*), or 30-min chase (lanes *4*, *8*, and *12*) in the presence of 50 ng/ml of actinomycin D. RNA from ~2 × 10^6 nuclei was subjected to electrophoresis in a 1% agarose-formaldehyde gel. (*b*) Histogram of autoradiograph shown in *a*. Each bar represents the percent of total area (determined by densitometry of various exposures of the film) in the lane that corresponds to each band.

generate the 5.8S rRNA plus the 28S rRNA. No qualitative differences could be seen in pre-rRNA processing for disomic (lanes *1–4*), trisomic (lanes *5–8*), and tetrasomic (lanes *9–12*) CEFs. In all three genotypes, the 45S precursor is almost immediately cleaved to generate a 43S intermedi-

ate, which is visible even in the 10-min pulse samples (lanes *1*, *5*, and *9*). In other systems, the rate-limiting step in rRNA processing is the conversion of the 32S rRNA to 28S plus 5.8S rRNA (22, 25, 26). This is evidenced here by the accumulation of the 32S species. The relative amounts of the various RNA species were quantitated by densitometry and are illustrated in histogram form (Fig. 2 *b*). No quantitative differences could be seen in the processing profiles of the three genotypes. (Although it appears that there are slightly larger amounts of the 32S and 28S RNAs for the tetrasomic cells after a 10-min pulse [panel 9], this was not observed in replicate experiments.)

Transcription of rRNA in Isolated Nuclei

The above experiments indicate that the amount of 45S pre-rRNA and the rate of processing of the precursor are similar in normal and aneuploid CEFs. However, it is possible that more precursor is synthesized in aneuploid cells, and any extra transcript is rapidly degraded. We have addressed this possibility by measuring the synthesis of pre-rRNA in the CEFs by run-on transcription in isolated nuclei. When incubated under conditions of low ionic strength, and in the presence of α-amanitin (an inhibitor of RNA polymerase II and III, but not polymerase I), isolated nuclei will continue to elongate rRNA transcripts that were initiated in vivo (17, 19). The incorporation of [^{32}P]UTP into rRNA by identical numbers of disomic and tetrasomic nuclei was quantitated by TCA precipitation (Table II). For the two trials shown, no difference in incorporation for normal and aneuploid nuclei can be seen.

The amount of rRNA synthesized in vitro was also quantitated by hybridizing the ^{32}P-labeled rRNA to an excess of filter-bound cloned rDNA corresponding to the ETS and to the 28S regions of the gene (data not shown). The amount of hybridization to the filter-bound DNA was the same for the disomic and tetrasomic samples.

Chromatin Structure in Normal and Aneuploid Cells

The above data indicate that the amount of pre-rRNA transcribed in CEFs is independent of the rRNA gene copy number. Two possible mechanisms for regulation of rRNA transcription in these cells can be envisioned: (*a*) all of the rRNA genes are active in the cells with additional copies, but the amount of transcript produced from each gene is reduced, or (*b*) the same number of genes are turned on in each cell, and any additional genes are inactive.

*Table II. Incorporation of [^{32}P]UTP into rRNA by Isolated Nuclei**

Genotype	Incorporation (cpm per 5 × 10^6 nuclei)	
	Trial 1	Trial 2
Disomic	210,000 (7.35)	405,000 (7.08)
Tetrasomic	199,000 (6.97)	450,000 (7.88)

* Values represent number of counts per minute of ^{32}P per reaction as determined by TCA precipitation. Each reaction contained 5 × 10^6 nuclei in 500 μl which were incubated for 10 min at 37°C. All reactions were performed in duplicate. Numbers in parenthesis indicate number of picomoles of UTP incorporated per reaction. (The concentration of UTP was 2 μM; 25 μCi of [^{32}P]UTP was added to each reaction for Trial 1, and 50 μCi was added to each reaction for Trial 2.)

Transcriptionally active genes are more sensitive to digestion by various nucleases than inactive genes, due to differences in chromatin structure (11, 30, 53, 56). We compared the sensitivity of rDNA in normal and aneuploid CEFs to digestion by DNase I to probe the chromatin structure of the extra rRNA genes in the aneuploid cells. Identical numbers of nuclei from disomic and tetrasomic CEFS were digested with DNase I (5 μg/ml) for various times. Genomic DNA then was purified and digested with Pst I. A 5.1-kb Pst I fragment, which contains the site for initiation of transcription, was detected by hybridization to a nick-translated probe, pETS (Fig. 3 *a*). The intensity of the band was quantitated by densitometry, and the percent reduction in the signal com-

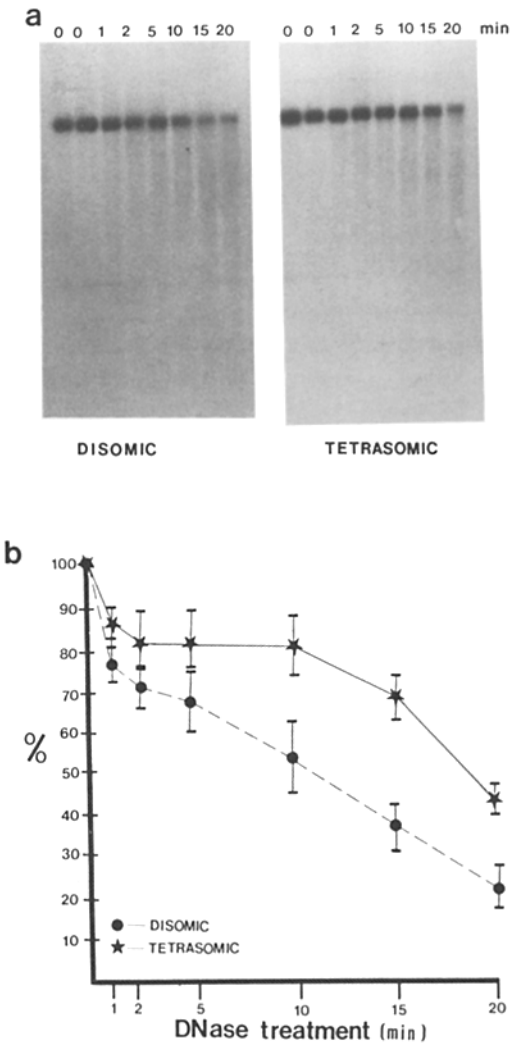

Figure 3. DNase I sensitivity of rDNA. (*a*) Representative time course of DNase I digestion of the 5.1-kb Pst I fragment that contains the transcription initiation site. Nuclei from disomic and tetrasomic CEFs were digested with DNase I (5 μg/ml in a total reaction volume of 100 μl, containing 1 × 10^6 cells) for the times indicated. Genomic DNA was purified and digested with Pst I. The filter was hybridized using the ETS probe. (*b*) Graph of relative rate of degradation of fragment in *a* showing increased resistance of rRNA in tetrasomic nuclei to digestion by DNase I. Points represent the percent of signal remaining as compared with the 0-min controls, as determined by densitometry of two separate blots, with duplicate samples on each blot (i.e., four individual values).

pared with the controls was determined. The values obtained for the representative blot shown, and two other blots, were averaged and are displayed graphically (Fig. 3 *b*). We consistently found that the rDNA from disomic nuclei was more rapidly degraded by DNase I than the rDNA from tetrasomic nuclei. This was true for at least three different nuclear preparations, and for fragments corresponding to other portions of the coding region (data not shown). The increased resistance of rDNA to digestion by DNase I in the tetrasomic cells supports the second possibility presented above, namely, that a larger proportion of rRNA genes in aneuploid cells is transcriptionally inactive compared with the rRNA genes in disomic cells.

Mapping and Methylation Analysis in the Region of the rRNA Start Site

Before we could proceed to further characterize the rDNA in the normal and aneuploid CEFs, it was necessary to map the site for initiation of transcription for the chicken. Using S1 protection analysis in which total RNA was hybridized to the 1.5-kb Bam HI-Sma I fragment (Fig. 4 *b*), we found a protected band that was ~300 bp smaller than the full length

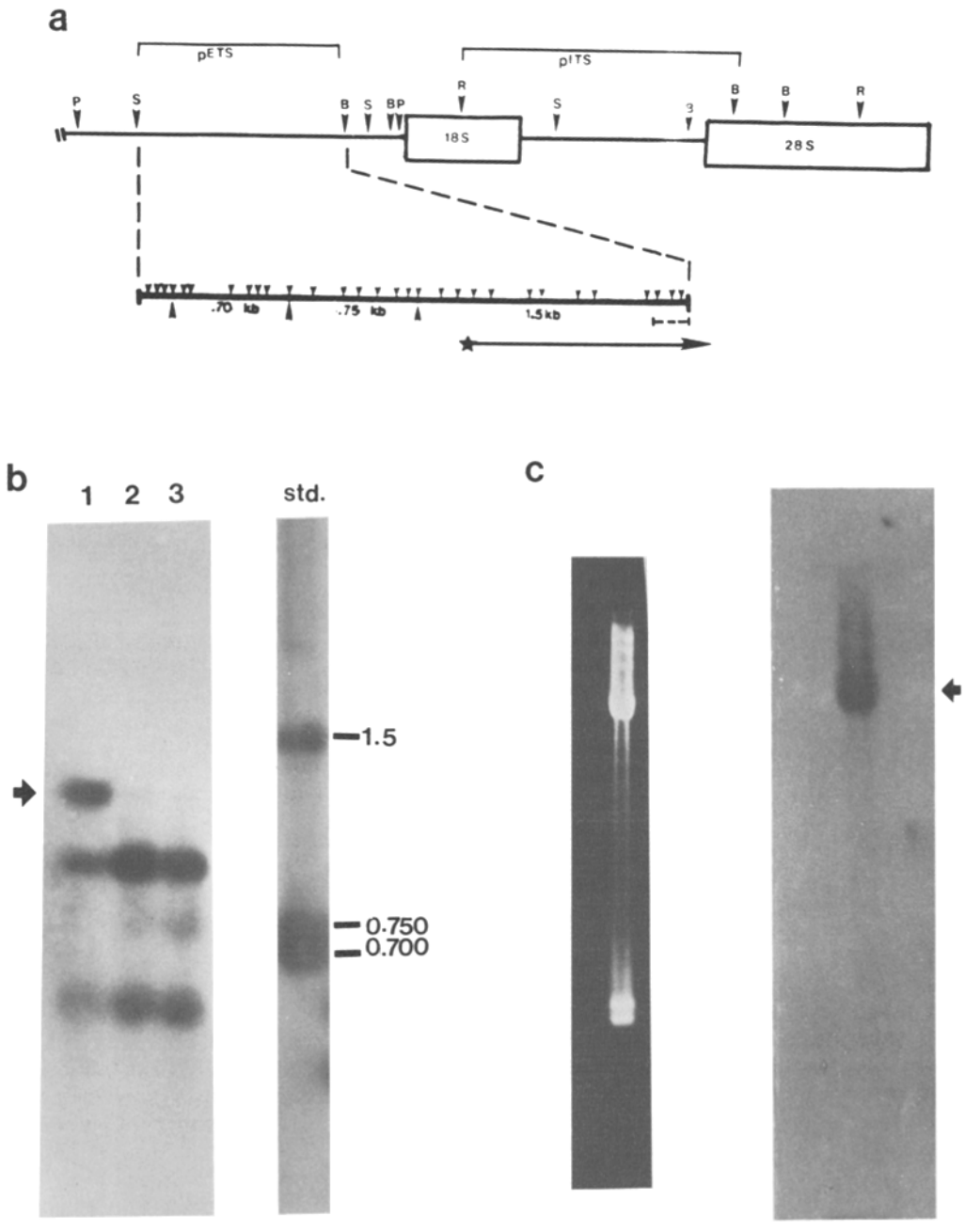

Figure 4. Map of chicken rDNA and determination of the pre-rRNA start site. (*a*) A partial restriction map of chicken rDNA is shown. The fragments corresponding to pETS and pITS are indicated by brackets. Restriction sites for Pst I (*P*), Sal I (*S*), Bam HI (*B*), and Eco RI (*R*) are indicated in upper map. The lower map shows details of the 3.2-kb Bam HI-Sal I fragment.Small arrows (above the line) indicate the location of the Hpa II/Msp I sites and large arrows (below the line) correspond to the Sma I sites. The approximate location of the site for initiation of transcription is indicated (★) and the probe used in the indirect end labeling for S1 mapping (below) is also shown (|---|). (*b*) Blot of S1 mapping experiment for determination of site of initiation of transcription. pETS was digested with Sma I plus Bam HI to generate the three fragments shown in standard lane (*std.*). Plasmid DNA was hybridized to total cellular RNA as described in Materials and Methods, and digested with 10 (lane *1*), 100 (lane *2*), and 1,000 (lane *3*) units of S1 nuclease. Fragments were subjected to electrophoresis in a 1% alkaline-agarose gel, transferred to blotting membrane, and hybridized to the probe indicated in *a*. Standards correspond to a lane in the same blot that was hybridized to pETS to detect all three Sma I fragments. The largest protected fragment (lane *1*, *arrow*) is ~300 bp smaller than the 1.5-kb Bam HI-Sma I fragment. Its 5′ end is presumed to correspond to the site of initiation of transcription. Further digestion with S1 nuclease (lanes *2* and *3*) results in the generation of small fragments that probably correspond to early processing intermediates. (*c*) Nuclear run-on experiment in which ^{32}P-labeled RNA that was synthesized by isolated nuclei in vitro was hybridized to pETS that was digested with Sma I, run on a 1% agarose gel (*left*) and transferred to blotting membrane. Only the fragment containing the 1.5-kb Bam HI-Sma I fragment (*arrow*) hybridizes to the RNA probe.

Bam HI-Sma I fragment (Fig. 4 *b*, lane *1*). This band decreased in intensity at higher S1 concentrations (lanes *2* and *3*), while other bands, which we assume result from rRNA processing intermediates, became the major species. Because of the rapid processing of the rRNA primary transcript, accurate S1 mapping using genomic RNA has posed some difficulties in other species, resulting in the mistaken identification of early processing sites as the initiation site (40). Although we cannot say with absolute certainty that the 5′ end of the larger fragment corresponds with the actual initiation site, we feel that, at least at the level of resolution shown here, it is a good estimate of the start site. This conclusion is supported by a second experiment (Fig. 4 *c*) in which ^{32}P-labeled RNA that was synthesized in isolated nuclei (as previously described) was used as a probe to filter-bound ETS plasmid that had been digested with Sma I. Neither upstream Sma I fragment could be detected on the resulting autoradiography, whereas the Bam HI-Sma I fragment showed a strong signal.

Recently, it was reported that sequences in the "nontranscribed spacer" DNA, upstream of the major rRNA transcription start site, are transcribed in *Xenopus* and mouse (20, 23, 37, 50). The RNAs derived from this region apparently are highly unstable and are believed to be only 1–10% as abundant as the rRNA primary transcript. We did not find evidence for transcription of upstream sequences in the chicken. However, it is likely that our nuclear run-on experiment was not sensitive enough to detect such low levels of RNA.

Because methylation of rDNA has been shown to be inversely correlated with gene activity in some species, we decided to see if any differences in methylation could be detected in the normal and aneuploid CEFs. In vertebrates, methylation of DNA occurs almost exclusively in the cytosine residue of the dinucleotide CpG. The restriction enzymes Hpa II and Msp I both recognize the sequence CCGG, but Hpa II will not cleave the DNA if the second cytosine is methylated. Msp I will cut both methylated and unmethylated sequences. By performing parallel digestions of genomic DNA with these two enzymes, it is possible to determine the extent of methylation of a sample. We found that the recognition sequence CCGG is highly repeated in the 3.2-kb Bam HI-Sal I fragment that contains the initiation site (Fig. 4 *a*). Thus, it appeared possible that methylation of these sequences might be involved in regulating expression of the rDNA. Blotting hybridization of disomic and tetrasomic DNA that was digested with either Msp I (Fig. 5 *a*, lanes *a* and *b*) or Hpa II (lanes *c* and *d*) and hybridized to a mixture of the two "upstream" Sma I fragments revealed that much of the DNA in this region was methylated. This is evidenced by the smearing present in the Hpa II lanes, resulting from a series of partial digests by the enzyme. This smearing is absent from the Msp I lanes. When this same filter was washed and re-probed with the 1.5-kb Bam HI-Sma I fragment (most of which is transcribed), no partial digest products were observed (Fig. 5 *b*). Thus, the methylated rDNA sequences appear to be restricted to sequences upstream from the start site, and absent from transcribed sequences. We have also found an identical methylation pattern in the rDNA of a second cell type, the mature erythrocyte (data not shown). However, for both the CEF and the erythrocyte rDNA, no differences in methylation between normal and aneuploid cells were observed.

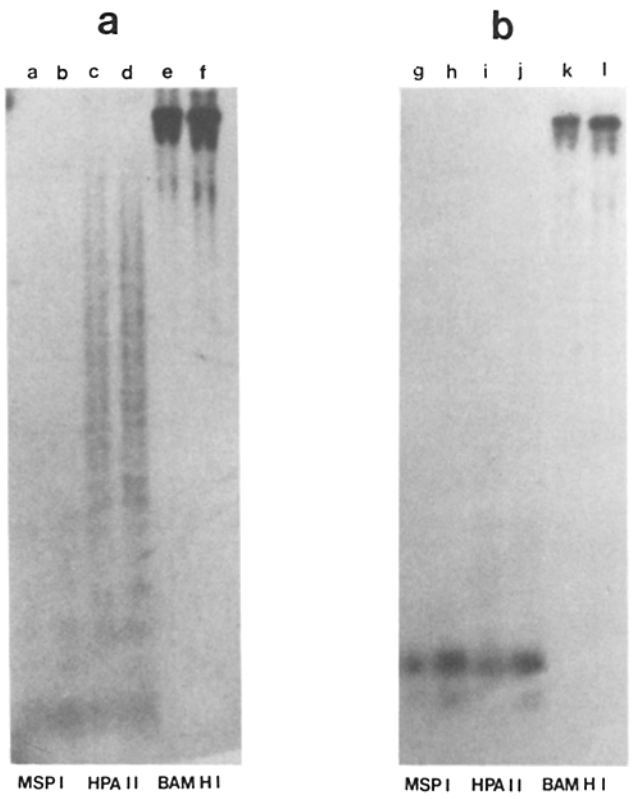

Figure 5. Methylation of chicken rDNA. (*a*) Approximately 10 μg of DNA from disomic (lanes *a*, *c*, and *e*) and tetrasomic (lanes *b*, *d*, and *f*) CEFs was digested with Msp I, Hpa II, or Bam HI, as indicated. (For Bam HI, 2 μg were run in each lane.) In all samples lambda DNA was added as carrier to ensure that complete digestion was obtained. The filter was hybridized to the 0.75-kb plus 0.70-kb upstream Sma I fragments (Fig. 4). A ladder of bands due to partial digestion by Hpa II, which does not cleave DNA at methylated sequences, can be seen (lanes *c* and *d*). No partial digest products are seen in the digest by Msp I, which is not sensitive to DNA methylation. (*b*) Same blot as in *a* after removal of first probe and reprobing with the 1.5-kb Sma I-Bam HI fragment. No partial digest products are present with either enzyme, indicating that unlike the upstream Sma I fragments, the Hpa II/Msp I sites in this region are not methylated.

Localization and Quantitation of Topoisomerase I on rDNA from CEFs

Because topoisomerase I is abundant on actively transcribed genes, we studied the distribution of this enzyme on the rDNA of normal and aneuploid CEFs. When cells are exposed to the compound camptothecin, a potent and highly specific inhibitor of topoisomerase I (14, 24), and then lysed with SDS, the enzyme remains covalently bound to the 3′ phosphoryl end of the DNA, resulting in the generation of single-strand DNA breaks. Treatment of the purified DNA with S1 nuclease, which cleaves the DNA strands opposite the topoisomerase I–induced nick, results in the generation of a double-strand break that can be easily detected by conventional electrophoresis and blotting hybridization (6, 16). Because topoisomerase I is particularly abundant in tran-

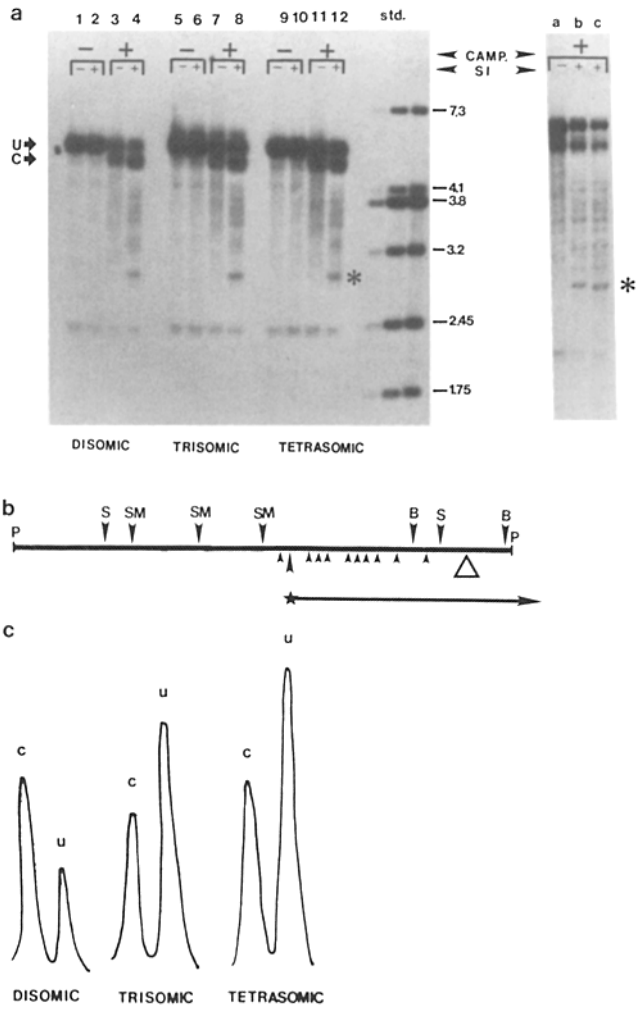

Figure 6. Camptothecin-induced cleavages in the ETS region of disomic, trisomic, and tetrasomic CEFs. (*a*) Equivalent amounts of genomic DNA (5 μg) from disomic (lanes *1–4*), trisomic (lanes *5–8*), and tetrasomic (lanes *9–12*) CEFs were digested with Pst I, subjected to electrophoresis in a 1% agarose gel, transferred to blotting membrane, and probed with pETS. Samples from cultures that were either treated with camptothecin (*+ CAMP.*; lanes *3, 4, 7, 8, 11*, and *12*) (20 μM for 5 min) or untreated controls (*− CAMP.*; lanes *1, 2, 5, 6, 9*, and *10*) are indicated. Samples in lanes *2, 4, 6, 8, 10*, and *12* were also treated with S1 nuclease (10 U/50 μl reaction for 30 min at 37°C). Cleaved (*C*) and uncleaved (*U*) Pst I fragment that was used for subsequent quantitation is also indicated. Size standards are also shown (*std.*). Insert panel (*right*) shows blot with higher resolution of single-strand cleavages in the ETS. Camptothecin-treated DNA samples from the same experiment were digested with 0 (lane *a*), 5 (lane *b*), and 10 (lane *c*) units of S1 nuclease. This and other blots probed with appropriate fragments for indirect end labeling were used to determine the sites for camptothecin-induced cleavages in the ETS which are shown in *b*. (*b*) Map of Pst I fragment in blot shown in *a* is shown, with restriction sites for Sal I (*S*), Bam HI (*B*), and Sma I (*SM*). Closed arrows indicate location of single-strand topoisomerase I cleavage sites. Large, closed arrow indicates position of the most prominent, single-strand site. A band corresponding to cleavage at this site is indicated by an asterisk in *a*. The large, open triangle corresponds to the double-strand cleavage site which reduces the 5.1-kb Pst I fragment by ~0.5 kb. The ratio of the cleaved (*C*) and uncleaved (*U*) fragment due to cutting at this site was used in the quantitation in Table III. (*c*) Densitometer scans of the cleaved (*C*) and uncleaved (*U*) Pst I fragments corresponding to lanes *4, 8*, and *12* (disomic, trisomic, and tetrasomic samples, respectively) from a lighter exposure of the autoradiograph shown in *a*. A similar amount of cleaved fragment is present for all three genotypes. In contrast, an increasing amount of uncleaved fragment is present in the two aneuploid samples.

scribed genes and may be present on both strands of the DNA, camptothecin induces double-strand breaks in addition to single-strand breaks (14). For the experiments described below, CEFs were exposed to 20 μM camptothecin for 5 min. Higher levels of the compound or longer exposure times did not enhance the amount of cleavage detected (data not shown). To detect single-strand breaks, DNA was also treated with 10 U of S1 nuclease for 30 min at 37°C. Under these conditions, S1 nuclease treatment alone did not cleave the rDNA with any specificity (Figs. 6 *a* and 7 *a*, lanes *2, 6*, and *10*).

An analysis of camptothecin–topoisomerase I cleavages in the ETS revealed that both double- and single-strand DNA breaks were generated in the rDNA. A prominent, double-strand cleavage site that maps ~0.7-kb upstream of the 18S coding region was detected in the samples treated with camptothecin only (lanes *3, 7*, and *11*). Cleavage at this site reduced the size of the original 5.1-kb Pst I fragment by ~0.5 kb, and accounts for the reduction of intensity of this fragment after camptothecin treatment. The single-strand breaks, which are visible only in the samples that were treated with camptothecin plus S1 nuclease (Fig. 6 *a*, lanes *4, 8*, and *12*), begin in the region of the transcription start site and continue into the ETS (Fig. 6 *b*). These single-strand cleavages represent a small proportion of the total amount of topoisomerase-induced cleavage of this fragment. (Although we did not detect cleavages in the upstream sequences in the CEFs, we did observe a few sites of campothecin-induced cleavage in the spacer region of MSB-1 cells, a cell line of Marek's virus–transformed T lymphocytes.)

When DNA samples from the same experiment were digested with Bam HI and probed with pITS (Fig. 7), little specificity of cleavage was detected. However, the dramatic reduction of the signal in the camptothecin-treated samples indicated that topoisomerase I was present in the coding region. Additional blotting experiments using indirect-end labeling of various restriction fragments revealed that there were several double-strand cleavage sites throughout the coding region (Fig. 7 *b*).

The amount of cleavage of the Pst I fragment from the ETS region was quantitated in disomic, trisomic, and tetrasomic cells by comparing the ratio of the uncleaved fragment to cleaved fragment within each sample. By visual inspection of this autoradiograph (Fig. 6 *a*), it is apparent that there is a greater amount of uncleaved fragment (*u*), compared with the cleaved fragment (*c*), in the trisomic (lanes *7* and *8*) and tetrasomic (lanes *11* and *12*) samples compared with the disomic samples (lanes *3* and *4*). Densitometry of this blot (Fig. 6 *c*) confirmed that proportionally more uncleaved fragment is present in the aneuploid cells. In addition, the amount of cleaved fragment is approximately the same in all three genotypes.

The amount of cleavage of the internal Bam HI fragment was also compared in the normal and aneuploid cells (Fig.

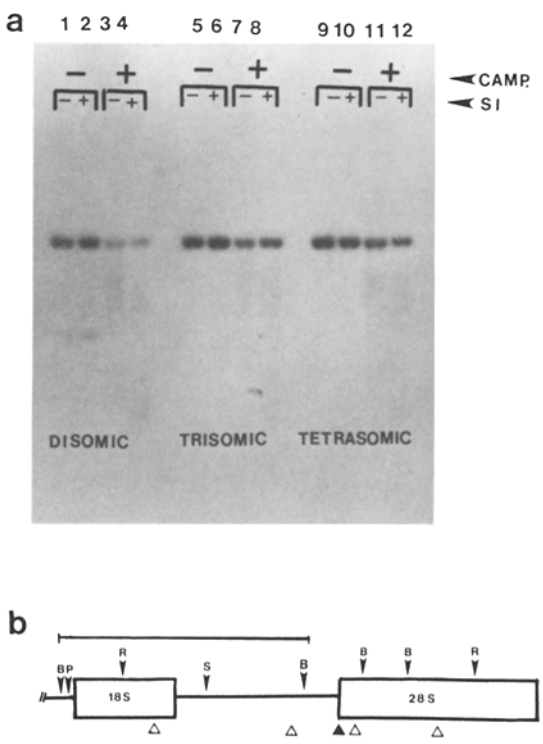

Figure 7. Camptothecin-induced cleavages in the internal region for disomic, trisomic, and tetrasomic CEFs. (*a*) DNA samples from same experiment described for Fig. 6 were digested with Bam HI and hybridized to the pITS. Treatment of samples in each lane is also the same as that for the previous figure. Extensive degradation of this fragment is illustrated by the lack of site-specific cleavages in the camptothecin treated samples (lanes *3*, *4*, *7*, *8*, *11*, and *12*). (*b*) Map of internal portion of the pre-rRNA coding region showing the site for Bam HI (*B*), Pst I (*P*), and Sal I (*S*). The Bam HI fragment in the blot shown in *a* is indicated by brackets. Additional blots obtained by indirect end labeling of fragments in this region indicated the presence of several topoisomerase I cleavage sites in this coding region. The closed triangle corresponds to a single-strand cleavage site, and the open triangles correspond to double-strand cleavage sites. (*c*) Densitometer tracings of autoradiograph pictured in *a* showing increased resistance to degradation of the Bam HI fragment in the aneuploid samples. The corresponding lane in the blot is indicated at the base of each peak. To normalize for the differences in the rDNA gene copy number among the three genotypes, the peaks in the control lanes (lanes *1*, *5*, and *9*) were adjusted to yield signals of equivalent intensity, and all other lanes for each genotype were scanned relative to the control peak.

7 *a*), by measuring the total amount of fragment present in the camptothecin-treated samples and in the untreated samples. Densitometry of this blot (Fig. 7 *c*) revealed that the reduction in signal due to camptothecin treatment was proportionally less in the trisomic (lanes *5–8*) and the tetrasomic (lanes *9–12*) samples, as compared with the disomic samples (lanes *1–4*).

We used the quantitative data obtained in the above experiments, plus data obtained from two additional trials, to arrive at an estimate for the number of transcriptionally active rRNA genes in the CEFs (Table III). These estimates are based on rRNA gene copy numbers of disomic, trisomic, and tetrasomic individuals that were determined in a previous study (44), in which the variation in copy number for individual animals measured within ±15% of the average copy number for each genotype. We believe that this variation may be, in part, due to actual differences in the number of rRNA genes among individual animals. Since the calculations in the present study are based on these numbers, we assume that they are also accurate within ±15%. We found that approximately the same number of genes (i.e., ~200 copies) are used in all three genotypes, despite the increase in total rDNA content in the trisomic and tetrasomic cells. These estimates are based on the assumption that rRNA genes with camptothecin-induced cleavage sites are active, and those without cleavage sites are inactive. That similar values were obtained for the ETS region and for the ITS region reinforces our interpretation that topoisomerase I cleavage sites occur throughout the transcriptional unit of the actively transcribed genes.

Discussion

We have previously shown that even though cells from trisomic and tetrasomic chickens have elevated levels of rDNA, the amount of mature rRNA per cell is regulated to diploid levels. Although regulation of initiation of transcription is the primary factor in the control of rRNA production in other cell types, posttranscriptional regulation also occurs (22, 25, 26). In the first portion of this study, we analyzed pre-rRNA synthesis and processing to determine at what level(s) rRNA production is regulated in aneuploid cells. We demonstrated that the amount of pre-rRNA per cell, as determined by blotting hybridization, is the same for all three genotypes. To ensure that rapid processing of the primary transcript does not account for the apparently similar levels of pre-rRNA, we analyzed pre-rRNA processing by pulse-chase labeling of the CEFs. The rate-limiting events in rRNA processing are: (*a*) the conversion of the 32S processing intermediate to the 28S and 5.8S rRNAs, and (*b*) the formation and transport of the large ribosomal subunit (22, 25, 26). Quantitation of the various rRNA intermediates in the CEFs indicated that no qualitative or quantitative differences in pre-rRNA processing are occurring in the trisomic and tetrasomic cells. These results do not exclude the possibility that excess pre-rRNA is synthesized in the aneuploid cells, but is rapidly degraded and therefore cannot be detected on gels. We determined the relative rates of rRNA synthesis by measuring the incorporation of label into rRNA by isolated nuclei that were incubated in vitro in the presence of α-amanitin. These results showed that normal and aneuploid CEFs incorporate

*Table III. Cleavage of rRNA Genes by Camptothecin**

Genotype	Total No. of genes per cell	Cleaved		Estimated No. of genes cleaved per cell	
		ITS	ETS	ITS	ETS
		%	%		
Disomic	290	61	70	177	203
Trisomic	420	49	40	206	168
Tetrasomic	570	38	40	217	228

* Values represent averages of two determinations (i.e., two independent blots) each, for the probe for the internal portion of the coding region (pITS) as shown in Fig. 7, and the region containing the ETS, as shown in Fig. 6. The values for percent cleaved for the ITS samples were determined by comparing the signal from the camptothecin-treated samples to the signal from the untreated control samples. The value for percent cleaved for the ETS samples was determined by comparing the amount of cleaved vs. uncleaved Pst I fragment within each lane for the camptothecin-treated samples. Total number of rRNA genes per cell was previously determined (44) and is accurate within ±15% of the values shown.

label into rRNA at similar rates. Together, the above experiments indicate that transcriptional regulation, not posttranscriptional regulation, of rRNA synthesis is occurring in the aneuploid cells.

There are two possible ways in which this transcriptional regulation can be achieved. The first alternative is that all of the rRNA genes in the aneuploid cells are transcribed, but the number of transcripts produced from each gene is reduced so that normal (i.e., disomic) amounts of pre-rRNA are made. Studies on mutants of *X. laevis* in which the rRNA gene copy number was reduced to less than the haploid level by partial deletions of the NORs showed that the amount of rRNA synthesized in mutant embryos was disproportionally less than expected (27, 39). The interpretation of these results was that the rRNA genes in the mutant embryos were transcribed less efficiently than those in the wild-type embryos. The second alternative is that the same number of rRNA genes are transcribed in disomic, trisomic, and tetrasomic cells, at similar rates, and the extra genes present in the aneuploid cells are inactive. This possibility could be achieved by the inactivation of the additional NORs in trisomic and tetrasomic cells. Such selective inactivation of extra NORs has been described in an aneuploid strain of *Drosophila* (29). However, the cytological results that we previously reported (4, 5, 44) preclude this possibility; all of the NORs are transcriptionally active in cells from aneuploid chickens and remain active in the cultured CEFs. Therefore, if the aneuploids contain a larger number of inactive rRNA genes than the disomic cells, the inactive genes must be distributed among all of the NORs. We have distinguished between these two models by comparing the chromatin structure of the rRNA genes in normal and aneuploid cells, and by determining the relative amount and distribution of topoisomerase I on the rDNA of disomic, trisomic, and tetrasomic CEFs.

Transcriptionally active sequences exhibit an increase in sensitivity to digestion by various nonspecific endonucleases such as DNase I and micrococcal nuclease, as compared with nontranscribed sequences (11, 30, 53, 56). This is presumably due to differences in the chromatin structure of active vs. inactive genes. Such nuclease sensitivity appears to be an all-or-none phenomenon. For example, genes that are transcribed at low levels in the chick oviduct display DNase I digestion profiles similar to that for the ovalbumin gene, which is transcribed at a high rate (11). If the first possibility described above were true, we would expect that all of the rRNA genes in normal and aneuploid cells would possess a chromatin structure characteristic of transcribed genes, and therefore, would be equally accessible to digestion by DNase I. We have consistently found that the rDNA of aneuploid CEFs is more resistant to digestion by DNase I than the rDNA from disomic CEFs. This suggests that a fraction of the rRNA genes is transcriptionally inactive in the aneuploid cells.

We compared two features of the rDNA in normal and aneuploid cells that could potentially provide further evidence for this hypothesis: the methylation of rDNA and the distribution of topoisomerase I on rDNA. Methylation of rDNA is inversely related to gene activity. For example, methylated rDNA in nuclei isolated from mouse liver was found to be more resistant to digestion by DNase I than the non-methylated fraction, suggesting that a different chromatin configuration is associated with the methylated sequences (7). Heavily methylated rDNA was also observed in a rat hepatoma cell line that possesses a 10-fold amplification of rDNA (41, 51). Despite an increase in rDNA content, the hepatoma cells had diploid levels of rRNA. An analysis of the amplified chromosomal region by staining with silver nitrate (which only stains transcribed rDNA) and by fluorescent antibody staining using an antibody to 5-methyl cytosine, showed that there is a reciprocal relationship between the silver-positive region of the NOR and regions that are stained by the antibody. Blotting analysis of the rDNA confirmed that most of the rDNA in the hepatoma cells is heavily methylated.

We found that the recognition sequence for Hpa II/Msp I (CCGG) is highly repeated in the region near the initiation of transcription for chicken rDNA. Blotting analysis of Hpa II–digested DNA revealed a ladder consisting of a heterogeneous array of fragments. That this was not present in the Msp I digests suggests that these fragments were the result of partial digests of DNA by Hpa II due to the presence of 5-methyl cytosine. Probing these digests with different restriction fragments around the rRNA start site revealed that the methylated sequences are present upstream from the initiation site, not in the transcribed region. However, the distribution of methylated sequences is quantitatively and qualitatively the same in normal and aneuploid CEFs, indicating that the excess genes in aneuploid cells are not hypermethylated. In many cases, it appears that demethylation of DNA is only one of several factors necessary for the expression of a gene (8). Thus, the lack of methylation in the coding region of all of the rRNA genes in the aneuploid cells may indicate that these genes have the potential to be expressed, or may

have been expressed in some cells, or at some point in development. This is supported by our observation that rDNA in the mature erythrocyte, a cell that does not transcribe rRNA, shows a methylation pattern that is identical to the CEFs.

Based on recent evidence that the presence of topoisomerase I on a gene is directly correlated with the transcriptional activity of that gene, we decided that a potentially useful approach to distinguish between active and inactive genes in our system would be to determine the distribution of topoisomerase I on the rDNA. Topoisomerase I is present on actively transcribed DNA sequences. Immunofluorescence studies in *Drosophila* (10) and in chicken cells (43) have shown that the enzyme is particularly abundant in the nucleolus, presumably due to the high level of transcription and high copy number of the rRNA genes. The localization of topoisomerase I on *Drosophila* heat shock genes has been achieved using two techniques: (*a*) SDS-induced cleavage of topoisomerase–DNA complexes in camptothecin-treated cells (14) and (*b*) UV-crosslinking followed by immunoprecipitation of topoisomerase–DNA complexes (15). Both of these studies showed that topoisomerase I is absent, or present in very low amounts, in noncoding regions of these genes, and from the coding regions in non-heat shocked cells. The latter study has shown that although there is not necessarily a one-to-one correlation between the number of molecules of polymerase and topoisomerase on a particular gene, topoisomerase I may be as abundant as polymerase.

We have shown that camptothecin treatment of CEFs results in the generation of multiple- and double-strand breaks throughout the rDNA coding region, as well as a large decrease in the intensity of the original signal, corresponding to the uncleaved band in the autoradiograph. Therefore, topoisomerase I appears to be an abundant enzyme in the coding region of the chicken rDNA.

We used the camptothecin-induced cleavage of topoisomerase I–DNA complexes in the CEFs to compare the relative proportion of cleaved, and presumably transcribed genes, to uncleaved, and presumably inactive genes, per cell. Camptothecin cleaves ~10% of the topoisomerase I–DNA complexes in vitro (24). We do not know the efficiency of cleavage by camptothecin alone, or by camptothecin plus S1 nuclease, in vivo. We assume that our values represent a minimum estimate of the number of topoisomerase I–containing genes per cell.

We found that the same number, ~200 rRNA genes, are cleaved per cell, regardless of the genotype. Thus, for disomic cells, ~70% of the total number of rRNA genes are cleaved, whereas in tetrasomic cells, ~40% of the rRNA genes are cleaved. The observation that a third of the rRNA genes in the disomic cells are not cleaved by camptothecin may indicate that some of the topoisomerase I–containing genes are not cleaved in our experiments. However, it may also mean that even in the rapidly dividing CEFs, not all of the rRNA genes are used. This is not surprising since viable individuals are obtained when up to half of the rDNA has been deleted in *Drosophila* (47) and in *Xenopus* (46).

These results provide evidence for the hypothesis that regulation of rRNA synthesis is achieved by the selective activation of a defined number of rRNA genes. This hypothesis is strongly supported by data obtained in vitro showing that specific factors must bind to the rRNA promoter before transcription by RNA polymerase I can occur (50). The use of topoisomerase I–DNA complexes as an indicator for gene activity provides a useful means to assess the transcriptional activity of this, and possibly other, multigene families in vivo. To this end the trisomic strain of chickens used in this study provides a unique opportunity to study the regulation of rRNA genes in vivo in cells with varying rDNA contents.

The authors thank Drs. I. Mattaj and A. Skalka for providing the chicken rDNA clones, Dr. D. Gilmour for sharing results with us before publication, and the Drug Synthesis and Chemistry Branch, National Cancer Institute, National Institutes of Health, for providing the camptothecin. We also thank Ms. Ellen Willand for technical support and Ms. Diane Colf for editorial assistance.

This research was supported by grants from the Cornell University Biotechnology Program, the United States Department of Agriculture (NY-157433), and the National Institutes of Health (GM-31577).

Received for publication 18 March 1987, and in revised form 29 June 1987.

References

1. Abelson, H. T., L. F. Johnson, S. Penman, and H. Green. 1974. Changes in RNA in relation to growth of the fibroblast. II. The lifetime of mRNA, rRNA and tRNA in resting and growing cells. *Cell.* 1:161–165.
2. Barry, J., and J. Gorsky. 1971. Uterine ribonucleic acid polymerase: effect of estrogen on nucleotide incorporation into 3′ chain termini. *Biochemistry.* 10:2384–2390.
3. Bieker, J. J., P. L. Martin, and R. G. Roeder. 1985. Formation of a rate-limiting intermediate in 5S gene transcription. *Cell.* 40:119–127.
4. Bloom, S. E. 1979. Linkage relationships: chicken. *Fed. Proc. Am. Soc. Exp. Biol.* 2:620–622.
5. Bloom, S. E., and L. D. Bacon. 1985. Linkage of the major histocompatibility (B) locus and the nucleolus organizer in the chicken: Assignment to a microchromosome. *J. Heredity.* 76:146–154.
6. Bonven, J. B., E. Gocke, and O. Westergaard. 1985. A high affinity topoisomerase I binding sequence is clustered at DNase I hypersensitive sites in *Tetrahymena* R-chromatin. *Cell.* 41:541–551.
7. Davis, A. H., T. L. Reudelhuber, and W. T. Garrad. 1983. Variegated chromatin structures of mouse ribosomal RNA genes. *J. Mol. Biol.* 160:133–155.
8. Doerfler, W. 1983. DNA methylation and gene activity. *Annu. Rev. Biochem.* 59:193–224.
9. Fetherston, J., E. Werner, and R. Patterson. 1984. Processing of the external transcribed spacer of murine rRNA and site of action of actinomycin D. *Nucleic Acids Res.* 12:7187–7198.
10. Fleischmann, G., G. Pflugfelder, E. K. Steiner, K. Javaherian, G. C. Howard, J. C. Wang, and S. C. R. Elgin. 1984. *Drosophila* DNA topoisomerase I is associated with transcriptionally active regions of the genome. *Biochemistry.* 81:6958–6962.
11. Garel, A., and R. Axel. 1977. The structure of transcriptionally active ovalbumin genes in chromatin. *Cold Spring Harbor Symp. Quant. Biol.* XLII:701–708.
12. Gargiulo, G., R. Farzana, and A. Worcel. 1984. Assembly of transcriptionally active chromatin in *Xenopus* oocytes requires specific DNA binding factors. *Cell.* 38:511–521.
13. Gausing, K. 1977. Regulation of ribosome production in *E. coli*: synthesis and stability of ribosomal protein messenger RNA at different growth rates. *J. Mol. Biol.* 115:335–354.
14. Gilmour, D. S., and S. R. C. Elgin. 1987. Localization of specific topoisomerase I interactions within the transcribed region of active heat shock genes using the inhibitor camptothecin. *Mol. Cell Biol.* 7:141–148.
15. Gilmour, D. S., G. Pflugfelder, J. C. Wang, and J. T. Lis. 1986. Topoisomerase I interacts with transcribed regions in *Drosophila* cells. *Cell.* 44:401–407.
16. Gocke, E., B. J. Bonven, and O. Westergaard. 1983. A site and strand specific nuclease activity with analogies to topoisomerase I frames the rRNA gene of *Tetrahymena*. *Nucleic Acids Res.* 11:7661–7678.
17. Grummt, I. 1975. Synthesis of RNA molecules larger than 45S by isolated rat-liver nucleoli. *Eur. J. Biochem.* 57:159–167.
18. Grummt, I. 1982. Nucleotide sequence requirements for specific initiation of transcription by RNA polymerase I. *Proc. Natl. Acad. Sci. USA.* 79:6908–6911.
19. Grummt, I., and R. Lindigkeit. 1973. Pre-ribosomal RNA synthesis in isolated rat-liver nucleoli. *Eur. J. Biochem.* 36:244–249.
20. Grummt, I., A. Kuhn, I. Bartsch, and H. Rosenbauer. 1986. A transcriptional terminator located upstream of the mouse rDNA initiation site affects rRNA synthesis. *Cell.* 47:901–911.
21. Grummt, I., V. A. Smith, and F. Grummt. 1976. Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase. *Cell.* 7: 436–445.

22. Hadjiolov, A. A., and N. Nikolaev. 1976. Maturation of ribosomal ribonucleic acids and the biogenesis of ribosomes. *Prog. Biophys. Mol. Biol.* 31:95–144.
23. Henderson, S., and B. Sollner-Webb. 1986. A transcriptional terminator is a novel element of the promotor of the mouse ribosomal RNA gene. *Cell.* 47:891–900.
24. Hsaing, Y.-H., R. Hertzberg, S. Hecht, and L. F. Liu. 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *J. Biol. Chem.* 260:14873–14878.
25. Johnson, L. F., H. T. Abelson, H. Green, and S. Penman. 1974. Changes in RNA in relation to growth of the fibroblast I: Amounts of mRNA, rRNA, and rRNA in resting and growing cells. *Cell.* 1:96–100.
26. Johnson, L. F., R. Levis, H. T. Abelson, H. Green, and S. Penman. 1976. Changes in RNA in relation to growth of the fibroblast IV. Alterations in the production and processing of mRNA and rRNA in resting and growing cells. *J. Cell Biol.* 71:933–938.
27. Knowland, J. S., and L. Miller. 1970. Reduction of ribosomal RNA synthesis and ribosomal RNA genes in a mutant of *Xenopos laevis* which organizes only a partial nucleolus. I. Ribosomal RNA synthesis in embryos of different nucleolar types. *J. Mol. Biol.* 53:321–328.
28. Kohorn, B. D., and P. M. M. Rae. 1982. Accurate transcription of truncated ribosomal DNA templates in a *Drosophila* cell-free system. *Proc. Natl. Acad. Sci. USA.* 304:179–181.
29. Krider, H. M., and W. Plaut. 1972. Studies on nucleolar RNA synthesis in Drosophila melanogaster I: the relationship between the number of nucleolar organizers and rate of synthesis. *J. Cell Sci.* 11:675–687.
30. Lacy, E., and R. Axel. 1975. Analysis of DNA isolated chromatin subunits. *Proc. Natl. Acad. Sci. USA.* 72:3978–3982.
31. Learned, R. M., and R. Tjian. 1982. *In vitro* transcription of human ribosomal RNA genes by RNA polymerase I. *J. Mol. Appl. Genet.* 1:575–584.
32. Macleod, D., and A. Bird. 1983. Transcription in oocytes of highly methylated rDNA from *Xenopus laevis* sperm. *Nature (Lond.).* 306:200–203.
33. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY.
34. Mattaj, I. W., A. Mackenzie, and J.-P. Jost. 1982. Cloning in a cosmid vector of complete 37 kb ribosomal DNA repeat units from the chicken. *Biochim. Biophys. Acta.* 698:204–210.
35. McClements, W., and A. M. Skalka. 1977. Analysis of chicken ribosomal RNA genes and construction of lambda hybrids containing gene fragments. *Science (Wash. DC).* 196:195–197.
36. McKnight, S. L., M. Bustin, and O. L. Miller, Jr. 1977. Electron microscopic analysis of chromosome metabolism in the *Drosophila melanogaster* embryo. *Cold Spring Harbor Symp. Quant. Biol.* XLII:741–754.
37. McStay, B., and R. H. Reeder. 1986. A termination site for *Xenopus* RNA polymerase I also acts as an element of an adjacent promotor. *Cell.* 47:913–920.
38. Miesfeld, R., and N. Arnheim. 1984. Species-specific rDNA transcription is due to promotor-specific binding factors. *Mol. Cell Biol.* 4:221–227.
39. Miller, L., and J. Knowland. 1972. The number and activity of ribosomal RNA genes in *Xenopus laevis* embryos carrying partial deletions in both nucleolar organizers. *Biochem. Genet.* 6:65–73.
40. Miller, K. G., and B. Sollner-Webb. 1981. Transcription of mouse rRNA genes by RNA polymerase I: *In vitro* and *in vivo* initiation and processing site. *Cell.* 27:165–174.
41. Miller, O. J., R. Tantravahi, D. A. Miller, L.-C. Yu, P. Szabo, and W. Prensky. 1979. Marked increase in ribosomal RNA gene multiplicity in a rat hepatoma cell line. *Chromosoma (Berl.).* 71:183–195.
42. Mishima, Y., L. Financsek, R. Kominami, and M. Muramatsu. 1982. Fractionation and reconstitution of factors required for accurate transcription of mammalian ribosomal RNA genes: identification of a species-dependent factor. *Nucleic Acids Res.* 10:6659–6670.
43. Muller, M. T., W. P. Pfund, V. B. Mehta, and D. K. Trask. 1985. Eukaryotic type I topoisomerase is enriched in the nucleolus and catalytically active on ribosomal DNA. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1237–1243.
44. Muscarella, D. E., V. M. Vogt, and S. E. Bloom. 1985. The ribosomal RNA gene cluster in aneuploid chickens: evidence for increased gene dosage and regulation of gene expression. *J. Cell Biol.* 101:1749–1756.
45. Perry, R. 1963. Selective effects of actinomycin D on the intracellular distribution of RNA synthesis in tissue culture cells. *Exp. Cell Res.* 29: 400–406.
46. Reeves, R. 1977. Structure of *Xenopus* ribosomal RNA gene chromatin during changes in genomic transcription rates. *Cold Spring Harbor Symp. Quant. Biol.* XLII:709–722.
47. Ritossa, F. M., K. C. Atwood, and S. Spiegelman. 1966. A molecular explanation of the bobbed mutants of *Drosophila* as partial deficiencies of rDNA. *Genetics.* 54:819–834.
48. Rudland, P. S., S. Weil, and A. R. Hunter. 1975. Changes in RNA metabolism and accumulation of presumptive messenger RNA during transition from the growing to the quiescent state of cultured mouse fibroblasts. *J. Mol. Biol.* 160:133–146.
49. Schlissel, M. S., and D. D. Brown. 1984. The transcriptional regulation of *Xenopus* 5S RNA genes in chromatin: the roles of active stable transcription complexes and histone H1. *Cell.* 37:903–913.
50. Sollner-Webb, B., and J. Tower. 1986. Transcription of cloned eukaryotic ribosomal RNA genes. *Annu. Rev. Biochem.* 55:801–830.
51. Tantravahi, R. V., R. V. Guntaka, B. F. Erlanger, and O. J. Miller. 1981. Amplified ribosomal RNA genes in a rat hepatoma cell line are enriched in 5-methyl cytosine. *Proc. Natl. Acad. Sci. USA.* 78:489–493.
52. Todaro, G., and H. Green. 1963. Quantitative studies on the growth of mouse embryo cells in culture and their development to established lines. *J. Cell Biol.* 17:299–313.
53. Weisbrod, S. 1982. Active chromatin. *Nature (Lond.).* 297:289–295.
54. Wellauer, P. K., and I. B. Dawid. 1973. Secondary structure maps of RNA-processing of HeLa rRNA. *Proc. Natl. Acad. Sci. USA.* 70:2827–2831.
55. Wellauer, P. K., I. B. Dawid, D. E. Kelly, and R. P. Perry. 1974. Secondary structure maps of ribosomal RNA II: Processing of mouse L cell rRNA and variations in the processing pathway. *J. Mol. Biol.* 89:397–407.
56. Wu, C., C. Y. Wong, and S. C. R. Elgin. 1979. The chromatin structure of specific genes II: distribution of chromatin structure during gene activity. *Cell.* 19:807–814.