Regulation of Ribosomal Ribonucleic Acid Levels in Growing, ^{3}H-Arrested, and Crisis-Phase WI-38 Human Diploid Fibroblasts†

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ABSTRACT: When the growth of WI-38 cells slowed in confluent cultures, the levels of RNA and protein per cell remained constant. In contrast, when cell division was completely arrested by incubation of cells in serum factor depleted medium, by ^{3}H irradiation, or by repeated passage of cells into the nondividing senescent phase, the RNA and protein contents of each cell increased up to 3.5-fold and then stabilized. We analyzed how the balance of synthesis and turnover could produce the higher levels of RNA. The flow of methyl label into nascent 45S pre-rRNA and rRNA and the turnover of methyl- or uridine-labeled cytoplasmic rRNA were measured. Computer-assisted modeling of data from gel electrophoretic separations of RNA [Wolf, S. F., & Schlessinger, D. (1977) *Biochemistry 16*, 2873] was used to quantitate rates of synthesis, processing, and nuclear turnover. In addition to the RNA species found in growing cells, phenol–detergent extracts from nongrowing cells contained a previously undetected, rapidly methyl-labeled fraction that was formed even in the presence of actinomycin D and was excluded from the analyses. The rates of initial labeling of 45S pre-rRNA varied at most by 50% in growing and nongrowing cells. The rates of formation of 18S and 28S rRNA were very similar, and the half-life of cytoplasmic RNA was 3–4 days in all growth states. In senescent cells, quantitation showed that at least 30% of the 18S and 28S rRNA was turned over in nuclear precursors and no 32S pre-rRNA was observed. In that case, wastage affected the accumulation of RNA and the processing pathway may be altered. In growing and ^{3}H-arrested cells, however, no nuclear turnover of 18S or 28S rRNA sequences was detected and all processing intermediates and levels were the same. Thus, the level of rRNA in diploid fibroblasts increases when division stops primarily because dilution of RNA into daughter cells ceases.

Cellular ribosome content can be regulated by (1) variable rates of rRNA transcription (Abelson et al., 1974; Johnson et al., 1976), (2) variable rates of rRNA processing (Johnson et al., 1976), (3) variable amounts of newly formed rRNA that are discarded completely and never arrive in completed ribosomes ("wastage"; Bowman & Emerson, 1977; Cooper, 1973; Gausing, 1977), and (4) variable rates of breakdown of mature cytoplasmic ribosomes (Abelson et al., 1974; Bowman & Emerson, 1977; Hirsch & Hiatt, 1966; Melvin et al., 1976; Weber, 1972).

For an assessment of the relative importance of these control points, both nuclear processing and the stability of the resulting cytoplasmic RNA must be analyzed quantitatively. Recently, we have found that [^{3}H]methylmethionine labeling can be used to measure all the relevant parameters and to improve the quantitation of results (Wolf, 1977; Wolf & Schlessinger, 1977).

Fibroblasts provide a case where the analysis can be applied throughout the natural life span of cells. The cells have a limited mitotic capacity in culture and then reach a nondividing "senescent" state (the "crisis phase"; Hayflick & Moorhead, 1961). They can also be rendered nonmitotic by two other means, ^{3}H irradiation (Ehmann et al., 1975; Liebhaber & Schlessinger, 1975; Marz et al., 1977) and growth in factor-depleted serum (Liebhaber et al., 1978).

Here we report that in all three types of arrested cells, the level of RNA increased sharply. The level of RNA increased primarily because the dilution of rRNA into daughter cells stopped. In addition, in senescent fibroblasts many of the nuclear precursors to 28S and 18S rRNA were degraded.

Experimental Procedures

Cells and Cell Culture. WI-38 human diploid fibroblasts were obtained from the American Type Culture Collection at passage 18 and grown at 37 °C in 5% CO_2 in minimum Eagle's medium (MEM) α supplemented with 10% fetal calf serum (Kansas City Biologicals). They were often used at passage 25 ("P-25 cells").

Early passage cultures doubled at a rate which gradually slowed from less than 40 h to every third day. At approximately passage 47, the cultures' doubling times sharply increased to several weeks, and by approximately passage 50–53, as expected (Hayflick & Moorhead, 1961), the cells no longer doubled ("P-50 cells").

To obtain early passage cells that had also stopped dividing, as a control, we arrested WI-38 cells either by labeling them for 24 h in growth medium containing [^{3}H]methylmethionine (10.5 Ci/mM from New England Nuclear) at 20–80 μCi/mL, in the presence of 20 mM sodium formate (Ehmann et al., 1975; Liebhaber & Schlessinger, 1978; Marz et al., 1977), or, in experiments to measure the subsequent rate of accumulation of methyl label in RNA, by labeling them for 24 h in normal medium containing [^{3}H]leucine (12 Ci/mM from New England Nuclear; 20 μCi/mL). DNA was simultaneously labeled with 0.1 μCi/mL [2-^{14}C]thymidine (57 mCi/mM; New England Nuclear). These cells showed no further increase in cell count but could reattach to Petri dishes after trypsinization, exclude trypan blue, and lose less than 1% of prelabeled [^{14}C]DNA per day.

To arrest cells in factor-depleted medium, we used a medium containing 1% fetal calf serum after it had been in contact with a confluent monolayer of WI-38 cells for 3 days [see Liebhaber et al. (1978)].

Determination of ^{3}H-Labeled rRNA Decay. To study rRNA decay during exponential growth or in senescent or serum factor depleted medium, we labeled cells for 24 h in

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medium with 0.2 μCi/mL [5-^{3}H]uridine (28 Ci/mmol, New England Nuclear) and DNA with 0.003 μCi/mL [2-^{14}C]-thymidine (57 Ci/mol, New England Nuclear). Labeled cells were washed twice with phosphate-buffered saline, trypsinized, resuspended in unlabeled medium containing 1–10 mM uridine and 0.1–5 mM cytidine, and plated out in 60-mm Petri dishes, usually at 2×10^5 cells/dish. [For ^{3}H-arrested cultures, labeling was with [^{3}H]uridine (10 μCi/mL) for 24 h in order to both arrest cells and label their RNA.]

On the indicated days, cells from two plates were trypsinized into 3 mL of 0.25 M sucrose, counted on a hemocytometer, precipitated with ice-cold 5% CCl_3COOH, and counted in toluene-based scintillation fluid. Decay rates of total ^{3}H-labeled RNA were corrected for cell loss (usually less than 1%/day) by plotting all values as $^3H/^{14}C$. To measure the decay rates of rRNA species, we brought 2.5 mL of the cell suspension to 100 mM Tris–acetate, pH 5.4, 10 mM EDTA, and 0.5% sodium dodecyl sulfate and extracted RNA once at 55 °C for 5 min with phenol and a second time at 0 °C with 0.5 volume of phenol–chloroform–isoamyl alcohol (1:1:0.01). The final aqueous phases were stored in 70% ethanol at −20 °C.

In preparation for polyacrylamide gel electrophoresis, each sample was centrifuged and the RNA pellet was dried by a stream of air at 4 °C and resuspended in 65 μL of 0.1 M sodium acetate, 0.001 M EDTA, and 0.5% sodium dodecyl sulfate in 90% formamide. A 5-μL sample was counted ("phenol-extracted counts") in 5 mL of 10% protosol (New England Nuclear) in a toluene-based scintillator. To the remainder of the sample was added 0.1% bromophenol blue and marker ^{14}C-labeled HeLa or *Escherichia coli* rRNA. Samples were warmed at 55 °C for 5 min, chilled to 0 °C, and layered on the polyacrylamide gels.

Determination of Nuclear Metabolism of rRNA. A detailed description of the protocol for labeling and extracting rRNA labeled with [^{3}H]methylmethionine in the presence of adenosine, guanosine, and formate is given in Wolf & Schlessinger (1977). To summarize, the washed cells were allowed to preincubate in the fresh low methionine medium for 1 h. The preincubation medium was aspirated off and 1.5 mL of the same medium containing [^{3}H]methylmethionine (New England Nuclear) was added. For the kinetic analysis of pre-rRNA processing, three sets of plates were used in order to avoid any perturbations in the cells due to the low methionine media (Wolf & Schlessinger, 1977). At the indicated times, labeling was terminated by aspirating off the medium and placing the Petri dish on ice. The monolayer was then rinsed with ice-cold solution A (0.2 M NaCl, 5 mM KCl, 16 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). The cells were lysed in extraction buffer (0.05 M EDTA and 0.5% sodium dodecyl sulfate in 0.1 M Tris–acetate, pH 5.4), and the RNA was extracted several times with warm phenol. The material purified by phenol extraction was precipitated with 2.5 volumes of 95% ethanol and stored overnight a −20 °C.

To determine the rates of synthesis of 45S pre-rRNA, we preincubated cells in low methionine medium (1.0 μg/mL methionine) as described above and then labeled them for 15 min in methionine-free medium containing [^{3}H]methylmethionine (11 Ci/mmol) at a concentration of 0.1 mCi/mL.

Polyacrylamide Disc Gel Electrophoresis and Quantitation. The technique used for acrylamide gel electrophoresis was essentially that of Weinberg & Penman (1968), with the modifications described before (Wolf & Schlessinger, 1977). After electrophoresis, gels were frozen and sliced. The slices were dissolved in vials containing 5% protosol in a toluene scintillator. The counts per minute was then summed up for each RNA species.

On the basis of the assumption of stochastic processing, simulation, analysis, and modeling (SAAM) 23 program determines the net rate at which label accumulates in any species from the difference between the sum of the rates of its formation and the rates of its processing into all products.

A processing model is submitted to the SAAM 23 program by specifying (1) an initial estimate of the rate constants, (2) initial conditions, (3) the accumulation data for the experiment, and (4) error estimates for the data. When desired, wastage pathways are included by submitting a rate constant for a pathway with no normal product. The program then iteratively adjusts the rate constants to minimize the sum of squares of the difference between the calculated and observed values. The interdependent fitting of the accumulation curves of each of the pre-rRNA and rRNA species yields much smaller error estimates than would be possible if data for individual rRNA species could only be analyzed independently of data for the other species [see Wolf & Schlessinger (1977) for further details].

Determination of Methionine Pool Sizes. Cultures of cells were incubated in low methionine medium (1.0 μg/mL methionine) for 1 h. The medium was then aspirated off, and macromolecules in the monolayer were precipitated and washed with 0.2 N perchloric acid at 0 °C. The perchloric acid extract was neutralized with potassium hydroxide, and the potassium perchlorate was removed by centrifugation. The supernatant was lyophilized, resuspended in a small volume, and analyzed on a Beckman Model 120B amino acid analyzer under standard conditions. From the elution profiles, the amount of methionine per cell was the same in all cultures.

Results

Levels of RNA and Protein in Growing and Nongrowing Cells. Growing WI-38 fibroblasts usually contain ~16–23 μg of RNA/10^6 cells, a figure similar to that reported for HeLa and other cultured cells (Littlefield, 1976). Cells that had become confluent (day 4 in Figure 1) slowed their rate of growth progressively but maintained a constant RNA content (Figure 1, open symbols). In contrast, in cultures that had stopped growing, whether by senescence, by ^{3}H labeling, or by incubation in serum factor depleted medium, we repeatedly found levels as high as 70 μg of RNA/10^6 cells. The increase in RNA was rapid and reached a new steady state up to 3.5-fold higher after ~7–8 days (Figure 1).

As passaged fibroblasts approached senescence, their content of RNA increased progressively with the population doubling time [as reported by Schneider & Shoer (1975) and Pochron et al. (1978)], but the increase in RNA was still appreciable during the final accelerated decline of growth rate (Figure 1 and unpublished data). The final level of RNA was again about threefold higher than that in growing cells.

Increases in the protein content proportional to that of RNA were found in all the cultures so that the ratio of RNA to protein remained the same in nongrowing and growing cultures (Figure 1, right panels). Increases in protein could most simply arise from the continued function of larger numbers of ribosomes, but the level of RNA could alter through changes in rates of synthesis, processing or wastage, or turnover. We analyzed further the RNA metabolism in growing, ^{3}H-arrested, and senescent cells.

Methyl Labeling of Nuclear RNA Species. Methyl label is a useful tracer to detect rRNA transcription, processing, and any nuclear wastage. Exogenous methionine rapidly equilibrates with the cellular pool in these cells (Wolf &

Table I: Summary of Measured Kinetic Data[a]

cells	cells/culture ($\times 10^{-5}$)	synthesis: μg of RNA extracted per culture (±10%)	synthesis: cpm in 45S RNA per μg of RNA	synthesis: cpm in 45S RNA per 10^5 cells	processing: pathway	processing: wastage (%)
		12	340	567		
P-25	7.2 ± 6%	11	312	477	normal	0
		13	303	547		
^{3}H-arrested P-25	6.9 ± 6%	19	219	603		
		19	205	565	normal	0
		6	138	690		
P-50	1.2 ± 15%	7	130	758	no vis pool of 32S	50 ± 36

[a] The number of cells per cultures was determined by a hemocytometer count of a sample of cells from a Petri dish treated in parallel with the labeled dishes. The ^{3}H-arrested cells were tested 2 days after the stoppage of growth, when the rRNA level per cell has not yet attained its maximum (Figure 1). Labeling, RNA extraction and quantitation, and kinetic analysis were performed as described under Experimental Procedures. The data related to "synthesis" are from parallel samples in one experiment. The processing pathways and percent wastage were determined from the best fit in the computer analysis. Error estimates of wastage were determined by the computer program.

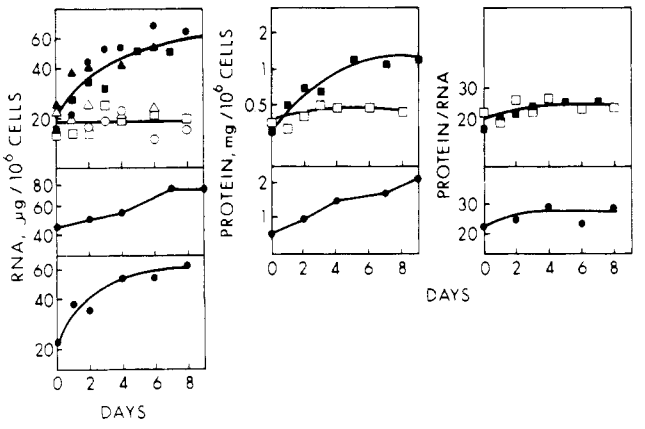

FIGURE 1: RNA and protein levels in WI-38 cells after ^{3}H arrest of cell division, in serum factor depleted cultures, and in senescent cells. For the top panels, 4 days before day 0, confluent cultures of passage no. 26 WI-38 cells were trypsinized and plated in 40 75-cm^2 Costar tissue culture flasks at 1.5×10^5 cells/flask. For ^{3}H-arrested cultures, 1 day before day 0, 20 mL of MEM α containing 10% fetal calf serum and 10 μCi/mL [5-^{3}H]uridine was added to each of 20 flasks to produce ^{3}H arrest. Twenty-four hours later, the medium was aspirated off all the flasks, the monolayers were rinsed with warm solution A, 20 mL of unlabeled medium was again added to each flask, and incubation was resumed at 37 °C. On the indicated days, pairs of flasks of ^{3}H-arrested cells (filled symbols) or growing cells (open symbols) were trypsinized. The number of cells from each flask was determined in a hemocytometer, and RNA was then extracted and hydrolyzed from each sample by two incubations at 90 °C, each in 0.5 mL of 5% trichloroacetic acid for 15 min. The precipitated protein was collected by centrifugation, and RNA was determined on the 1-mL supernatants by the method of Webb (1956). Protein was determined by the method of Lowry et al. (1951). The top left curve shows the expected accumulation of RNA ("R") in a ^{3}H-arrested culture in which dR/dt = (rate of synthesis) − k_2R (see text), with a half-life of rRNA of 96 h. The other curves were drawn by eye. The top panels show data from three different experiments (●, ▲, and ■). The middle panels show data for senescent cells (P-50); the bottom panel shows data for cells maintained in serum factor depleted medium [prepared as described under Experimental Procedures; cf. Liebhaber et al. (1978)]. Comparative data for growing cultures of control cells are shown in open symbols.

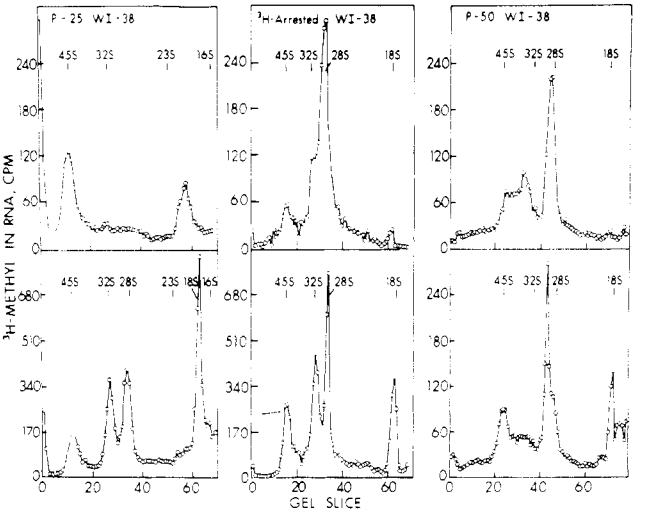

FIGURE 2: Polyacrylamide gel profiles of ^{3}H-labeled rRNA. Cultures were treated and labeled with [^{3}H]methylmethionine, and the RNA was extracted and run on polyacrylamide gels as described under Experimental Procedures. The top panels show profiles of RNA extracted after labeling for 5 min, and the lower panels show profiles after labeling for 75 min. ^{14}C-Labeled marker 23S and 16S *E. coli* rRNA were coelectrophoresed with the growing WI-38 (P-25) samples. ^{14}C-Labeled marker 28S and 18S HeLa rRNA were coelectrophoresed with the ^{3}H-arrested and senescent (P-50) samples.

Schlessinger, 1977); the bulk of rRNA methylation occurs during or shortly after transcription (Zimmerman & Holler, 1967), and all methyl groups are conserved during rRNA processing (Maden & Salim, 1974).

The accumulation of the [^{3}H]methyl label in the individual pre-rRNA and rRNA species was determined from polyacrylamide gel profiles (as in Figure 2) of purified total RNA extracted from logarithmically growing, ^{3}H-arrested, and crisis-phase cells during continuous labeling. In all these cultures, as in HeLa cells (Wolf & Schlessinger, 1977), the labeling of cellular RNA and 45S pre-rRNA with [^{3}H]-methylmethionine was linear for the first 15 min of labeling. Therefore, the initial rates of uptake into pre-rRNA (Table I) provide an estimate of the rate of synthesis, since the specific activity of the methionine pool was comparable in all the cultures. [A single lot of medium with ^{3}H-labeled methionine was used, and the methionine pool size in the cells was the same and very small in all three cultures [see Liebhaber et al. (1978) and Experimental Procedures]]. The actual data and relative rates of incorporation into 45S pre-rRNA in a 10-min pulse label are given in Table I for a particular experiment. If the rate of incorporation per cell in growing P-25 cells is set equal to 1.0 (±10%), then the relative rates in ^{3}H-arrested and senescent cells varied by no more than 50% (1.2 ± 10% and 1.3 ± 20%, respectively).

Figure 2 shows typical gel profiles of labeled RNA extracted from cells in the three growth states after 5 and 75 min of labeling. In the gel profiles of growing and ^{3}H-arrested cells, the 45S precursor, 32S intermediate, and 28S and 18S rRNA products could be clearly seen by 75 min; the 20S pre-rRNA,

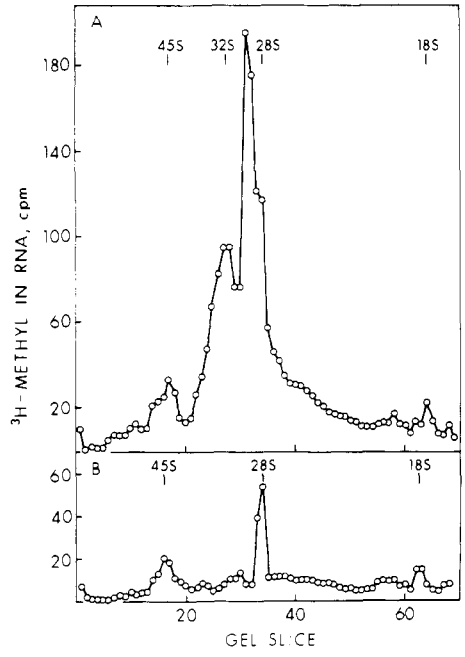

FIGURE 3: Polyacrylamide gel profiles of ^{3}H pulse-labeled material before and after a chase period. Cultures of passage 50 WI-38 cells were preincubated for 1 h in MEM α (containing only 0.5 μg/mL methionine and supplemented with 10% dialyzed fetal calf serum). The top panel shows the gel profile from material labeled for 15 min with [^{3}H]methylmethionine (8 μCi/mL, 11 Ci/mmol). The lower panel shows the gel profile of material from a duplicate culture labeled in parallel and then washed once with normal MEM α containing 10% fetal calf serum and incubated 20 min further in the normal medium before phenol extraction.

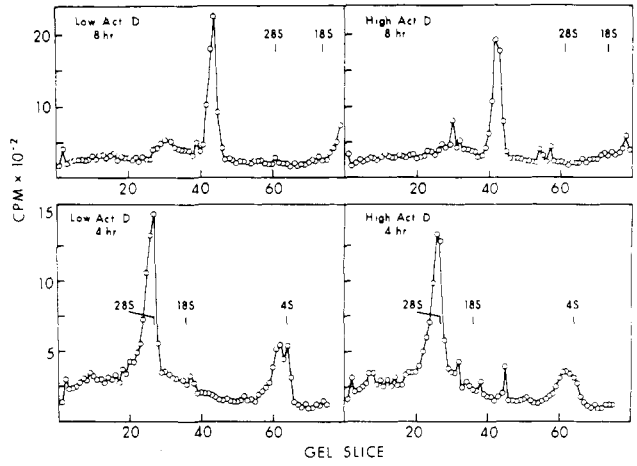

FIGURE 4: Polyacrylamide gel profiles of material labeled with [^{3}H]methylmethionine in crisis-phase cells in the presence of actinomycin D. Cultures of passage 49 WI-38 cells in 60-mm Petri dishes were preincubated for 30 min in low methionine medium (see Experimental Procedures) and with actinomycin D at a concentration of 0.04 or 0.4 μg/mL as indicated. The cultures were further incubated for 15 min in the presence of [^{3}H]methylmethionine at 60 μCi/mL (11 Ci/mmol). The upper panels show profiles of phenol-extracted material (as described under Experimental Procedures) from gels electrophoresed for 8 h; the lower panels show profiles for parallel gels electrophoresed for only 4 h.

as expected, was always low in its content of label. After 5 min of labeling, some radioactive 18S rRNA was observed in growing cells, resulting from the methylation of six residues in cytoplasmic 18S rRNA.

In the two types of nondividing cells, however, additional material was observed at 28S to 36S. It was nonribosomal because (1) in both ^{3}H-arrested and senescent cells, it was labeled to saturation within 10 min [faster than the 45S pre-rRNA (Figure 2 and unpublished data)] and during a chase period in the absence of labeled methionine little if any of this material became rRNA (Figure 3), (2) unlike known pre-rRNA and rRNA species, the mobility of this material relative to other peaks changed with the time of electrophoresis (Figure 4), (3) the synthesis of the material, unlike that of rRNA, was not inhibited by 0.04 or even 0.4 μg/mL actinomycin D (Figure 4), and (4) it could not be labeled with [^{3}H]uridine (data not shown).

This fraction may represent small amounts of methyl-labeled DNA synthesized in short chains (Wolf, 1977) and may represent a feature of DNA turnover or abortive synthesis in nongrowing cells. In the analysis of RNA metabolism it introduces uncertainty in the levels of 28S RNA and 32S pre-rRNA at early times in the nongrowing cells. The unusual electrophoretic behavior of the material (Figure 4) permits it to be distinguished from true rRNA precursors by comparison of replicate gels run for different times, but similar accumulation data (Figure 5) were derived by subtracting the 10-min values for the 32S–36S material from the RNA patterns of nongrowing cells labeled for longer times.

Computer-Assisted Testing for Rates of Processing and Wastage. The order and efficiency of pre-rRNA processing steps can be accurately analyzed with a simulation, analysis, and modeling program (SAAM) (Berman, 1966; Wolf & Schlessinger, 1977). The program simultaneously obtains a least-squares fit to the accumulation data for all of the RNA species, thereby determining rate constants for (1) synthesis of the primary 45S transcript, (2) processing of each of the pre-rRNA intermediates, and (3) any appreciable degradation of the pre-rRNA intermediates. The program also calculates the standard deviation of each rate constant.

Table II: Pre-rRNA Half-Lives and Cellular Pool Sizes[a]

RNA	$t_{1/2}$ (min) P-25	^{3}H-arrested P-25	P-50
45S	16 (±5%)	13 (±7%)	12 (±15%)
32S	37 (±8%)	39 (±10%)	
20S	8 (±17%)	10 (±18%)	
RNA	**pool sizes (relative to 45S RNA) P-25**	**^{3}H-arrested P-25**	**P-50**
45S	1.0 (±5%)	1.0 (±7%)	1.0 (±10%)
32S	2.5 (±8%)	2.0 (±10%)	0
20S	0.5 (±17%)	0.4 (±18%)	0

[a] The half-lives were computed from the calculated rate constants as described under Experimental Procedures. The percentage errors in parentheses are those arrived at by the SAAM program. Relative pool sizes were calculated by solving the differential equation for the model at the steady-state limit. At that time the rate of change of label content equals zero and the appropriate equations reduce to simple algebraic relationships [see Wolf (1977)]. The level of 45S pre-rRNA was arbitrarily set equal to 1.0, and the other pool sizes were calculated relative to the 45S species.

Computer analysis of the accumulation data for growing and ^{3}H-arrested cells resulted in the closest fitting accumulation curves (Figure 5) when the well accepted processing observed in HeLa cells was used: 45S to 32S and 20S rRNA and then to 28S and 18S rRNA [see Hadjiolov & Nikolaev (1976) for a review]. The intermediates were also processed with very similar half-lives in both growing and nongrowing cells (Table II).

In the "crisis state" (P-50), 28S and 18S rRNA did appear and the half-life of 45S pre-rRNA remained very similar (Table II) but little or none of the normal 32S pre-rRNA

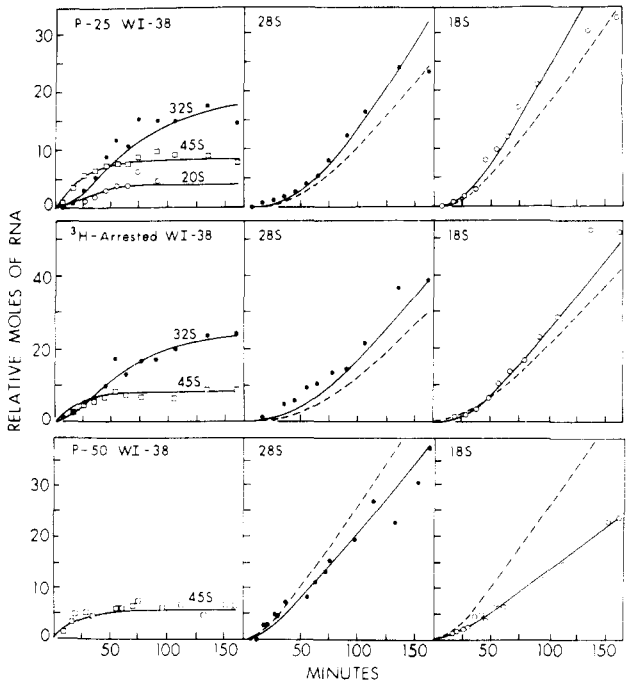

FIGURE 5: [^{3}H]Methyl-labeled pre-rRNA and rRNA accumulation curves. The accumulation of label into pre-rRNA species was quantitated and analyzed as desired in the text. The points represent the actual data. The solid lines represent the calculated accumulation curves for the best computer fit to the data. For P-25 cells and ^{3}H-arrested P-25 cells, the dashed lines illustrate theoretical accumulation curves assuming the same rates of synthesis and processing arrived at in the "best fit" but including 20% wastage of the synthesized 32S and 20S pre-rRNAs instead of no wastage. For P-50 cells, the solid lines include 50% wastage of 18S rRNA sequences and 20% wastage of 28S rRNA sequences. The dashed lines for P-50 cells indicate the calculated accumulation curves assuming no wastage. Relative moles were arrived at by dividing the accumulated counts in a species by the number of methyl groups per RNA chain of that species.

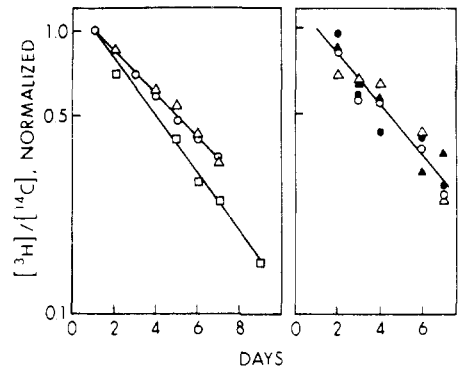

FIGURE 6: Decay of ^{3}H-labeled RNA from growing (P-25), ^{3}H-arrested, and senescent (P-50) WI-38 human diploid fibroblasts. Cells were labeled at the indicated passage for 24 h with 0.1 μCi/mL [5-^{3}H]uridine and 0.002 μCi/mL [2-^{14}C]thymidine (or with 5 μCi/mL [^{3}H]uridine and 0.05 μCi/mL [2-^{14}C]thymidine to arrest them). After the pulse period, cells were washed twice with phosphate-buffered saline. The growing cells, which were labeled in a T flask, were then trypsinized into unlabeled medium containing 10 mM uridine and 5 mM cytidine and plated out in 60-mm Petri dishes. The senescent or ^{3}H-arrested cells, which were already in Petri dishes during the labeling, were washed as described above, and the chase medium was added. Cells were incubated at 37 °C, and the medium was changed every 48 h. On the indicated days, cells from two plates were trypsinized and the radioactivity in individual RNA species was measured as described under Experimental Procedures. Decay rates for total RNA or for 28S and 18S rRNA, whose label content was determined from polyacrylamide gel profiles, were corrected for cell loss (usually less than 1%/day) by plotting all values as ^{3}H/^{14}C, normalized to 1.0 at day 1. The left panel shows total RNA in growing cells (○), ^{3}H-arrested cells (△), and senescent cells (□). The right panel shows 28S (●) and 18S (▲) rRNA in growing cells and 28S (○) and 18S (△) rRNA in senescent cells.

intermediate was detected. Either the 32S pre-rRNA does not form in P-50 cells or it has a very short half-life compared to its half-life in growing cells.

The computer-assisted analysis of [^{3}H]methyl-labeled RNA accumulation data was used to test for wastage of pre-rRNA species. Wastage was looked for by including a pathway for wastage of each of the pre-rRNAs in the model used for the data analysis. In growing cells possible pathways for wastage of 45S, 32S, and 20S pre-rRNA were eliminated by the computer program as it reached the best fit to the data. For example, the solid lines in Figure 5 (top panels) show the best computer fit to the measured accumulation data. The dashed lines represent predicted accumulation curves of 28S and 18S rRNA if as much as 20% wastage was imposed on 32S and 20S pre-rRNA. It is clear that that much wastage is excluded. In repeated experiments at least 90% of the rRNA sequences which started in 45S pre-rRNA eventually appeared in 28S and 18S rRNA.

In the ^{3}H-arrested cells, wastage was again probably inappreciable (Figure 5, middle panels), although a slight amount of wastage of 20S pre-rRNA (12%) was consistent with the data.

In contrast, for senescent cells, wastage of 50% of the 18S rRNA sequences and at least 20% of the 28S rRNA sequences was inferred when the computer analysis was used to compare the accumulation of label in 45S pre-rRNA to the accumulation of 28S and 18S rRNA (Figure 5).

Thus, in the case of P-50 senescent cells, and only in that case, a major fraction of newly synthesized rRNA sequences was destroyed and its destruction occurred at some point after initial processing of 45S pre-rRNA. In senescent cells, as in the other cultures, the computer modeling also showed that rapid turnover of 45S pre-rRNA or 18S or 28S rRNA was not compatible with the kinetic data.

Cytoplasmic Turnover Rates. Turnover was assessed by the decrease in the radioactivity of RNA prelabeled with [^{3}H]methyl groups or [^{3}H]uridine. Total RNA and cytoplasmic 28S and 18S rRNA, as well as 4S RNA, decayed with first-order kinetics, with a half-life of 72–96 h in all growth states (Figure 6 shows data for total RNA and for 18S and 28S rRNA labeled with [^{3}H]uridine in growing and senescent cells; cf. Liebhaber et al. (1978) for more data on P-25 cells).

Discussion

Control Points for rRNA Levels. In the mammalian systems studied thus far, changes in growth state affect rRNA metabolism in different ways. In regenerating rat liver, for example, rRNA synthesis increases to provide more ribosomes for new cells (Blobel & Potter, 1968). But during compensatory renal hypertrophy, rRNA synthesis remains constant and rRNA accumulation increases because the rate of cytoplasmic turnover decreases. As for cultured cells, in fusing quail myoblasts, for example, transcription continues at the same rate as in growing cells but the turnover of rRNA increases to limit the rRNA content of the cells (Bowman & Emerson, 1977). In growth-arrested mouse 3T3 cells and chicken fibroblasts, the rate of rRNA turnover also increases sharply (Abelson et al., 1974; Weber, 1972), but in mouse fibroblast lines (Johnson et al., 1976) and resting lymphocytes (Cooper, 1973; Purtell & Anthony, 1975), qualitative changes in rRNA synthesis and in the efficiency of processing have also been suggested.

The present study had two aims: to attempt a more quantitative assessment of every step in RNA metabolism in a model case and to analyze a number of growth states for a single cell type. Diploid WI-38 fibroblasts were studied during active growth, as well as after growth arrest by ^3H irradiation, by incubation in factor-depleted medium, or by senescence.

It became clear early on that when growth slowed, at least two different responses of RNA metabolism could occur. In WI-38 cells in which growth slowed down at confluence (day 4 for the growing cultures illustrated in Figure 1), the RNA content of the cells remained the same and the half-life of cytoplasmic RNA also remained very similar (Liebhaber et al., 1978). These results suggested that in this case the rate of rRNA synthesis drops, as has been reported for confluent cultures of 3T6 mouse fibroblasts (Abelson et al., 1974).

Rather different results were found when growth was completely stopped by ^3H arrest, senescence, or factor-depleted medium (Figure 1). The level of rRNA per cell increased and reached a new steady-state level up to 3.5-fold higher after ~1 week.

To understand how the RNA levels changed, we analyzed the kinetics of RNA metabolism. Two nongrowing states, one spontaneous (senescence) and the other forced (^3H arrest), were chosen for comparison to growing cells. [Confluent cultures of WI-38 cells were not used because they are hard to study precisely; growth slows gradually but does not stop (Littlefield, 1976).]

The flow of methyl label was followed to assess steps in nuclear rRNA metabolism. In all the growth states, methyl label appeared in 45S pre-rRNA and cytoplasmic 18S and 28S rRNA at very similar rates (Tables I and II and Figure 5). Because many data points were taken, the analysis became accurate enough to show clearly the consistency of the data with first-order processing steps. [Very rapid ordered events within the pool of molecules in a single electrophoretic species could be occurring (Hadjiolov & Nikolaev, 1976) but only at a rate rapid compared to the observed half-life of that species.] The rate constants, derived from the measured pool sizes and the measured incorporation rates (Table II and Figure 5), are unique within the errors of the determinations.

As discussed elsewhere (Wolf, 1977), the computer-assisted analysis sharpens the estimates of rates because the rates are interdependent. The rates are consistent with the generally accepted scheme for processing (Hadjiolov & Nikolaev, 1976) and show (1) that processing is not a point of differential regulation of rRNA synthesis in the different growth states (Table II) (however, an alternate processing scheme cannot be excluded for crisis-phase cells, in which no 32S pre-rRNA was detected) and (2) that in growing or ^3H-arrested cells all or nearly all of the methyl-labeled precursor chains arrive as cytoplasmic rRNA, i.e., there is no wastage (see the text, Figure 5, and Table I).

The unanticipated rapidly methylated component in some gels (Figure 2) weakens the inference that no wastage of 28S occurs at early labeling times in ^3H-arrested cells. Although this fraction may be an interesting feature of nongrowing cells, it is a nuisance when quantitating the label content of the 32S and 28S RNA species. Nevertheless, (1) the determinations of the label content of 18S rRNA and the resulting inferences are unambiguous and (2) the estimated levels of label in 28S and 32S rRNA are relatively good at later times. Even at early times, good agreement was observed for estimates from gels electrophoresed for different times (as in Figure 4) and for estimates made by subtracting from the 28S rRNA peak the radioactivity in the odd methylated species labeled for 10 min (to saturation) in the presence or absence of actinomycin D (as in Figure 3).

The reliability of the determinations is also strongly supported by the ability of the rates of synthesis and cytoplasmic decay to account for the steady-state levels of RNA. If synthesis and turnover are indeed the predominant controls of the rRNA level when growth is arrested and they remain constant, the level of RNA in the cells will not therefore be constant. Rather, because no dilution of RNA into new daughter cells is occurring, the level of rRNA in each cell will rise. An estimate of the expected rate and extent of increase is shown in the top left curve in Figure 1.

This curve was calculated by using a form of the standard equation (Kafatos, 1972; Palmiter, 1973) that relates dR/dt in each cell to the balance of synthesis (K_0), decay (k_2R), and dilution of rRNA by cell division. In the steady state in growing cells, $K_0 = (k_D + k_2)R$, where k_D is the growth constant and k_2 is the cytoplasmic decay constant. In nongrowing cells, $dR/dt = K_0 - k_2R$. Thus

$$\int \frac{dR}{K_0 - k_2R} = \int dt \qquad (1)$$

Knowing that at $t = t_0$, and $R = R_0$, one can integrate and evaluate the integral between R and R_0 and t and t_0, yielding

$$-\frac{1}{k_2}[\ln (K_0 - k_2R) - \ln (K_0 - k_2R_0)] = t \qquad (2)$$

Because the rate of transcription is very similar in nongrowing and growing cultures (Table I), K_0 in nongrowing cells equals $(k_D + k_2)R_0$. Letting $k_D + k_2 = k_1$ and substituting k_1R_0 for K_0 in eq 2

$$\frac{R}{R_0} = \frac{k_1}{k_2} - \frac{k_1 - k_2}{k_2}e^{-k_2t} \qquad (3)$$

Equation 3 implies that as time passes, the value of R approaches $(k_1/k_2)R_0$. From the actual data on the range of synthetic rates and half-lives of rRNA, the expected final ratio in different experiments would range from 3 to 3.6 and would be attained over ~7 days. In the experiments and calculations for Figure 1, the culture doubling time was ~36 h and the half-life of rRNA was ~96 h in all cases. As expected, a new homeostatic level of rRNA, about threefold higher than in growing cells, was reached after ~7 days.

Wastage and rRNA Levels in Crisis-Phase Cells. Nuclear wastage has often been noted as a means of regulating the accumulation of rRNA (Abelson et al., 1974; Bowman & Emerson, 1977; Cooper, 1973). However, wastage was not found in early passage human fibroblasts, whether growing or nongrowing. In contrast, the present data, although too inaccurate to quantitate the amount precisely, clearly demonstrate that in senescent cells a significant fraction of rRNA sequences in 45S pre-RNA, ~20–50%, is wasted.

Chien-Kiang et al. (1980) have shown that when labeled methionine is added to adenovirus 2 infected HeLa cells, the *S*-adenosylmethionine pool requires 60 min to become saturated with methyl label. This raises the possibility that the results presented here underestimate the initial levels of labeled 45S pre-rRNA. The extent of pre-rRNA wastage could then be even higher than we have estimated. [Such a possibility has not been excluded, but two observations suggest that the *S*-adenosylmethionine pool is probably rapidly equilibrated with label in the cells we have used: (1) in every experiment, both with HeLa cells in suspension culture or grown on plates

(Wolf & Schlessinger, 1977) and with the primary fibroblast cultures, the incorporation of methyl label into both protein and RNA showed no lag, increasing linearly within 5 min of the initiation of labeling (Figure 5 and unpublished data) and (2) computer models which included a pool prior to 45S pre-rRNA (equivalent to a slowly equilibrating labeled precursor) did not improve the computer fit to the data.]

Despite the wastage detected, because the rate of rRNA transcription is somewhat greater (Table I) and the half-life of cytoplasmic 28S and 18S rRNA in senescent cells is about the same, the final "steady-state" level of rRNA in crisis-phase cells is comparable to that in ^{3}H-arrested cells. The balance of synthesis and turnover is sufficient to explain the progressive increase of RNA content already reported in senescing cells (Schneider & Shoer, 1975; Pochron et al., 1978).

The mechanism of wastage of rRNA sequences in senescent cells is not known. One speculative possibility is that defective precursors, which may begin to be formed in crisis-phase cells, fail to meet the requirements for processing, such as a full complement of functional ribosomal proteins (Maden et al., 1969; Warner et al., 1966), and are exposed to nucleases. That the cytoplasmic ribosomes still produced in senescent cells are indeed fully functional is supported by two observations: (1) the protein/RNA ratio in senescent cells remains the same as that in growing cells (Figure 1) and (2) the incorporation of ^{3}H-labeled amino acids per ribosome in a pulse label remains the same in senescent cells (work in progress).

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