Ribosomal RNA Synthesis in Soybean Suspension Cultures Growing in Different Media

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ABSTRACT

The transcription of ribosomal RNA has been studied in suspension tissue cultures of soybean Glycine max L. Merr. cv. Mandarin cells (SB-1). A large precursor molecule was synthesized which contains RNA homologous to the 25 and 18S cistrons. Transcription was from one strand and appeared to start adjacent to the 18S cistron and to proceed through the 18S DNA, a <0.6-kilobase transcribed spacer and the 25S cistron. A nontranscribed spacer region was identified. When cells grew rapidly in sucrose (24 hours doubling time) they contained 7 times as many ribosomes as when they grew slowly in maltose (200 hours doubling time). Upon transfer from maltose to sucrose, cells began to accumulate ribosomes at a rapid rate (80-fold more rapid synthesis than in maltose medium) within 2 hours at 33°C. The 2-hour lag is due in large part to a longer processing time during which newly synthesized RNA is packaged into ribosomes. Therefore, the increase in transcription rate may occur within a few minutes of the transfer to sucrose.

In bacteria and yeast, the number of ribosomes per cell is directly proportional to growth rate (5, 6, 17, 18). This is reasonable since the number of ribosomes determine the rate of protein synthesis.

The ability to grow plant cells in suspension cultures presents an opportunity to study the ribosome content of plant cells growing at different rates and to determine how RNA synthesis is adjusted when cells are transferred from slow to rapid growth conditions.

Such a study was made feasible by the availability of soybean cell suspension cultures which grow exponentially: rapidly in sucrose medium with a 1-day doubling time; and slowly in maltose medium (in which maltose replaces sucrose as an energy-carbon source) with an 8-day doubling time. The slow growth in maltose has been shown to be the result of a permeability barrier which inhibits the uptake of maltose (8). Heritable variants which grow rapidly in maltose medium are also available (8) as are cell lines in which the number of ribosomal genes has been reduced.

In addition, a λ Charon-4A library (4) of soybean genes had been constructed and, as a result, clones of ribosomal genes were available. Using such clones, it was possible to determine the characteristics of rRNA transcription leading to a rRNA precursor. This in turn was used to determine the time required to process the precursor RNA into ribosomes.

From the studies described below, we have determined that soybean cells vary their rRNA content in response to growth conditions similar to bacteria or yeast.

MATERIALS AND METHODS

Cell Lines and Growth. SB-1 cells derived from roots of Glycine max L. Merr., cv Mandarin were provided by Dr. O. L. Gamborg. The M-24 cell line was derived from SB-1 in this laboratory (8). At 33°C, SB-1 grows exponentially with a 24-h doubling time in sucrose. M-24 grows with a doubling time of 22 h in maltose or sucrose. When SB-1 cells are grown in maltose, they lose 30% of their rRNA cistrons (rDNA) (4) and divide every 200 h (8). Upon return to sucrose, the number of RNA genes does not increase again; however, the cells divide every 24 h. We refer to such cells, which have permanently lost a portion of rRNA genes after propagation in maltose medium, as the M-200 cell line. (M-24 cells were selected after slow growth in maltose and also lack 30% rRNA cistrons.) The growth rates of these cell lines are summarized in Table 1.

Murashige-Skoog media were obtained from Flow Laboratories Inc., Inglewood, CA (2, 10). Conditions for cell growth have been described previously as well as the packed volume method for measuring cell growth rates (1, 2). All cell suspensions were maintained at a density between 10⁶ and 10⁷ cells/ml. Maltose, uncontaminated with either sucrose or glucose, was obtained from Baker Chemical Co., Philadelphia, NJ. Transfer of cells from maltose to sucrose media was accomplished by diluting cells in maltose medium with an equal volume of prewarmed filter-sterilized sucrose media.

Isotopic Labeling of Soybean RNA. RNA was labeled with [32P]phosphate by addition of 100 µCi/ml carrier-free [32P] phosphoric acid. Cells grow well in this radioactive medium. Indeed we have been able to grow cells for more than two generations in medium containing up to 10 times as much [32P] [1 mCi/ml.] RNA was labeled with [3H]uridine by addition of 10 µCi/ml 5-[3H]uridine (>25 Ci/mmol) to the medium. Both radioactive isotopes were obtained from New England Nuclear Corp.

Nick Translation of DNA. This procedure was a modification of that used by Rigby et al. (12). [32P]dCTP (60 µCi) (New England Nuclear Corp.) plus 1.5 µl each 1 mM dATP, 1 mM dGTP, and 1 mM dTTP (Schwarz/Mann) were evaporated under vacuum. One-half µg DNA to be labeled was added to this plus 5 µl buffer (5 M Tris-HCl [pH 7.5], 1 mM MgSO₄; 100 mM DTT, 5 mg/ml BSA) and distilled H₂O to a total volume of 37 µl. Ten µl of a solution of DNase I (0.1 µg/ml; Sigma Chemical Company) was added and the solution was incubated for 1 min at room temperature; 3 µl Escherichia coli DNA polymerase I (11,000 units/ml; New England Biolabs, Beverly, MA) then was added and the mixture was incubated at 14°C for 3 h. One volume stop buffer (20 mM Na₂EDTA, 2 mg/ml sonicated calf thymus DNA, 0.2% [w/v] SDS) was added and the mixture was heated at 65°C for 5 min. DNA...
was precipitated by addition of 2.5 volumes 2-propanol and was collected by centrifugation for 2 min in the microcentrifuge. The DNA pellet was resuspended in one-half of the original culture volume of sterile, ice-cold extraction buffer (50 mM Tris-HCl [pH 7.2], 0.15 M NaCl, 25 mM Na₂EDTA). This suspension was passed through a handheld homogenizer (VWR Scientific Co., Salt Lake City, UT) directly into an ice-cold extraction buffer (50 mM Tris-HCl, 25 mM Na₂EDTA) and the flask in which the RNA was precipitated was washed extensively to remove up to 60% RNA found to be adhering to the walls of the flask. The RNA was stored at -20°C. No degradation could be detected in any experiments, a yield of 80 to 83% of the total cellular RNA was obtained.

**Isolation of Ribosomes and Extraction of rRNA.** Fifty ml exponentially growing SB-1 cells at a concentration of 10⁶ cells/ml were collected by centrifugation at 100g for 1 min. The cell pellet was resuspended in 50 ml sterile, ice-cold, 0.5 M sucrose solution containing 5 mM MgCl₂, 16 mM KCl, and 50 mM Tris-HCl (pH 7.6). This suspension was passed through a handheld homogenizer. Twelve ml ice-cold 20% (v/v) Triton X-100 was added and the homogenate centrifuged at 25,000g for 10 min. The supernatant was collected and centrifuged at 4°C for 90 min at 105,000g to sediment the ribosomes (conditions which sediment >90% of the ribosomes). Taking care to avoid resuspension, the ribosomal pellet was washed with 5 ml solution containing 0.5 M sucrose, 5 mM MgCl₂, 16 mM KCl, and 50 mM Tris-HCl (pH 7.6). The ribosomes were then resuspended in 2 ml solution containing 3 mM MgCl₂, 0.5 M KCl, 10 mM Tris-HCl (pH 7.4), and then loaded onto sucrose step gradients consisting of equal volumes (bottom to top) of 34, 29, 24, 19, 14, and 10% sucrose solutions containing 3 mM MgCl₂, 0.5 M KCl, and 10 mM Tris-HCl (pH 7.4). The gradients were centrifuged in a Beckman SW 41 rotor at 20,000 rpm for 17 h at 4°C. Fractions were collected from the gradients into ice-cold, sterile tubes, and assayed for the presence of RNA. The appropriate fractions were resuspended in a solution of 50 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl and 2.5 mM Na₂EDTA and extracted with phenol to obtain RNA.

**Gradient Centrifugation of rRNA.** Purified RNA was sedimented through 15 to 30% (w/v) sucrose gradients in sterile NETS buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl, 10 mM Na₂EDTA, 0.2% [w/v] SDS) by centrifugation for 6.5 h at 38,000 rpm in a Beckman SW 41 rotor. Fractions were collected (usually 38) and analyzed for OD₂₆₀ and/or radioactivity. (All radioactivities were measured by counting in a Beckman scintillation counter. OD₂₆₀ was measured in a Zeiss PMQ spectrophotometer.) Fractions from preparative centrifugations were pooled (where appropriate [40S, 25S, or 18S]) precipitated with alcohol, redissolved in extraction buffer, and stored at -20°C.

**DNA Isolation from SB-1 Nuclei.** Cells were converted to protoplasts (14). No more than 4 × 10⁷/ml protoplasts were suspended in 3 ml ice-cold solution containing 0.26 M sucrose, 12.5 µg/ml DTT, 0.4 µl/ml 2-ethyl-1-hexanol, and 200 µg/ml Mes buffer (Sigma Chemical Co.), then adjusted to a pH of 6.4 with NaOH. After 1 min, 1 ml 0.2% (w/v) Triton X-100 dissolved in the same solution was added and the suspension was shaken vigorously. After 2 min at 0°C, 4 ml RS-KCl buffer (0.2 M sucrose, 12.5 mM Mes, 0.025 mg/ml DTT, 0.08% 2-ethyl-1-hexanol, 12.5 mM KCl, pH adjusted to 6.4 with NaOH) was added to the suspension. Nuclei were then purified by repeated centrifugation through gum arabic step gradients (2). Following the second centrifugation, the nuclear pellet was resuspended in 1 ml sterile solution of 50 mM Tris-HCl (pH 7.8) containing 0.15 M NaCl and 25 mM Na₂EDTA and heated to 65°C for 2 min. One ml 2% SDS in the same buffer was then added to lyse the nuclei. After 2 additional min, 0.2 ml pronase solution (15 mg/ml grade B pronase, self-digested for 1 h at 65°C to remove nucleases; Calbiochem-Behring Corp.) was added and the resulting viscous lysate was incubated at 65°C for 30 min. The hot nuclear lysate was then added to 8.72 g CsCl and the total weight of the CsCl solution was brought to 15.42 g by the addition of distilled H₂O. The solution was centrifuged in a Beckman Ti-50 fixed angle rotor for 40 h at 40,000 rpm. The gradients were fractionated and the fractions assayed for the presence of DNA. Fractions containing DNA were dialyzed overnight at 4°C against 50 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl and 0.1 M Na₂EDTA. This was then mixed with an equal volume of 80% distilled phenol suspended in the same buffer. Following 15 min of extraction at room temperature, the phenol and aqueous phases were separated by centrifugation. The aqueous phase was collected and extracted with ether to remove phenol.

DNA from A Chiron 4A-RB115. A λ clone of the SB-1 rRNA cistron (RB115) has been used. Its isolation in this laboratory is described elsewhere (4). The structure of the clone is shown in Figure 4. DNA from the clone was prepared as follows.

Confluent plates of lyzed bacteria were flooded with 3 ml SM buffer (0.1 M NaCl, 0.01% [w/v] gelatin, 4 mM MgSO₄, 50 mM Tris-HCl [pH 7.5]) and the soft agar-buffer mixture collected. Several drops of CHCl₃ were added and the suspension incubated at 37°C for 30 min. Agar and bacteria were removed by centrifugation and the supernatant, containing the phage, was collected. Fifty mg/ml NaCl were dissolved in this and the centrifugation was repeated. The supernatant was collected and 70 mg/ml polyethylene glycol 6000 powder (Fisher Scientific Co.) was added. After 1 h on ice, the suspension was centrifuged and the supernatant was removed from the pellet. The latter was resuspended in 10 mM Tris-HCl (pH 7.4), containing 0.1 M NaCl and 10 mM MgCl₂.

The phage suspension was layered onto CsCl step gradients containing 0.5 ml each of 4.8 and 4.0 M CsCl, and 1 ml each of 3.2 and 2.4 M CsCl (in 10 mM Tris-HCl buffer [pH 8.0] containing 1 mM MgSO₄ and 0.1 M Na₂EDTA). These gradients were centrifuged for 1 h at 30,000 rpm in a Beckman SW 39L rotor. The phage band was removed with a syringe mixed with an equal volume of saturated CsCl solution and then placed on the bottom of another CsCl step gradient, which also was centrifuged and the phage band collected. This was dialyzed against a solution containing 50% (v/v) formamide, 200 mM Tris-HCl buffer (pH 8.5), and 20 mM Na₂EDTA for 24 h at room temperature and then for 48 h at 4°C against four 500-ml changes of a solution containing 100 mM NaCl, 50 mM Tris-HCl buffer (pH 7.4) and 0.1 mM Na₂EDTA.

**Analysis of DNA Digested with Restriction Endonuclease.** DNA was digested with Bgl I endonuclease (New England Biolabs) in the buffer and under the conditions suggested by the supplier. DNA fragments were separated by electrophoresis through 1% (w/v) agarose gels (1% agarose boiled in electrophoresis buffer containing 1 mM EDTA, 50 mM NaOH, and 40 mM Tris-Base adjusted with glacial acetic acid to pH 7.8). Gels were run at 80 amp for 4 to 5 h.

DNA was transferred from agarose gels to nitrocellulose filters using the methods of Southern (15). For RNA hybridization, filters were placed in ziplock plastic bags and bathed in a solution of 50% formamide, 5% SSC (SSC contains 0.15 M NaCl and 0.015
RESULTS

Ribosomes Content of Cells with Different Growth Rates. The number of ribosomes per cell was determined using cells from slowly and rapidly growing cultures. Total RNA was extracted from a known number of protoplasts and sedimented through 15 to 30% sucrose gradients. The amount of RNA in each fraction of the gradient was determined. The values for the 18S and 25S rRNAs were used to determine the number of ribosomes present in each cell. (Approximately 24% TCA-precipitable \( ^{3} \text{H} \)uridine was lost during rRNA purification. This loss was corrected in the calculations.) The results, which are compared with the growth rates in Table I, indicate that the number of ribosomes per cell is roughly proportional to the division rate of the cells. Moreover, when cells which grow rapidly (M-24) or slowly (M-200) in maltese medium are grown rapidly in sucrose, they have approximately the same number of ribosomes as the number found in the parental cell line. Since these types of cells have fewer rRNA cistrons than SB-1 cells (4), the reduction in number of cistrons (from 2200-1500) does not limit the amount of rRNA synthesized.

Transcription of rRNA Precursor Molecule. A heterogeneous population of rapidly labeled RNA molecules would be demonstrated by sedimenting radioactive RNA isolated from SB-1 cells (Fig. 1). For this purpose, SB-1 cells were labeled with radioactive \( ^{32} \text{P} \)phosphate for 15 min. The bottom 14 fractions of the gradient in Figure 1 (gradient fractions greater than 30S) were collected. This RNA hybridized to Bgl I DNA restriction fragments from both 18S and 25S rRNA structural genes (Fig. 2a). Hybridization of this same RNA to rDNA fragments in the presence of an excess of unlabeled 18S rRNA resulted in a specific hybridization pattern characteristic of 25S rDNA sequences (Fig. 2b), while hybridization in the presence of a large excess of unlabeled 25S rRNA results in hybridization to fragments (2.4 and 1.8 kilobases) characteristic of 18S rDNA sequences (Fig. 2c). Since this RNA fraction hybridizes to both 18S and 25S rDNA sequences, it must contain a common precursor of these rRNAs similar to the 40S RNA precursor described for other eukaryotic systems (3, 7, 9, 13).

In eukaryotes, the 18 and 25S rRNA structural genes are arranged in tandem. The two genes alternate with one another and are separated by two DNA spacer sequences. One of these spacers is not transcribed. The other is transcribed as part of the rRNA precursor molecule. Digestion of Charon 4A-RB115 with Bgl I generates a 1.15-kilobase DNA fragment (Fig. 2f) which does not hybridize to either 18S or 25S rRNA (Fig. 2, d and e) and lies within the large DNA spacer sequence. The result in Figure 2a indicates that the RNA from the precursor fractions in the experiment in Figure 1 also does not bind to this sequence. Therefore, this DNA fragment must be part of the nontranscribed spacer sequence separating the 18S and 25S structural genes.

Transcription of rRNA Occurs from One DNA Strand. The two
DNA strands of phage λ can be separated by equilibrium centrifugation in alkaline CsCl. To determine which rDNA strand is used for transcription, the two strands of Charon 4A-RLB15 were separated in CsCl. The fractions of the gradients were assayed for the presence of DNA and a mixture of 32P-radioactive labeled 18S plus 25S rRNAs was then hybridized to each of the different fractions. Radioactive cloned DNA fragments of Charon 4A-RLB15 were also hybridized to fractions from another identical gradient. Figure 3 indicates that the rDNA fragments from Charon 4A-RLB15 hybridize to both DNA strands, but the 18S and 25S rRNAs hybridize to only the light DNA strand. This indicates that both rRNAs are transcribed from one strand. From the polarity of the two strands of λ DNA (19) and the structure of Charon 4A-RLB15, it was possible to assign a polarity to the rDNA sequences (Fig. 4). The larger, nontranscribed, spacer sequence lies to the 3′-side of the 18S gene (with respect to the transcribed DNA strand). RNA polymerase reads the DNA coding strand in the 3′ to 5′ direction as it synthesizes RNA in 5′ to 3′ direction. Transcription must begin at the 5′-end of the nontranscribed spacer region. This suggests that the RNA promoter for transcribing the rDNA sequences is probably located within the nontranscribed spacer region between a Bgl I cleavage site close to the 18S structural gene and the beginning of the 18S structural sequence (see Fig. 4). Therefore, the 18S gene should be the first sequence transcribed during synthesis of the rRNA precursor molecule, as is observed in other organisms (3, 11).
Rates of rRNA Synthesis during Rapid and Slow Growth. The number of ribosomes within each cell is determined by the ribosome synthesis and degradation rates and the division rate of the cells. If the degradation rate is slow compared to the synthesis rate, the number of ribosomes per cell will be determined primarily by the rates of ribosomal synthesis and of cell division. After transfer from maltose into sucrose, SB-1 cells resume a 24-h doubling time and the first cell division occurs almost synchronously, 26 h following this transfer (data not shown). To determine the rate of ribosomal RNA synthesis, M-200 cells growing in maltose were labeled with $[^{32}P]$phosphate for different periods of time. Cells were harvested and RNA was extracted from the cells. Extracted RNA was sedimented through 10% to 30% sucrose gradients by centrifugation at 38,000 rpm in an SW 41 rotor. Gradient fractions were assayed for RNA and $^{32}P$ content and these values were utilized to determine the specific activity of the 25S rRNA: (O--O), M-200 cells growing in maltose with a 200-h generation time; (A--A), M-200 cells transferred from maltose medium to sucrose medium plus $[^{32}P]$phosphate; and ($\Delta$--$\Delta$), M-200 cells transferred from maltose medium to sucrose medium 2 h prior to addition of $[^{32}P]$phosphate.

FIG. 5. Synthesis of rRNA in cells with different growth rates. Cells were labeled with $[^{32}P]$phosphate for different periods of time and RNA was extracted from the cells. Extracted RNA was sedimented through 10% to 30% sucrose gradients by centrifugation at 38,000 rpm in an SW 41 rotor. Gradient fractions were assayed for RNA and $^{32}P$ content and these values were utilized to determine the specific activity of the 25S rRNA: (O--O), M-200 cells growing in maltose with a 200-h generation time; (A--A), M-200 cells transferred from maltose medium to sucrose medium plus $[^{32}P]$phosphate; and ($\Delta$--$\Delta$), M-200 cells transferred from maltose medium to sucrose medium 2 h prior to addition of $[^{32}P]$phosphate.

FIG. 6. Processing of rRNA in rapidly dividing cells. SB-1 cells with a 24-h generation time were labeled with $[^{32}P]$phosphate for 15 min then transferred to medium containing 100 mM phosphate buffer. Ribosomes were isolated from the cells following: (a) 0, (b) 15, (c) 30, (d) 45, (e) 60, (f) 90 min of growth and the rRNA extracted from these. The rRNA was sedimented through 10% to 30% sucrose gradients by centrifugation at 58,000 rpm for 3.5 h in an SW 60 rotor and fractions were assayed for $^{32}P$ radioactivity.

FIG. 7. Processing of rRNA in cells transferred from slow to rapid growth conditions. SB-1 cells growing with a 200-h generation time in maltose were transferred to sucrose medium containing $[^{32}P]$phosphate for 30 min. They were then transferred to fresh sucrose medium containing 100 mM phosphate buffer and ribosomes were isolated following (a) 0, (b) 30, (c) 60, and (d) 90 min of growth. RNA was extracted from the ribosomes and sedimented through 10% to 30% sucrose gradients by centrifugation for 3.5 h at 58,000 rpm. Fractions of the gradients were collected and assayed for $^{32}P$ radioactivity.
these cells than in cells previously grown in sucrose in which they divide every 24 h. This result suggests that the apparent delay of ribosomal RNA synthesis seen in M-200 cells transferred from maltose to sucrose (Fig. 5) is almost entirely due to rRNA processing. Thus, although rapid synthesis of RNA may begin within 30 min of adding sucrose, this RNA does not appear in ribosomes for another 60 to 90 min.

**DISCUSSION**

SB-1 cells, growing in sucrose with a 1-day doubling time have 7 times as many ribosomes as cells growing in maltose with an 8-day doubling time. The use of maltose as an energy-carbon source does not of itself affect the ribosome content since M-24 cells growing rapidly on maltose have a ribosome content equal to M-24 or SB-1 cells growing rapidly on sucrose.

The data in Figures 1 to 4 suggest that a precursor molecule is synthesized by transcribing the 18 and 25S structural genes together with an intervening 0.6-kilobase (or smaller) spacer. Transcription, as in other systems appears to begin with the 18S gene and proceed through the 25S gene (Fig. 5). This precursor molecule is processed into ribosomal RNA at different rates depending on the growth conditions. During continued growth in sucrose, this takes 30 to 45 min or about 2 to 3% doubling time. After growth in maltose, however, the time required for processing in sucrose is greater.

An 80-fold increase in the rate of ribosomal RNA synthesis is measured (after a 2-h lag) when cells are shifted from maltose to sucrose media. This lag corresponds to the processing time necessary to convert precursor molecules into 18 and 25S RNA, which suggests that after the shift to sucrose, the rate of RNA transcription may increase almost 100-fold within a few min. This is in contrast to the change in RNA synthesis which occurs when plant cell suspensions are transferred from stationary to exponential growth conditions (16). In this case, the rapid synthesis of messenger RNA is not immediately accompanied by an increase in ribosomal RNA synthesis. Instead, rate of cell division lags for 3 days and ribosomal RNA synthesis increases only 24 h after the change to exponential growth conditions.

During rapid growth, 10^7 ribosomes are synthesized from 1500 genes in 24 h at an average rate of 5 ribosomal RNA molecules per gene per min. If the average rate of precursor RNA synthesis is 3 min or as in other systems (9), this would mean that at any moment some 15 transcripts are being copied per 7.9-kilobase cistron. In contrast, M-200 cells growing in maltose medium need only make one transcript per cistron using one-fifth of the available rRNA cistrons. (Even fewer cistrons may be used if more than one transcript is synthesized per cistron at any moment.) If this is the case, cistrons which are normally not transcribed will become active within minutes of adding sucrose. An alternative regulatory mechanism could be that the rate of RNA chain elongation is 50 to 100 times slower in maltose medium than in sucrose. In either case, the soybean suspension culture system presents an ideal opportunity to study regulation of transcription in plants, an opportunity which we are currently pursuing.

**LITERATURE CITED**