MOLECULAR AND CELLULAR BIOLOGY, Mar. 2003, p. 1558–1568 Vol. 23, No. 5
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.5.1558–1568.2003

In Exponentially Growing *Saccharomyces cerevisiae* Cells, rRNA Synthesis Is Determined by the Summed RNA Polymerase I Loading Rate Rather than by the Number of Active Genes

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Received 26 August 2002/Returned for modification 3 October 2002/Accepted 13 December 2002

Genes encoding rRNA are multicopy and thus could be regulated by changing the number of active genes or by changing the transcription rate per gene. We tested the hypothesis that the number of open genes is limiting rRNA synthesis by using an electron microscopy method that allows direct counting of the number of active genes per nucleolus and the number of polymerases per active gene. Two strains of *Saccharomyces cerevisiae* were analyzed during exponential growth: a control strain with a typical number of rRNA genes (~143 in this case) and a strain in which the rRNA gene number was reduced to ~42 but which grows as well as controls. In control strains, somewhat more than half of the genes were active and the mean number of polymerases/gene was ~50 ± 20. In the 42-copy strain, all rRNA genes were active with a mean number of 100 ± 29 polymerases/gene. Thus, an equivalent number of polymerases was active per nucleolus in the two strains, though the number of active genes varied by twofold, showing that overall initiation rate, and not the number of active genes, determines rRNA transcription rate during exponential growth in yeast. Results also allow an estimate of elongation rate of ~60 nucleotides/s for yeast Pol I and a reinitiation rate of less than 1 s on the most heavily transcribed genes.

The regulation of rRNA synthesis is intimately tied to regulation of cell growth rate, and synthesis of rRNA by RNA polymerase I is the major transcriptional activity of the cell, accounting for 60% of total transcription in rapidly growing yeast cells (56). In spite of this critical role, certain aspects of Pol I transcription are still poorly understood (reviewed in references 12, 30, 33, 34, 43, and 56). The situation is complicated by the fact that the genes encoding rRNA are multicopy. Thus, rRNA synthesis could be modulated by varying the transcription rate per gene or by varying the number of active genes (1). Electron microscopy (EM) visualization of active rRNA genes from many different cell types by the Miller spreading method typically shows genes rather heavily packed with polymerases and nascent rRNA transcripts in the familiar Christmas tree configuration (27, 28, 49). Since these active rRNA genes are highly transcribed, it appears, that once a gene is open for transcription, there is no shortage of Pol I for initiation. Based largely on EM observations, two recent review articles have indeed concluded that Pol I transcription does not appear to be limited by initiation rate (30, 43). There is additional direct support for this idea from other EM studies (24, 38). For example, polymerase density is uniformly high on any rRNA gene that is activated after introduction into *Xenopus* oocytes, even when the introduced genes are in large excess over endogenous genes (38). These observations and others led to a model in which initiation-competent Pol I is present in excess in actively growing cells and in which rRNA synthesis is regulated by controlling the number of active genes (43). In this model, each gene is a "binary unit" that is either on or off and, if on, is producing rRNA at approximately the same rate as other active genes due to high Pol I loading.

Other evidence besides EM is consistent with rRNA transcription being regulated by changing the number of active genes. Exponentially growing cells use only half or fewer of their total complement of rRNA genes (6, 7, 17, 22), and it has been shown in mammalian and budding yeast cells that the number of active genes decreases when cells undergo the transition from log to stationary phase (7, 13). It was recently demonstrated that this gene inactivation in yeast (as determined by accessibility to psoralen cross-linking) is dependent on the histone deacetylase Rpd3 (46). In mammalian cells, the nucleolar remodeling complex, NoRC, recruits HDAC1 and DNA methyltransferases to inactive rRNA gene repeats (48). Combined with reports showing various correlations between chromatin modifications and the activity level of rRNA genes (e.g., references 16, 31, 40, 47, and 52), it is clear that epigenetic mechanisms either control or enforce the ratio of active to inactive genes. In NIH 3T3 cells, chemical inhibitors of DNA methylation and histone deacetylation result in an increase in endogenous rRNA transcription (16, 47). One interpretation of these findings is that the number of active genes, and not initiation by Pol I, is limiting for rRNA transcription, consistent with the model based on EM visualization of genes.

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On the other hand, a large body of evidence points to a specific Pol I transcription factor, Rrn3p in yeast or its mammalian homologue hRrn3 or TIF-1A, as being the growth regulatory factor for rRNA synthesis (3, 25, 29, 58). This protein functions by binding directly to Pol I (20, 58) in an interaction controlled by phosphorylation (5, 10) and renders Pol I competent for initiation (25, 58), apparently by bridging the interaction between Pol I and other components of the preinitiation complex (26, 39). Only a small fraction of both Pol I and Rrn3p is in the active complex, and the active Pol I-Rrn3p complex has been shown to be limiting for rRNA transcription in stationary or growth-arrested cells (3, 25, 50). Thus, at least during down-regulation, Pol I transcription is regulated by the concentration of the Pol I-Rrn3p complex and thus by initiation rate, rather than by the number of active genes. As predicted, this results in a lower average polymerase density on active rRNA genes in post-log-phase cells (46). What is not known is whether the concentration of the active Pol I-Rrn3p complex is also limiting for transcription in exponentially growing cells. A clear prediction of the binary-unit model is that the active Pol I-Rrn3p complex is present in excess in logarithmically growing cells such that the number of genes open for transcription is limiting.

We addressed this question in exponentially growing *Saccharomyces cerevisiae* cells. A published observation seemed difficult to reconcile with the prevailing model, in which each gene is a binary unit of activity. That is, experiments in yeast cells showed that it was possible to decrease the total number of rRNA genes from the typical number of ~150 to ~40 without affecting cell growth rate or rRNA synthesis rate (21). Since psoralen cross-linking assays indicate that about half of the total number of genes (or ~75 genes) are active in wild-type yeast cells (7), the prediction of the binary unit model would be that wild-type cells with ~75 active rRNA genes would have a higher rRNA synthesis rate than cells with only ~40 rRNA genes total. However, given uncertainties about the psoralen cross-linking assay discussed elsewhere (2), such as the possibility that a psoralen-accessible gene may be in an open chromatin configuration but not yet transcribed, it remained possible that the number of transcribing genes was regulating rRNA synthesis. We undertook a new approach to address this long-standing question. We directly counted the number of active genes per nucleolus and the number of polymerases per gene. When gene number was reduced, the available polymerases loaded with a higher density on fewer genes to produce the same amount of rRNA as in control cells. On some genes in the reduced copy strain, there was a polymerase every 41 nucleotides (nt), which is near the limit possible for polymerase packing. Our results show that rRNA synthesis in exponentially growing yeast cells is controlled by the ability of cells to load polymerases and not by the number of open genes.

MATERIALS AND METHODS

Yeast strains and media. The following strains were used.

(i) NOY886 (NOY886^{42C}). The genotype of NOY886 is *MAT*α *rpa135*Δ::*LEU2 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 fob1*Δ::*HIS3* pNOY117 (*CEN RPA135 TRP1*); the number of ribosomal DNA (rDNA) repeats is ≅42. The construction of this strain was described previously without giving the strain name (21) and is explained in Results.

(ii) NOY1051 (NOY1051^{143C}). NOY1051 has the same genotype as NOY886 except for the number of rDNA repeats (≅143) and was constructed from NOY408-1a by first introducing pNOY117 and allowing rDNA repeat expansion, followed by removal of the helper *GAL7-35S rDNA* plasmid used to support the growth of NOY408-1a (32) and disruption of *FOB1* to stabilize the expanded rDNA repeat number.

(iii) JS772. The genotype of JS772 is *MAT***a** *ura3*Δ*0 his3*Δ*0 leu2*Δ*0* (derivative of S288C [see reference 57]).

Yeast-peptone-dextrose (YPD) medium used for growing yeast cells was described previously (19).

Miller chromatin spreading and EM. Miller chromatin spreads (28) were prepared after brief digestion of the yeast cell wall with zymolyase followed by hypotonic lysis (42). Yeast was grown in YPD plus 1 M sorbitol to mid-log phase (A_{600} = 0.35 to 0.55) at 30°C. One-milliliter volumes of the culture were pipetted into prewarmed tubes containing 5 mg of zymolyase (T20; U.S. Biologicals), which were then shaken at 30° for 4 min to allow partial digestion of the cell wall. The cells were spun at maximum speed in a microcentrifuge for 6 s, and the pellet was resuspended in 1 ml of lysis solution (0.025% Triton X-100, pH 9), which was then further diluted with an additional 3 ml of lysis solution. The samples were swirled gently for 20 min to allow for dispersal of cellular contents before being fixed by the addition of 0.4 ml of 0.1 M sucrose–10% formalin. The lysates were then centrifuged onto carbon-coated EM grids, stained with phosphotungstic acid and uranyl acetate, and rotary shadow cast as described (37). Grids were examined in a JEOL 100CX electron microscope.

Analysis of EM data. Multiple chromatin spreads from multiple cultures of yeast cells were visualized for each strain, and two to four Miller spread preparations were quantitatively analyzed for each strain. For quantitative analysis, entire EM grids were scanned and all yeast cells captured on that grid were photographed along with their associated chromatin strands. For the two control strains, NOY1051^{143C} and JS772, the polymerase number/gene was determined by counting the number of RNA polymerases (or nascent RNA transcripts) on all rRNA genes that could be unambiguously followed from the 5′ to the 3′ end from the entire grid database. For NOY886^{42C}, the polymerase number was counted on 174 genes randomly selected from several different preparations.

Other methods. Analysis of chromosome XII by contour-clamped homogeneous electric field (CHEF) electrophoresis was carried out as described previously (35). Analysis of 5′ ends of precursor rRNA by primer extension was carried out as described previously (54). Total cellular RNA was determined by orcinol reaction (51) after extraction of RNA with 5% tricarboxylic acid at 100°C for 15 min.

RESULTS

Two yeast strains with ~140 and ~40 copies of the rRNA gene grow at similar rates. To investigate the relative role of active gene number versus the transcription rate per gene in determining total rRNA synthesis, we took advantage of a yeast strain in which the total rRNA gene number had been decreased to a number below the ~75 genes predicted to be active in wild-type cells. We first obtained a more accurate count of the gene number in this reduced-copy-number strain and also confirmed that the reduction in gene number did not change the cell's growth rate compared to the relevant control cells, as was shown previously for a similar pair of strains (21). The reduction in gene number originally occurred in cells that were unable to transcribe rDNA by Pol I due to disruption of the *RPA135* gene (NOY408-1a; see reference 32), which encodes the second largest subunit of yeast Pol I. In these cells, rRNA was transcribed by Pol II from a multicopy plasmid containing the 35S rRNA-coding region fused to the *GAL7* promoter. Strain NOY886 was constructed by first stabilizing the reduced rRNA gene numbers by disruption of the *FOB1* gene, followed by introduction of the intact *RPA135* gene on a *CEN* plasmid, restoring the intact Pol I system for rDNA transcription, and allowing curing of the multicopy *GAL7-35S rDNA* plasmid. The control strain for this study, NOY1051, was constructed from the same strain (NOY408-1a) with a reduced rRNA gene copy number by introducing the *CEN* plasmid carrying the *RPA135* gene first and allowing an rDNA

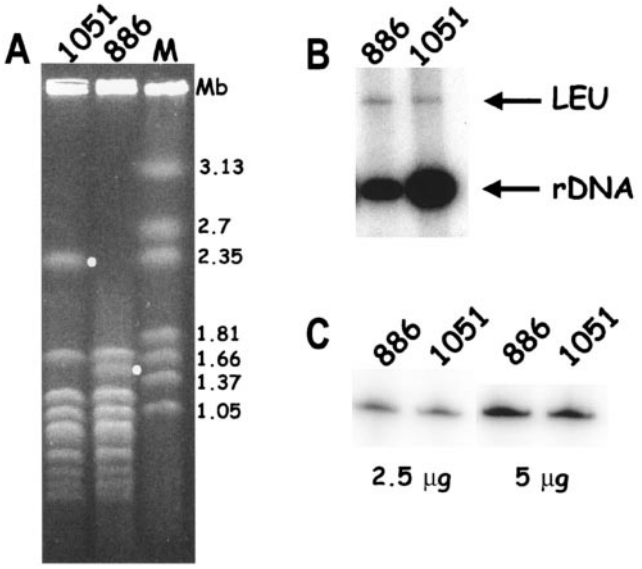

FIG. 1. Comparison of the reduced gene copy strain, NOY886, with a control strain, NOY1051, for size of chromosome XII (A), rDNA copy number (B), and rRNA synthesis rate (C). (A) Chromosomal DNA was isolated and analyzed by CHEF electrophoresis. An ethidium bromide-stained gel is shown. Positions of chromosome XII are indicated by a white dot. Size markers (lane M) are *Hansenula wingei* chromosomes. When these markers were used, the sizes of chromosome XII estimated for NOY1051^{143C} and NOY886^{42C} were approximately 2.4 and 1.48 Mb, respectively, which correspond to rDNA repeat numbers of 143 and 42, respectively. (B) For Southern analysis, DNA was isolated and digested with *Hin*dIII and *Pst*I and digests were subjected to agarose gel electrophoresis, followed by transfer to a nylon membrane, and were then subjected to hybridization first with a *LEU2* probe (a 598-bp *Cla*I-*Eco*RV fragment) and then with an rDNA probe (a 613-bp *Sma*I-*Eco*RV fragment). An autoradiogram is shown. Radioactivity in each band was quantified with a PhosphorImager. The ratios of the values for rDNA after normalization to the values for reference *LEU2* DNA (the *LEU2* gene used for disruption of *RPA135*) were calculated. The ratios of the amount of rDNA for NOY1051^{143C} to that for NOY886^{42C} were then calculated, and the average ratio from five independent experiments was 3.0 ± 0.3. (C) RNA was isolated from cells, and 2.5 and 5.0 μg were used to determine the amounts of the 5′ end of 35S rRNA by primer extension. Radioactive bands visualized by a PhosphorImager are shown. The ratios of the radioactivity in the band for NOY886^{42C} to that for NOY1051^{143C} were calculated. The average ratio from three independent experiments was 1.1 ± 0.1.

repeat number increase, followed by curing of the multicopy *GAL7-35S rDNA* plasmid and by deletion of *FOB1* to stabilize the increased rDNA copy number. Deletion of *FOB1* had no effect on growth rate or rRNA synthesis rate compared to the strain with *FOB1* intact (not shown).

Strain NOY886 but not NOY1051 was used in the previous study, and the rDNA repeat number of NOY886 was estimated to be ~40 (21). In order to confirm the reduction of rDNA repeat number in this strain and to compare its rDNA repeat numbers directly with those of the newly constructed control strain NOY1051, we carried out two types of measurements. First, we separated chromosomes by CHEF electrophoresis (Fig. 1A) and estimated the size of chromosome XII, which is the chromosome containing the single endogenous array of tandem repeated rRNA genes. In Fig. 1A, positions of chromosome XII for NOY1051 and NOY886 are shown by white dots. By using the size markers in lane M, chromosome XII sizes estimated for NOY1051 and NOY886 were approximately 2.4 and 1.48 Mb, respectively, which correspond to rDNA repeat numbers of 143 and 42, respectively.

TABLE 1. Comparison of growth rates and rRNA synthesis rates in two *FOB1* deletion strains in YPD medium

Strain	Doubling time (h)	Relative content of RNA[a]	rRNA synthesis rate[b]
NOY1051^{143C}	1.6	1.0	1.0
NOY886^{42C}	1.6–1.7	0.95 ± 0.11	1.05 ± 0.15

[a] Total RNA content was determined as described in Materials and Methods. The values normalized for cell density were compared between the two strains. The average ratio from five independent experiments was 0.95 ± 0.11. Since the number of cells per cell density was found to be approximately the same for the two strains, the relative values shown in this table should also be applicable to comparison per cell with an average size.

[b] The results of primer extension indicated that the amounts of the 5′ end of 35S rRNA, which is unstable and reflects in all likelihood the rate of rRNA transcription, gave the average value of 1.1 ± 0.1 for the ratio of NOY886^{42C} to NOY1051^{143C} based on an equal amount of total RNA (see Fig. 1C and its legend). Since the ratio of the total amount of RNA (per cell density) in NOY886^{42C} to that in NOY1051^{143C} is 0.95 ± 0.11, the ratio of the rate of rRNA synthesis normalized to an equal amount of cell density is 1.05(1.1 × 0.95) ± 0.15.

Second, Southern hybridization analysis (Fig. 1B) was used to confirm that a difference in the number of rRNA genes could account for the size difference in chromosome XII. DNA from NOY886 and NOY1051 was digested with *Hin*dIII and *Pst*I and subjected to electrophoresis followed by hybridization with an *LEU2* probe and an rDNA probe (see figure legend). After normalization of the rDNA signals to the values for *LEU2* DNA, the ratio of rDNA in the two strains was calculated. The average ratio calculated from five independent experiments was 3.0 ± 0.3, which is approximately the same as the value of 3.4 calculated from the above-mentioned CHEF electrophoresis experiments. We conclude that NOY1051 has significantly more chromosomal rRNA genes than NOY886. EM visualization of dispersed chromatin from NOY886 supports the conclusion that this strain has very close to 42 genes (see Fig. 3 and 4 below). The copy number of rDNA in NOY1051 is approximately 126 (42 × 3.0) to 143 (based on CHEF analysis). For discussion of the results to be described below, we use the values obtained by CHEF analysis, namely, ~42 and ~143 for rDNA repeat numbers in NOY886 and NOY1051, respectively. We will refer to these strains as NOY886^{42C} and NOY1051^{143C}.

We compared NOY886^{42C} to NOY1051^{143C} with respect to growth rate, RNA content, and rRNA synthesis rate (Table 1). We found that the growth rate of the ~42-copy strain was approximately the same as or only slightly slower than for the control ~143-copy strain. Similarly, the rate of rRNA transcription calculated from the primer extension analysis of the unstable 5′ end of primary rRNA transcripts (Fig. 1C), together with values for relative RNA content (Table 1), showed that rRNA synthesis rates in these two strains were approximately the same (Fig. 1 and Table 1 legends). As shown in Table 1, when values for NOY1051^{143C} were normalized to 1.0, the relative growth rate of NOY886^{42C} was 0.94 to 1.0, while the relative rRNA synthesis rate was 1.05. Thus, regardless of a 3- to 3.4-fold difference in rRNA gene number, the two strains make rRNA and grow at approximately the same rates.

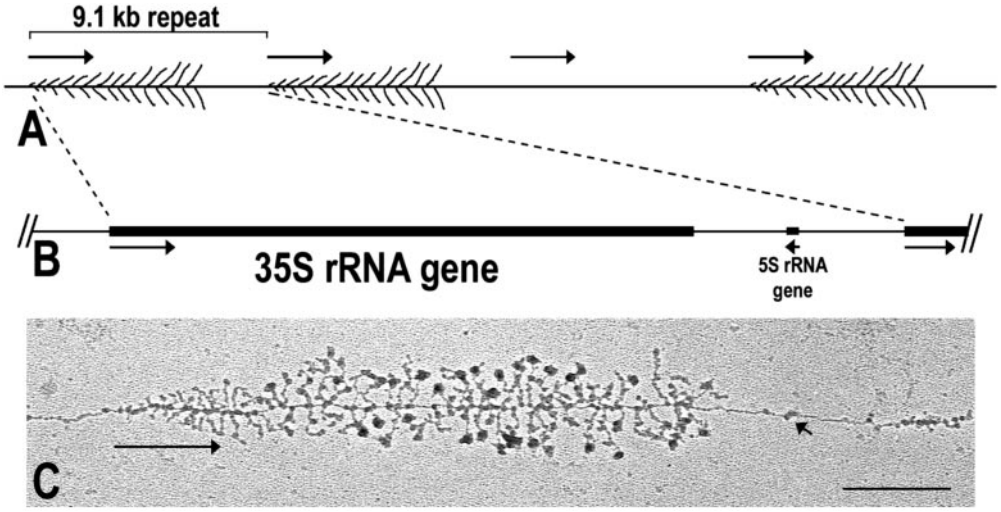

FIG. 2. The yeast rRNA gene repeat unit. (A) Schematic of four tandem rRNA gene repeats showing transcriptionally active and inactive units. The endogenous *S. cerevisiae RDN1* locus on chromosome XII contains ~150 of these repeats. (B) Schematic of one gene spacer unit, including the Pol I-transcribed 35S rRNA gene (long thick line), the Pol III-transcribed 5S rRNA gene (short thick line), and nontranscribed spacers (thin lines). (C) A representative electron micrograph of the rRNA gene spacer unit from the control JS772 strain is shown, aligned with the gene map in panel B. Long arrow indicates direction of transcription for the Pol I gene. It was identified as a Pol I gene by its tandem repetition (not shown), its expected length (corresponding to ~6.7 kb), and the characteristic terminal knobs at the 5′ ends of the nascent rRNA transcripts. The short arrow indicates the structure frequently seen at the position of the 5S gene. Bar = 0.4 μm.

Identification of *S. cerevisiae* rRNA genes by Miller spread EM. In chromatin spreads of wild-type yeast strains, we identified rRNA genes by previously established criteria for identification of this gene family: tandem repetition, expected gene length, relatively high transcriptional activity, and presence of 5′ terminal knobs on nascent rRNA transcripts (27). Nucleolar masses containing many copies of these genes in an active configuration could be seen from disrupted yeast cells that were displayed on EM grids. Figure 2C shows a micrograph of a representative gene spacer unit from a control yeast strain aligned with the rDNA map in Fig. 2B. All of the expected features are present, including tandem repetition (Fig. 2A and subsequent figures). There are no other repeated genes of this length in yeast (11). In addition, a characteristic peanut-shaped structure (small arrow in Fig. 2C) that we interpret to be a Pol III transcription unit was frequently seen at the position of the 5S rRNA gene, which is located in the spacer between *S. cerevisiae* 35S rRNA genes.

Determination of active gene number by direct counts in individual nucleoli. In order to determine how NOY886^{42C} and NOY1051^{143C} make rRNA at similar rates even though they differ over threefold in rRNA gene number, two approaches were taken to determine the number of active genes/nucleolus for the two strains. In the first, direct counts of active genes were made. This was possible only in cases where chromatin spreads were particularly favorable, allowing an entire nucleolus to be seen in a reasonably well-dispersed configuration.

Figure 3 shows examples of dispersed nucleoli from two NOY886^{42C} cells (Fig. 3A and B) and from two NOY1051^{143C} cells (Fig. 3C and D), all shown at the same magnification. Figure 3A′ to D′ show a few genes from each of these nucleoli at a higher magnification (areas enclosed in rectangles in Fig. 3A to D). The NOY886^{42C} nucleolus in Fig. 3A displays 41 active genes. These genes are shown schematically in Fig. 3A-trace, in which each line represents a transcribed rRNA gene, visible by the row of Pol I molecules lined up along the DNA template. Counts made of active rRNA genes from 44 individual NOY886^{42C} cells yielded numbers that clustered around 40 and spread upwards to 80, as shown in the graph in Fig. 3E. We interpret this range as cells in various stages of the cell cycle, with those having ~40 active genes being in G_1 and those having significantly more than 40 genes being at various points in S to G_2, in agreement with previous reports that newly synthesized yeast rRNA genes can be transcribed very soon after DNA replication (23, 45). This interpretation was also supported by the budding status of cells in cases where it was possible to identify the lysed cell from which chromatin had been released. For example, the nucleolus shown in Fig. 3B has 62 active genes (Fig. 3B-trace) and was released from a medium-budded cell, seen on the right in the figure, indicative of G_2. Thus, this direct count method indicates that most if not all of the 40 to 80 genes are active in NOY886^{42C}.

Direct counts of active genes proved more difficult in the ~143-copy strain NOY1051^{143C} and also in two other ~150-copy strains that we have used for other studies (latter not shown). The nucleoli infrequently appear in complete, discrete clusters of active genes, presumably because of the larger number of genes and the transcriptional inactivity of many of these genes. However, in cases when individual nucleoli were seen, it was clear that the absolute number of active genes was higher in NOY1051^{143C} (Fig. 3C and D) than in NOY886^{42C} (Fig. 3A and B) as shown by the larger gene cluster size when all are shown at the same magnification. Approximate determinations of active gene counts were obtained from 28 of the NOY1051^{143C} cells. Two examples are shown in Fig. 3C and D, which we again interpret as possible G_1 and G_2 cells, with ~84 and ~147 active genes, respectively. The range in active gene

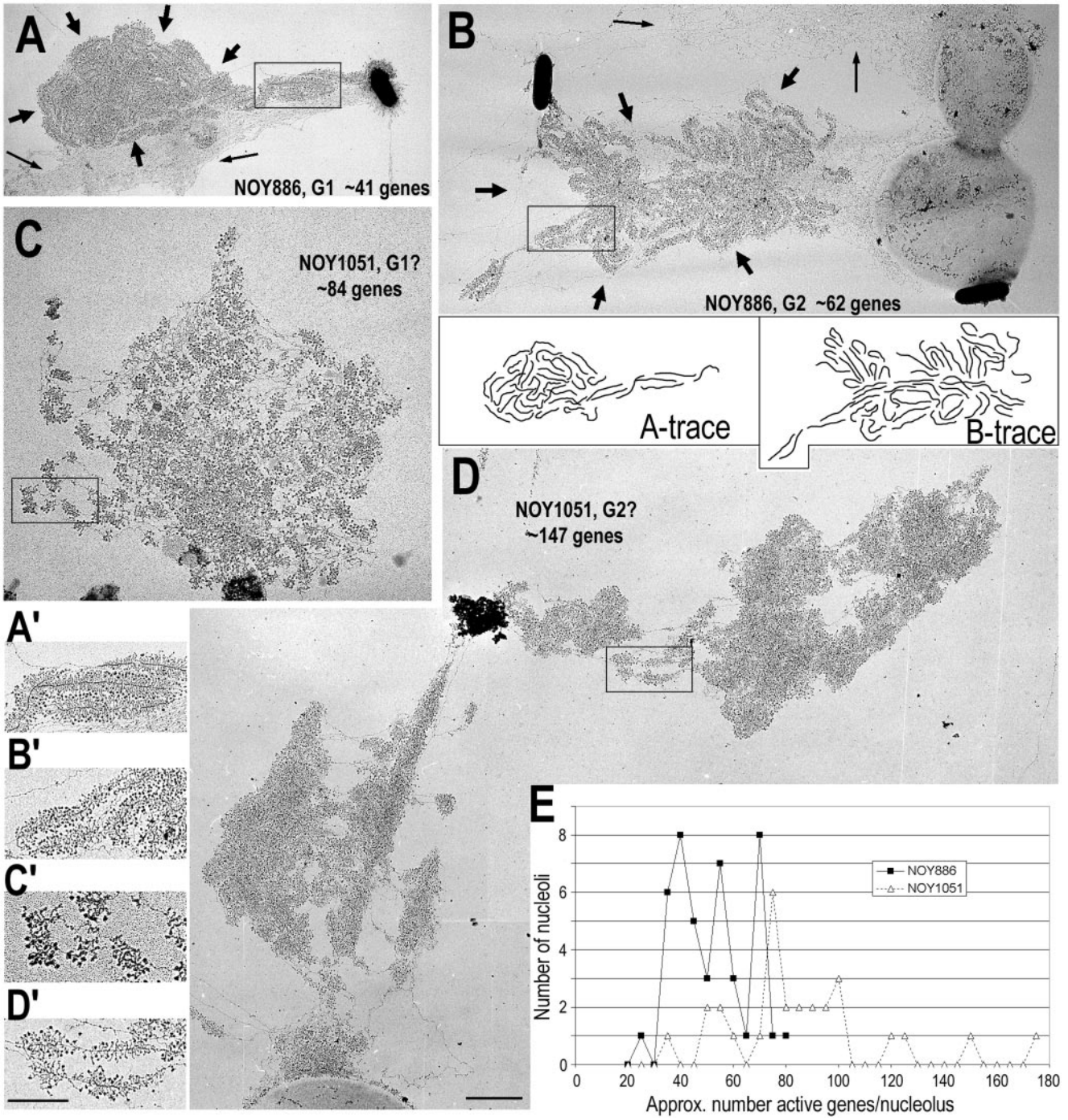

FIG. 3. More genes are active per nucleolus in the control strain, NOY1051^{143C}, than in the 40-copy strain, NOY886^{42C}, as determined by direct gene counts. (A to D) Four micrographs, at the same magnification, showing dispersed nucleolar contents from NOY886^{42C} (A and B) and NOY1051^{143C} (C and D) and from cells known or interpreted to be in G_1 (A and C) or G_2 (B and D). The chromatin in B has been released from a budded cell (seen at right in panel B), indicating that it is in the G_2 stage of the cell cycle. The rectangular boxed areas in panels A to D are shown at higher magnification in panels A′ to D′, better displaying examples of the characteristic active rRNA genes that are present in each nucleolar cluster. In panels A and B, some nonnucleolar chromatin is present (thin arrows) as well as nucleolar chromatin (thick arrows), the latter of which appears as a darker grey area in micrographs at this low magnification. (The dark fat rods in panels A and B are contaminating bacteria). A-trace and B-trace are schematic tracings of the DNA template of the active genes in the nucleoli in panels A and B. In panels C and D, essentially all of the dispersed chromatin shown is nucleolar. Note the increasing size of the nucleolar gene cluster as one goes from panels A to D. Bar in panel D = 2 μm; bar in panel D′ = 1 μm; panels A to D are at same magnification. (E) The active rRNA gene number per nucleolus was estimated for NOY1051^{143C} (n = 28) and for NOY886^{42C} (n = 44) by using micrographs shown in panels A to D and additional micrographs, and the results are shown graphically.

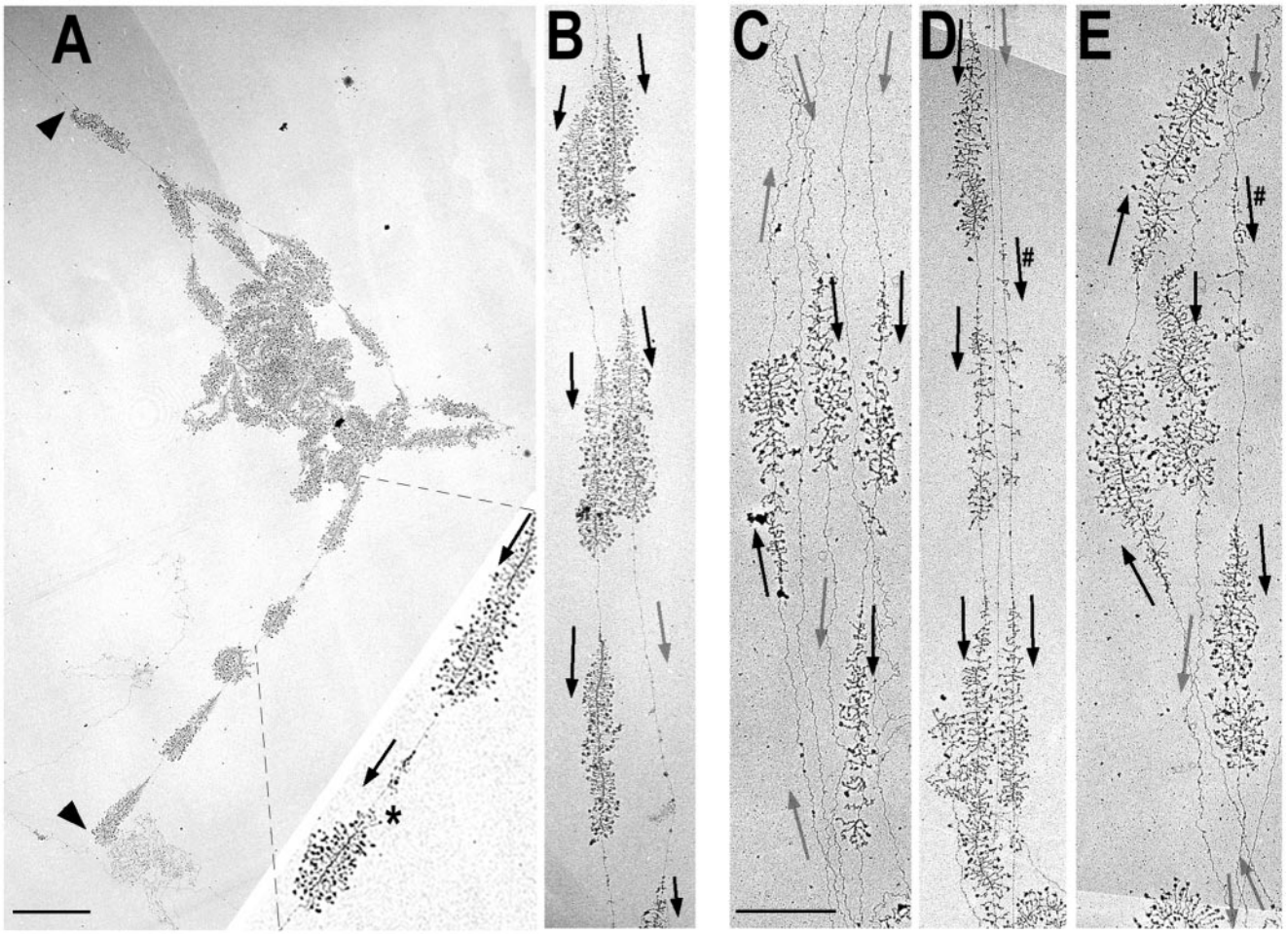

FIG. 4. A higher percentage of rRNA genes is active in NOY886^{42C} as determined by analysis of activity of contiguous gene repeats. Examples of genes repeated in tandem from NOY886^{42C} (A and B) and NOY1051^{143C} (C to E). The black arrows indicate active genes and direction of transcription. The grey arrows indicate inactive genes, which were identified as a nontranscribed region immediately upstream or downstream of an active gene. Inactive genes were very rarely seen in NOY886^{42C} (one example is shown by grey arrow in panel B) but were common in NOY1051^{143C} (grey arrows in panels C to E). More than 99% of rRNA genes were active in NOY886^{42C} by this analysis, while 67% of genes were active in NOY1051^{143C} (see text). The micrograph in panel A displays a complete NOY886^{42C} *RDN1* locus, containing the tandem rRNA genes from one chromosome XII, with both ends of the locus identifiable (arrowheads), and with ~42 active genes. The inset in panel A shows two of these genes at a higher magnification; the asterisk indicates a transcript-free gap in the middle of one gene. In both panels D and E, one gene is marked with a black arrow and pound sign. These are active genes with low polymerase density (discussed in text). Bar in panel A = 2 μm; bar in panel C = 1 μm; panels B to E are at approximately the same magnification.

number for 28 NOY1051^{143C} nucleoli was 33 to 171, with a peak between 75 and 80 (Fig. 3E). As in the ~42-copy strain, this first peak is about half of the highest count seen, consistent with cell cycle stage being responsible for the range. Thus (keeping in mind the caveats of small sample size and approximate gene counts), if we take the modal value of 78 to be representative of a G_1 stage cell, then ~78 of 143 total genes or ~54% of the rRNA genes are active in NOY1051^{143C} as estimated by this method.

Determination of active gene number by analysis of the activity of contiguous genes. A second approach was used to estimate the percentage of rRNA genes that were transcriptionally active in the two strains. All rDNA strands in which three or more tandem gene repeats were visible were scored as to whether each gene was on or off. Examples of these extended strands are shown in Fig. 4, with Fig. 4A and B from NOY886^{42C} and Fig. 4C to E from NOY1051^{143C}.

Figure 4A (which is shown at a lower magnification than the other panels in Fig. 4) displays a complete *RDN1* locus from a G_1-stage NOY886^{42C} cell with both unique ends of the locus on chromosome XII identifiable (arrowheads). In the better-dispersed regions, it is obvious that each rRNA gene is transcriptionally active. The total active gene count in this example is 42 or 43 (with some ambiguity in the central mass), again indicating that all genes are on. In other examples not shown, individual contiguous stretches of active genes of 10, 12, 13, 14, 17, and 23 genes were seen from this ~42-copy strain, as well as numerous shorter stretches. Rare examples were seen of inactive genes. One of these is shown in Fig. 4B (grey arrow). In all, 16 examples of inactive genes were seen in nucleoli from 141 cells, indicating that >99% of genes were active in NOY886^{42C} cells.

On the other hand, numerous examples of inactive genes were seen in dispersed regions of NOY1051^{143C} nucleoli. Figure 4C to E show examples of these stretches, with black arrows alongside active genes and grey arrows alongside inactive genes. For example, Fig. 4C shows four active genes and five inactive genes distributed on several DNA strands. For this analysis, results could be skewed if the distribution of active and inactive genes is not random in the repeat array, leading to a situation in which active regions are preferentially scored. However, a previous report (8) concluded that yeast rRNA genes are distributed randomly in the repeat array in terms of transcriptional activity, as judged from the results of a psoralen cross-linking analysis, and so far, our analysis of another control strain agrees with this finding (unpublished data). In all, 355 genes from 31 different cells were scored, in stretches ranging from 3 to 65 contiguous genes. Of these 355 genes, 67% were active. In one nucleolus, 106 genes in three contiguous stretches were able to be scored. Of these, 64% were active.

In summary, results from two methods, direct gene counts in Fig. 3 and contiguous-repeat analysis in Fig. 4, indicated that all or almost all of the genes in NOY886^{42C} were transcriptionally active in early log phase, regardless of cell cycle stage. For the ~143-copy strain, the direct-count method indicated that ~54% of the genes were active, while the contiguous-repeat analysis indicated that ~67% were active. Both of these estimates are near, though slightly higher than, the ~50% value previously estimated for log-phase *S. cerevisiae* cells by the psoralen cross-linking assay (7). Thus, ~42 genes (100% of 42) were active per G_1 cell in log-phase NOY886^{42C} cells, while ~78 to 95 genes (54 to 67% of 143) were active per G_1 cell in log-phase NOY1051^{143C} cells. If one knows that the rate of rRNA synthesis is approximately the same in the two strains (Table 1), these data alone indicate that the number of active genes is not the determining factor for rRNA synthesis in log-phase *S. cerevisiae* cells.

The average number of polymerases per gene is higher in the ~42-copy strain. Another transcriptional parameter, specifically, the average number of polymerases per active gene, was measured to see if differences in this number compensated for the difference in the number of active genes. Direct counts were made of polymerase molecules per gene for 136 active genes from NOY1051^{143C}, for 174 active genes from NOY886^{42C}, and for 121 genes from JS772, a standard laboratory strain not mutant in *FOB1*. For the analyses of the control strains, all rRNA genes displayed on random grids were included in order to avoid selection bias. Figure 5A shows the distribution of polymerase counts per gene for the three strains. The distributions and averages were very similar for JS772 and NOY1051^{143C} (both with 140 to 150 rRNA genes) but were significantly higher for NOY886^{42C} with ~42 gene copies. Specifically, the average number of polymerases/gene and standard deviations were 49 ± 17 for JS772, 51 ± 20 for NOY1051^{143C}, and 100 ± 29 for NOY886^{42C}. The modal, or most common, values for polymerases/gene were very close to the averages for JS772 (mode = 47) and NOY1051^{143C} (mode = 52), while the modal value for NOY886^{42C} was 114 polymerases/gene, a polymerase density not seen on even the most highly transcribed genes from the control strains in this analysis.

Examples of genes with a close to average number of polymerases are shown in Fig. 5B and C for NOY886^{42C} and NOY1051^{143C}, respectively. Figure 5D and E display portions of spread nucleoli from NOY886^{42C} (Fig. 5D) and NOY1051^{143C} (Fig. 5E) shown at the same magnification and with a similar number of genes and similar degree of compaction. Note the pronounced dark lines representing the "polymerase backbones" of the highly transcribed genes in the ~42-copy strain. These are detectable in the control strain but are not nearly as pronounced. An average gene in NOY1051^{143C} with 51 polymerases/gene has one polymerase every 132 nt of DNA. An average gene in the ~42-copy strain has one polymerase every 67 nt, while the most densely packed gene seen, with 165 polymerases on the 6.74-kb rRNA gene, has one polymerase every 41 nt. This is probably very near the packing limit for elongating RNA polymerases, which footprint ~35 nt of template DNA when arrested (53).

A surprising result was the wide range in number of polymerases transcribing each gene (2 to 102 for NOY1051^{143C} and 9 to 165 for NOY886^{42C}), in disagreement with the prevailing view that an active rRNA gene represents a full unit of activity, as proposed by the binary unit model. Note also that the two control strains have very similar distributions (Fig. 5A). In some cases, a lower-than-average polymerase density is due to polymerase-free gaps at random positions in the middle of the gene, an example of which is shown in the inset in Fig. 4A (asterisk). Such gaps might indicate promoter-level opening and closing of the gene, cooperative polymerase initiation, or a stalled polymerase. Many other genes, however, had a uniformly lower polymerase density across the length of the gene. Examples of these are shown in Fig. 4D and E (arrows with # symbols).

The combined results are the following: in the control strain, NOY1051^{143C}, ~78 to 95 of the 143 genes are active, with an average of 51 polymerases per gene. Thus, the total number of engaged polymerases per G_1 cell in this strain is in the range of 3,980 to 4,850. In NOY886^{42C}, all ~42 genes are active with an average of 100 polymerases per gene, resulting in ~4,200 engaged polymerases per G_1 cell. (Using the modal value of 114 polymerases/gene for this calculation results in an estimate of ~4,790 engaged polymerases for NOY886^{42C}.) Thus, the number of engaged polymerases is approximately the same in the two strains, as expected from approximately the same growth rates and rRNA synthesis rates, but there is a twofold difference in polymerase density per gene. As discussed, these findings are most easily explained by a difference in transcription initiation rate per gene in the two strains.

DISCUSSION

Combined data indicate that initiation is limiting for Pol I transcription in *S. cerevisiae*. The major conclusion from this study is that rRNA synthesis rate in log-phase *S. cerevisiae* cells is determined by the cells' ability to load and transcribe rDNA with polymerases, rather than by the number of transcriptionally active genes. We showed that cells with a 3- to 3.4-fold difference in gene number produced the same amount of rRNA by loading a similar total number of polymerases on different numbers of active genes. The parameter that we measure, polymerase number per gene, could be influenced by

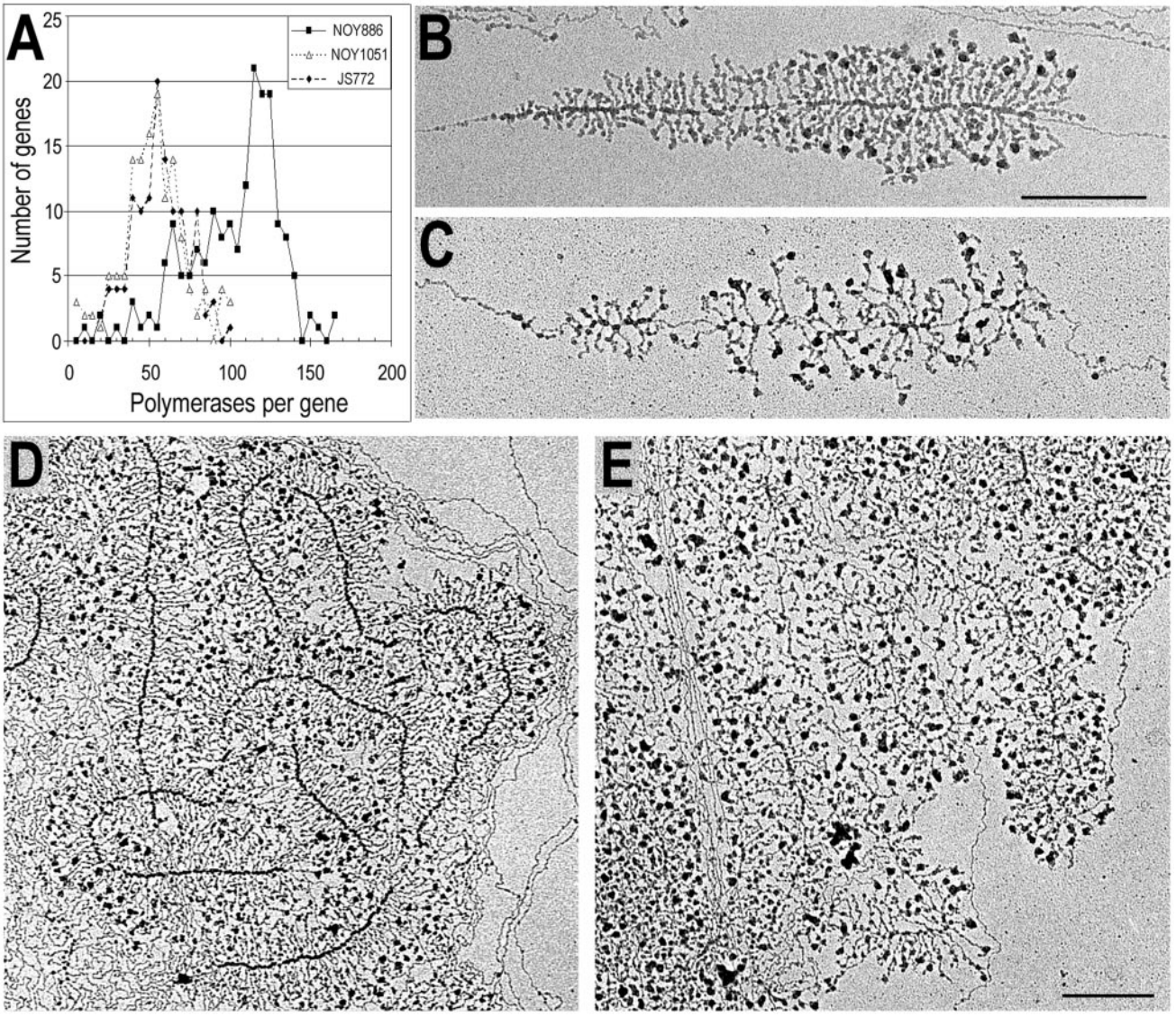

FIG. 5. The number of polymerases per gene is higher in the strain with fewer genes. (A) The average number of polymerases per gene is lower in the two control strains that have a typical number of genes (NOY1051^{143C} and JS772) than in the ~42-copy strain NOY886^{42C}. In all strains, there is a wide range in the number of polymerases per gene. (B and C) Representative rRNA genes from NOY886^{42C} (B) and NOY1051^{143C} (C) showing genes with an average number of polymerases. Bar = 0.5 μm. (D and E) Representative clusters of dispersed genes from NOY886^{42C} (D) and NOY1051^{143C} (E) showing the more prominent polymerase backbones on the genes in the ~42-copy strain, due to the very dense packing of polymerases on the genes. Bar = 0.5 μm.

both transcription initiation and transcription elongation rates. However, it should be noted that most exponentially growing yeast cells are in a steady state in the synthesis of rRNA. Under such conditions, regardless of steps limiting overall synthesis of rRNA, observed rates of loading of rRNA genes with Pol I must be the same as the rates of Pol I movement, i.e., the rates of elongation, which in turn must be the same as the rates of production of finished rRNA chains. Thus, results in hand make it unlikely that the elongation rate is different for the two strains or that it is limiting for transcription. Since the two yeast strains have approximately the same rRNA synthesis rates and the same numbers of transcribing Pol I molecules, these Pol I molecules must be elongating rRNA chains at the same rate, thus producing finished rRNA chains at the same rate. Also consistent with elongation not being limiting, the polymerases in the control strain are not piled up next to each other or distributed on genes in a pattern suggestive of elongation blocks.

There is a considerable body of evidence implicating the initiation-competent Pol I-Rrn3p complex or its mammalian homologue hRrn3/TIF-1A as being regulatory for rRNA transcription as growth becomes slowed (3, 25, 29, 50, 58). Experiments in cell extracts have shown that Rrn3p/TIF-1A is not necessary for formation of a stable preinitiation complex but is deposited anew with each Pol I initiation event and is necessary for formation of the first phosphodiester bond (1, 50, 58). Thus, a possible explanation of our findings is that the amount of initiation-competent Pol I is regulating rRNA syn-

thesis in log-phase conditions as well as during down-regulation.

Kinetic parameters of Pol I transcription in yeast. Measurements of the number of Pol I molecules engaged in rRNA gene transcription in exponentially growing yeast cells allow us to roughly estimate some parameters pertaining to Pol I transcription. Yeast cells growing in rich media with a generation time of ~100 min as in the present study contain, on the average, ~200,000 ribosomes per cell and hence are synthesizing ~2,000 ribosomes per min (56). Using the values obtained for the ~42-copy strain and assuming that every Pol I transcript is used for ribosome production, one can calculate that each of the 42 active genes produces ~48 (2,000/42) 35S rRNA molecules destined to become ribosomes per minute or one 35S rRNA molecule per 1.25 s. This also means that the initiation complex assembles and that the polymerase clears the promoter, on average, in 1.25 s for every polymerase. The ~42-copy strain averages 100 ± 29 polymerases/gene; therefore, the time interval represented by the first and last polymerase on a gene is ~125 s. Thus, at 30°C, the Pol I elongation rate on the 6,740-nt rRNA gene can be calculated to be 54 (6,740/125) nt/s. We argue above that elongation rates are similar in NOY886^{42C} and NOY1051^{143C} and that, thus, this rate would also apply to the ~143-copy strain.

This elongation rate agrees reasonably well with the value obtained by combining transcription parameters from *Escherichia coli* and mammalian cells to estimate the elongation rate at 30°C. The temperature dependence of the transcription elongation rate has been investigated in *E. coli*; elongation rates for stable RNA synthesis in rich media were determined to be 86 nt/s at 37°C and 59 nt/s at 30°C (44). A recent measurement of the mammalian Pol I elongation rate yielded 95 nt/s at 37°C (9). Using the temperature coefficient determined for *E. coli* RNA polymerase (44), and the value of 95 nt/s at 37°C for eukaryotic Pol I (9), one can estimate a Pol I elongation rate of ~60 nt/s at 30°C, which agrees well with the estimate of 54 nt/s arrived at above. We suspect that 60 nt/s is closer to the true elongation rate because two of our initial assumptions are likely to underestimate the rate. That is, some estimates for number of ribosomes per yeast cell are higher than the estimate of 200,000 that we used (e.g., up to 348,000 [55]), and also, the efficiency of conversion of 35S pre-rRNA to ribosomes is probably not 100%. Thus, if one uses what may still be a conservative estimate for the yeast Pol I elongation rate of 60 nt/s, together with our data showing one polymerase every 132 nt on average in NOY1051^{143C}, the estimated reinitiation interval for Pol I would be 2.2 s (132/60) in the control strain. A similar calculation yields a value of 1.1 s for an average polymerase to load and initiate in the ~42-copy strain, while polymerases on the most heavily transcribed gene (one polymerase/41 nt) would reinitiate in ~0.8 s. These values compare to a reinitiation interval of 1.4 s recently estimated for Pol I in mammalian cells (9).

Role of the two proposed levels of regulation for Pol I transcription in *S. cerevisiae*. It is frequently concluded that there are two potential levels of regulation for Pol I transcription: control of the number of active genes and control of the transcription rate per gene. As discussed in the introduction, there is much evidence for variations in both of these parameters and both vary as a function of growth rate in yeast (7, 18, 46). Recent results, however, point to transcription rate per gene as being of primary importance in regulating Pol I transcription in budding yeast. First, the results herein show this to be the case in exponentially growing cells. Although active gene number varied by twofold in the two strains, cells were able to increase the initiation rate per gene to ensure synthesis of normal levels of rRNA. What about the case during down-regulation, when cells leave exponential growth and are known to shut down half of the genes that were active during log phase? Recent results from Sandmeier et al. (46) yielded unexpected information that also indicates that the number of active genes has little to do with total rRNA synthesis. This study identified the histone deacetylase Rpd3 as being required for inactivation of genes as cells leave log phase. Interestingly, however, in *rpd3* null mutants, although the number of open genes did not decrease in post-log-phase cells as it did in control cells, rRNA synthesis was down-regulated the same as it is normally down-regulated in control cells (46). This resulted in a similar number of total polymerases being distributed on more open genes in the *rpd3* strain than in the control strain in post-log-phase cells. This is just the opposite of results herein, in which a similar number of polymerases was distributed on fewer open genes. Both results, however, indicate that the summed Pol I loading rate determines rRNA synthesis rather than the number of active genes. Since cells typically go to the trouble of changing the number of active genes and usually have at least twice as many genes as are active at any time (7), there clearly is some advantage to this level of regulation, even though it appears to be of secondary importance in regulating the rRNA synthesis rate in normal cycling yeast cells.

Are these results applicable to cells other than those from *S. cerevisiae*? Budding yeast appears to differ from other eukaryotes in certain components of the basal Pol I transcription machinery (30, 43). In addition, yeast rRNA genes are not regulated by the tumor suppressors, RB and p53, which may work at the level of preinitiation complex formation in mammalian cells (4, 59). On the other hand, yeast and mammals have similar regulatory features that adjust rRNA synthesis in response to environmental cues (12, 30, 33, 34, 43, 56) and both use Rrn3p for growth-dependent control of rRNA synthesis (25, 29, 50).

Although there are insufficient data from other organisms to determine whether the number of active genes is regulatory during normal growth, it is worth reconsidering the data that are typically cited to support the binary unit model. The strongest evidence for this model has been the many previous EM studies from various organisms in which Pol I density is typically high across each gene (reviewed in references 27 and 49). However, the cells most frequently used for these studies are not representative of normally multiplying cells, as are the yeast cells studied here, but rather are from the amplified rDNA of amphibian oocyte germinal vesicles or from oocytes or embryos of insects, which might be subject to a different type of regulation. The fact that amphibian oocytes specifically amplify their rRNA genes to meet their developmental needs (which makes these oocytes a favorite model system for EM analyses) agrees with the interpretation that the limiting factor in these cells is the number of genes. Furthermore, oocytes and early embryos are stocked with excess stores of molecules that ensure rapid growth after fertilization, again indicating that

trans-acting factors are unlikely to be limiting, as they may be in later developmental stages.

There are few EM studies of rRNA genes from cells not in an early developmental stage. However, average Pol I density has been measured on rRNA genes from CHO cells (41) and rat liver cells (15) and found to be 114 polymerases/gene and 101 polymerases/gene, respectively, which correspond to one polymerase/123 nt and one polymerase/139 nt. Likewise, less direct evidence indicates an average of ~110 polymerases/gene in HeLa cells (17) in agreement with a few micrographs (14), indicating one polymerase/123 nt in these human cells. These polymerase densities are similar to the average seen in the control yeast strain in our study: one polymerase/132 nt (Fig. 5). In comparison, *Xenopus* oocyte rRNA genes averaged one polymerase/104 nt (38), and the 42-copy yeast strain averaged one polymerase/67 nt. Although present data are insufficient to make definitive conclusions, the combined evidence indicates that active genes in multiplying cells are not maximally transcribed by the criterion of maximal polymerase packing and thus that our findings in yeast cells may be applicable to other cells.

Implications regarding rRNA gene number. By artificially decreasing the number of rRNA genes to a number less than the number typically active in normal yeast cells, we were able to address the question at hand. Based on the results obtained, one could predict that it may be possible to lower gene number a bit more and still obtain normal growth rate, since the average Pol I density per gene in the 42-copy strain (100/gene) was below both the modal polymerase density in this strain (114/gene) and the maximal Pol I packing seen (165/gene). We have recently constructed a strain with ~25 to 30 rRNA genes and found that its growth rate in rich media is slightly (~10%) slower than the control strains. Thus, 25 to 30 copies appear to be just slightly below the minimum number of genes that are required to obtain normal growth (L. Vu, K. Eliason, and M. Nomura, unpublished observations). Strains with 25 to 30 rRNA genes would be expected to require 140 to 168 polymerases/gene on average to attain the same rRNA synthesis rate as control cells.

In the ~42-copy strain, essentially all of the genes are active, thus breaking the previously noted pattern that half or fewer of the rRNA genes are active in normally growing cells (6, 7, 17, 22). It has been suggested that inactive or silenced regions of nucleolar chromatin may be involved in some of the newly appreciated roles for the nucleolus, such as cell cycle regulation (reviewed in reference 36). However, the fact that NOY886^{42C} cells grow robustly without maintaining any significant number of inactive rRNA genes indicates that a store of inactive genes is not required in the short term for growth in rich media. Lastly, given the large number of molecular signaling pathways that regulate rRNA synthesis, it is expected that some of these pathways operate by regulating the number of active genes via either preinitiation complex formation or epigenetic mechanisms. Based on results reported here, however, this level of regulation is not limiting overall rRNA synthesis rate under optimal growth conditions in yeast.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM63952 (to A.L.B.) and GM35949 (to M.N.) and by the Human Frontier Science Program Organization (HFSPO) grant RG0336 (to M.N.).

We thank Jeff Smith and Takehiko Kobayashi for strains; Melanie Oakes, David Auble, Tom Misteli, Marisol Santisteban, and Paul Adler for discussion and critical reading of the manuscript; and Martha Sikes and Kristilyn Eliason for excellent technical assistance.

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