JOURNAL OF BACTERIOLOGY, July 1972, p. 66-72

Vol. 111, No. 1
Printed in U.S.A.

Synthesis, No Synthesis, or Synthesis and Degradation of Ribosomal Ribonucleic Acid in Various *Escherichia coli* Strains Starved for an Amino Acid

E. CRAIG, D. SCHLESSINGER, AND C. GURGO

Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received for publication 8 March 1972

A standard stringent strain of *Escherichia coli* makes little or no ribosomal ribonucleic acid (RNA) during starvation for an essential amino acid, whereas the isogenic relaxed strain makes both ribosomal and messenger RNA. A third class of strains was found which continues to make ribosomal RNA during starvation, but the RNA made is apparently unstable. There is little accumulation of RNA in the third class of strains, and few complete newly formed chains of ^{3}H-ribosomal RNA are observed in sedimentation analyses, even after long labeling times.

The net increase of ribonucleic acid (RNA) in *Escherichia coli* strains starved for an essential amino acid is regulated differently in relaxed and stringent cells. Stringent strains do not accumulate RNA (14, 16); relaxed strains accumulate RNA at a nearly normal rate (2). However, even in stringent strains some RNA synthesis continues (5). Recent studies indicate that in stringent strains the synthesis of ribosomal RNA (rRNA) and transfer RNA (tRNA) is sharply curtailed, whereas messenger RNA (mRNA) synthesis continues at a slightly reduced rate (9).

Variations from the two extremes—none at all or normal accumulation of RNA—have been reported. Fiil and Friesen (4) showed that the rate of incorporation of ^{14}C-uracil into RNA during amino acid starvation varies widely between independently isolated "relaxed" mutants. Alfoldi et al. (1) noticed the same type of variation in recombinants of *rel*$^+$ and *rel* crosses.

Here we confirm the conclusion that the standard relaxed strain continues to make ribosomal and messenger RNA, whereas the stringent cells synthesize mainly mRNA. Furthermore, we find an intermediate type which makes both rRNA and mRNA during starvation, but the rRNA does not accumulate and seems to be highly unstable.

MATERIALS AND METHODS

Strains and cell growth. The stringent strain 58-161 *rel*$^+$ and its relative, the standard relaxed strain 58-161 *rel* (K-12 W6) (2) were kindly provided by A. Ryan and E. Borek, Columbia University. They were grown in minimal salts medium plus glucose and fortified with the required amino acid methionine.

S31 is a streptomycin-resistant methionine auxotroph of the strain *sud* 24 (11). It was grown in the nonfragile form on minimal salts medium plus glucose in 20% sucrose. It also retains the capacity of the parental strain to grow in fragile form in media described previously (10, 11). S98 is a methionine auxotroph of K-12 3000, a reisolated form of the parental strain of *sud* 24. AB301 is a methionine auxotroph derived from Hfr Hayes. All cultures were incubated with mild shaking at 30 C on a New Brunswick rotary shaker. Starvation for methionine was achieved by growing cells on limiting methionine (0.85 μg/ml) and permitting them to use up the supply in the medium. Addition of methionine led to immediate resumption of growth at a rate characteristic of exponential growth. In some experiments (see Table 1) starvation was induced for isoleucine by the addition of 300 μg of valine per ml (13).

Sucrose gradient centrifugation of RNA. RNA was extracted three times with phenol, precipitated with ethanol, and dialyzed against 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 7.3), 50 mM NaCl, and 0.5% dodecyl sulfate for 2 hr at 22 C before being layered on 15 to 30% sucrose gradients made up in the same buffer. The gradients were centrifuged at 15 C for 14 or 20 hr at 25,000

rev/min in an SB110 swinging-bucket rotor of a B60 International centrifuge. To avoid quenching by the dodecyl sulfate, the precipitation and washes of gradient fractions were carried out with a mixture of 60% ethanol in 5% trichloroacetic acid, followed by a final wash with 0.1 N HCl.

Cell lysis and polysome sucrose gradient centrifugation. Lysates were prepared from fragile cells with buffers containing 0.5% sodium deoxycholate, as previously described (11).

Zonal sedimentation in 15 to 30% sucrose gradients, collection of gradient samples, and plating and counting of radioactive RNA were as described (10). To avoid quenching by 5% trichloroacetic acid during scintillation counting, each filter bearing a sample of precipitated RNA was given a final wash with 2 ml of 0.1 N HCl and dried thoroughly. Samples that contained deoxycholate were given an additional wash with 70% ethanol.

Labeling of cellular RNA. Stable RNA was labeled as previously described with ^{14}C-uracil (1.8 μM and 0.05 μCi/ml final concentration) or ^{3}H-uracil (0.75 μM and 7.5 μCi/ml final concentration) except where otherwise indicated, from Schwarz-Mann (10). The ^{3}H-uracil without added carrier was at 16.8 Ci/mmole.

Other techniques. The RNA content of the cultures was determined by the orcinol reaction with adenosine monophosphate as a standard (3). Published procedures were used to hybridize RNA to deoxyribonucleic acid (DNA) immobilized on filters (8).

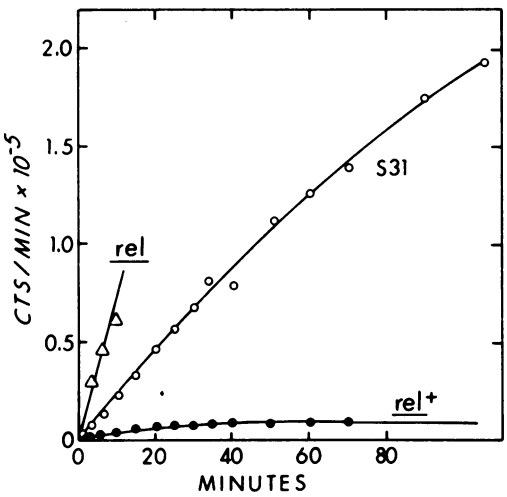

FIG. 1. *Cultures were grown on limiting methionine (0.85 μg/ml). At a cell concentration of about 5 × 10^{8} cells/ml, the methionine supply was exhausted and starvation began. Forty-five minutes later (time zero), 12.5 μCi of ^{3}H-uracil per ml (1 μg/ml) was added to permit continuous labeling of RNA. At the indicated times, 0.1-ml samples were precipitated with cold trichloroacetic acid, filtered, and counted. Symbols: ●, 58-161 rel^{+}; △, 58-161 rel; ○, S31.*

RESULTS

Labeling of RNA during starvation. RNA synthesis was measured by following the incorporation of ^{3}H-uracil into trichloroacetic acid-precipitable material. Figure 1 shows the incorporation of label into RNA from 60 to 165 min after the onset of starvation. In *rel*$^{+}$ cells the synthesis of ^{3}H-RNA is strictly limited, reaching a steady-state level at about 40 min. When methionine was added back to the culture the ^{3}H-uracil promptly began to be incorporated into RNA (data not shown).

Both S31 and the relaxed strain, 58-161, continued to accumulate ^{3}H-RNA for at least 2 hr after the addition of label. The rate of incorporation of label was fourfold greater in the relaxed strain than in S31.

Figure 2 shows the incorporation of ^{3}H-uracil over 30-min periods at different times after starvation had begun. In both the stringent strain and S31 the amount of ^{3}H-RNA accumulated in a 30-min interval sharply decreased with time after starvation. However, the amount of label incorporated in the 30-min interval was always much greater in S31 than in the stringent strain. The relaxed strain

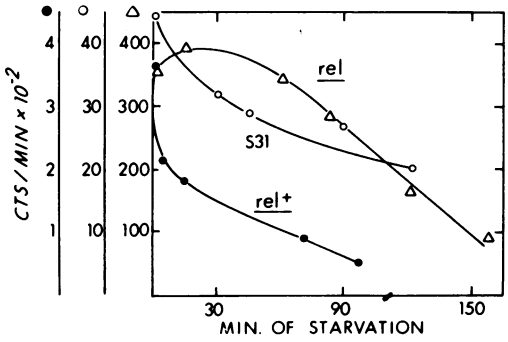

FIG. 2. *Samples (0.3 ml) of cultures grown on limiting methionine were labeled for 30-min intervals at different times after the onset of starvation. The three cultures were all labeled with 0.125 μCi/ml, but with different amounts of carrier uracil added in each case to insure that the label was not limiting during the 30-min interval. The rel^{+} strain was labeled with uracil at 0.066 μg/ml; rel at 16.5 μg/ml; S31 at 1.66 μg/ml. The ^{3}H counts per minute are normalized to the level that would be observed if the same specific activity of uracil was added to S31 and rel as was added to rel^{+}. Data are plotted on separated scales so that the basic shape of the curves can be easily compared as well as the differences in levels of incorporation. Symbols: ●, rel^{+}; △, rel; ○, S31.*

showed a constant accumulation of RNA for about 1 hr, and then a gradual decrease. Two and one-half hours after starvation began, labeling had declined to about 24% of the level at the start of starvation.

RNA accumulation by the orcinol method. From the labeling data, S31 did not seem to behave like either the relaxed or stringent strains. To circumvent problems of permeability and pool size inherent in labeling experiments, we looked at RNA accumulation directly, using the orcinol method. S98, a methionine auxotroph of the original parental strain of S31, K-12 3000, was also studied, to eliminate the possibility that the differences found between S31 and the stringent (and relaxed) strains were due to the mutation which allows S31 to grow in the fragile form.

Figure 3 shows the RNA content in different strains at increasing times after the onset of starvation. The stringent strain shows no increase in RNA from the onset of starvation, whereas the relaxed strain continues to accumulate RNA for at least 2.5 hr. The RNA content of both S31 and S98 increased slightly, about 10%, leveling off by 45 to 60 min after starvation. The RNA content of AB301 increased to a level 70% above that at the time of starvation, but the accumulation stopped at about 80 min.

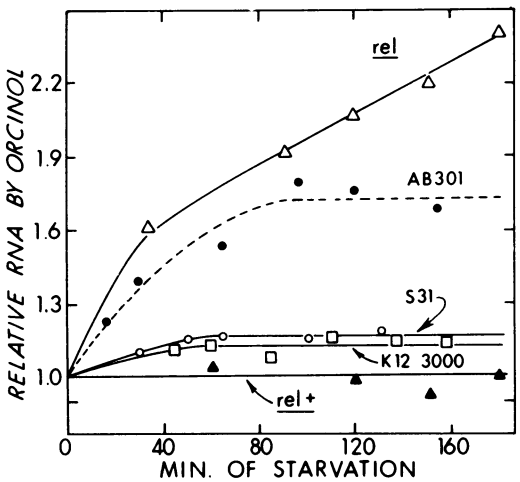

FIG. 3. *Orcinol determinations of cell cultures at different times after the onset of starvation. A 2-ml amount of cell culture was precipitated with an equal volume of ice-cold trichloroacetic acid and allowed to sit on ice for at least 30 min, and the precipitate was spun down. The precipitate was dissolved in 1 ml of 0.1 N NaOH, and the RNA content was determined by the orcinol method (3). RNA content at time zero (the onset of starvation) is plotted as 1.0, and the data are plotted as relative increase of RNA content with time of starvation. Symbols: ●, rel⁺; □, K-12 3000 met⁻; ○, S31; ●, AB301; △, rel.*

DNA-RNA hybridization. DNA-RNA hybridization experiments were carried out to determine the type of RNA being made in these strains during amino acid starvation. Two procedures were used to estimate the amount of ^{3}H-rRNA present in a preparation of RNA. (i) RNA samples were hybridized in the presence and absence of a 20-fold excess of cold rRNA. The per cent reduction of counts hybridized in the presence of cold rRNA is referred to as the per cent of rRNA in the sample. (ii) RNA samples were also hybridized over a wide range of RNA-to-DNA ratios, from 1/10 to 1/1,000, to determine the rRNA content by titrating the sites of abundant RNA species (7). At low RNA-to-DNA ratios (1/1000) all DNA sites are in excess and the efficiency of hybridization is high. When the RNA-to-DNA ratio is raised, the DNA sites for rRNA and tRNA become limiting and the per cent of ^{3}H-RNA hybridized falls. At an RNA-to-DNA ratio of 1/10, only about 3% of the rRNA will hybridize. Therefore, essentially all of the counts hybridized at this ratio will be in mRNA.

The value obtained for rRNA content by the titration curve method is consistently higher than that obtained by the competition method for samples from several strains under either starving or growing conditions. This discrepancy is probably due to the titration of the sites for tRNA over the range of ratios used. Transfer RNA accounts for between 10 and 20% of the RNA of growing cells. Since quantitative determinations of tRNA by hybridization under the conditions used is impossible, the result obtained by the competition method is a lower limit of rRNA content, and the value obtained by the titration curve is an upper limit.

Figure 4 shows a series of titration curves of RNA labeled for 3, 12.5, or 20 min during starvation. RNA from the stringent strain hybridizes with the same efficiency at high and low RNA-to-DNA ratios, when labeled either for short (3 min) or long (12.5 or 20 min) times. Therefore starving stringent cells made little or not detectable rRNA. This result is confirmed by competition experiments (Table 1); cold rRNA competes only 10 to 12% in all cases, which is near the limit of detectability of the assay. The result obtained with starved relaxed cells was strikingly different; 45 to 60% of the pulse label was in rRNA. With increasing times of labeling, the amount slowly increased, probably due to slow degradation of the mRNA.

S31, K-12 3000, and AB301 also make rRNA during starvation. The rRNA content of ^{3}H-RNA from S31 is 30 to 40% after various times of labeling. The per cent of label in rRNA after a 3-min pulse is 30 to 40% in K-12 3000 and 35 to 55% in AB301.

Zonal sedimentation of extracted pulse-labeled RNA. Since S31 does not seem to accumulate RNA during starvation, as determined by the orcinol test, but does seem to make rRNA, we wanted to see if 23*S* and 16*S* RNA accumulated during starvation.

Figure 5 shows sample sucrose gradient analyses of S31 and standard stringent and relaxed strains, with 16 and 23*S* RNA markers detected by their content of ^{14}C-uracil (panel C) or by their optical density at 260 nm (panels A, B, D). In controls with growing cells (panel A; also, cf. reference 15), 50% of the RNA made in a 1-min or 3-min pulse sediments at 16*S* or faster. As expected for the starved stringent strain (panel B), only 15 to 18% of the label in a 3-min pulse is in that region of the gradient. Instead, the pattern of 3-min-labeled RNA from the starved relaxed strain (panel C) is similar to that for growing cells, with 44% of the new RNA moving as fast or faster than 16*S* RNA. A direct comparison of S31 with the standard stringent and relaxed strains is shown in panel C: like the stringent strain, less than 18% of the RNA made in 3 min moves as fast as 16*S*. The similarity of the profiles for starved S31 and the starved stringent strain is further emphasized in a comparison of panels B and D: in both, very slow or no accumulation of 16 and 23*S* RNA was observed.

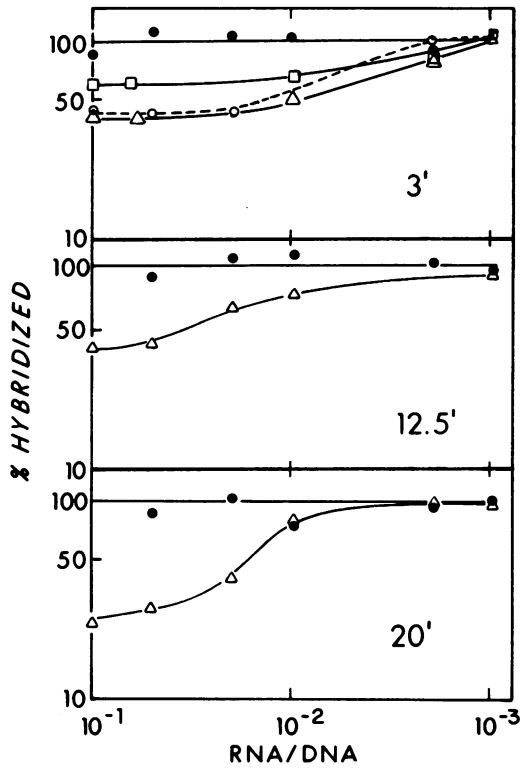

FIG. 4. *RNA from starved cultures (same RNA as in Table 1) was hybridized to DNA on filters at the RNA-to-DNA ratios indicated over a range of 10^{-1} to 10^{-3}. The amount of DNA on filters was always kept at 100 to 200 μg even at low ratios, to prevent a concentration effect which would decrease the efficiency of hybridization (8). Symbols: ●, rel$^+$; △, rel; ○, AB301; □, K-12 3000. The counts hybridized at an RNA-to-DNA ratio of 1/1,000 was set at 100%. The efficiency of hybridization varied between 50 and 70% with different filter preparations.*

Distribution of RNA during a continuous label. It has previously been shown (by hybridization analysis) that almost all of the pulse-labeled mRNA in starved cultures of S31 is in polysomes, sedimenting heavier than 50*S* (*manuscript in preparation*), and almost all rRNA sediments in the region of the gradient at 50*S* or less. Therefore the change in ratio of the amount of ^{3}H in the polysome region to the amount of label in the < 70*S* region with time during a continuous label should give an indication of the relative stabilities of messenger and ribosomal RNA in S31 during starvation.

Figure 6 shows the change in ratio of new ^{3}H-RNA free of polysomes to old ^{14}C-RNA free of polysomes and in the ratio of new ^{3}H-RNA in polysomes to old ^{14}C-RNA in polysomes with time after addition of label. Over the entire course of labeling, the distribution of newly formed RNA remained unchanged, so the ratio of ^{3}H-RNA in polysomes to that free of polysomes was constant, indicating similar stabilities for mRNA and rRNA.

DISCUSSION

The standard relaxed strain makes rRNA during starvation for an essential amino acid and accumulates it as large-molecular-weight species. The standard stringent strain makes only mRNA during starvation. Here we describe a new type of relaxed strain which does not accumulate RNA during amino acid starvation, but ribosomal RNA accounts for at least 35 to 40% of the total RNA being made (in a pulse label). The RNA is found as low-molecular-weight species. The rRNA appears to be about as unstable as the mRNA, because (i) it accounts for the same percentage of label as determined by hybridization after short and

TABLE 1. *Hybridization trials with RNA from starved cells*[a]

Strain	Expt	Labeling time after starvation (min)	Min of labeling	RNA/DNA	Input[b]	No rRNA[b]	rRNA[b]	Per cent rRNA
58-161 *rel*$^+$	1	50	3	10^{-3}	742	590	514	12.5
			12.5	10^{-3}	1,398	1,089	972	10
			25	10^{-3}	2,736	1,952	1,710	12.1
58-161 *rel*	1	50	3	10^{-3}	5,864	3,715	2,359	36.5
	2		12.5	10^{-3}	2,592	2,488	1,349	45.8
			25	10^{-3}	1,288	1,372	745	45.7
S31	1	70	5	10^{-2}	20,000	8,101	6,000	26
			25	2×10^{-3}	7,600	5,992	3,754	37
	2		5	10^{-3}	2,000	645	435	33
				3×10^{-3}	6,000	1,440	990	31
			25	10^{-3}	5,200	2,970	2,150	30
				3×10^{-3}	15,600	7,813	3,795	49
				10^{-2}	52,000	20,600	13,775	34
	3		2.5	1.6×10^{-2}	1,145	210	139	34
			12.5	10^{-3}	3,730	908	585	36
				5×10^{-4}	3,730	797	515	35
K-12 3000	1	90	3	10^{-3}	3,891	1,772	1,346	24.2
				10^{-3}	3,891	1,879	1,367	27.5
AB301	1	90	3	10^{-3}	1,290	525	317	39.7
				2×10^{-3}	1,290	715	500	30.2

[a] Cultures (10 ml) of cells were labeled with ^{3}H-uracil for the times indicated (starvation was achieved by treatment with L-valine in the case of S31 and by growth on limiting methionine in all other cases). The RNA was phenol-extracted from the washed pellet of cells and hybridized to 100 or 200 μg of DNA on filters at the RNA-to-DNA ratios indicated. In each case duplicate samples were hybridized in the presence of a 20-fold excess of unlabeled ribosomal RNA (see legend to Fig. 4).

[b] Counts per minute.

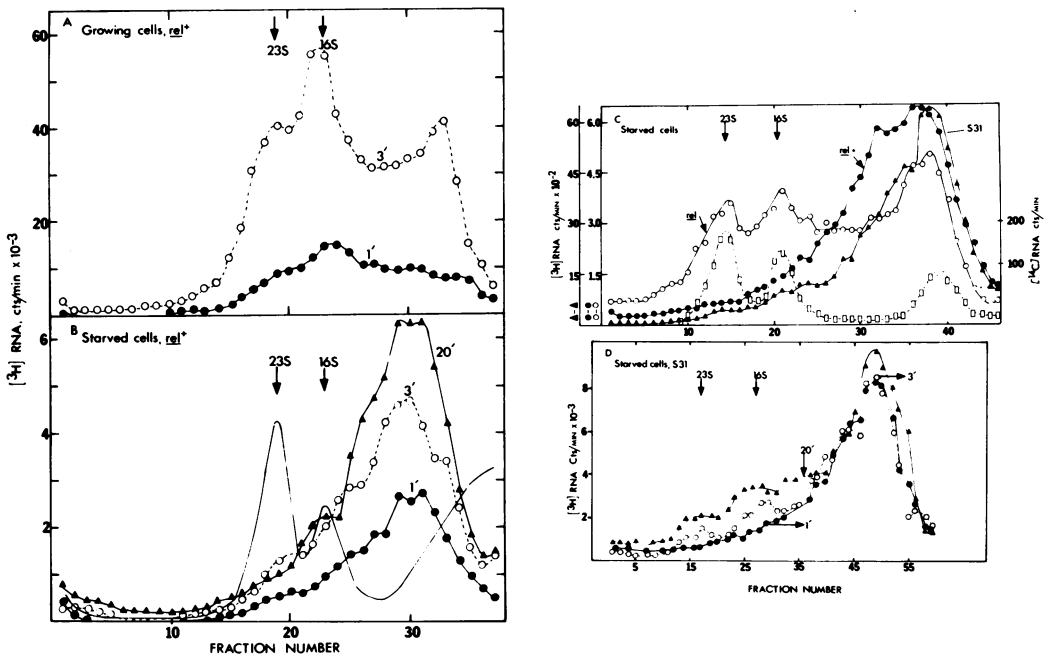

FIG. 5.

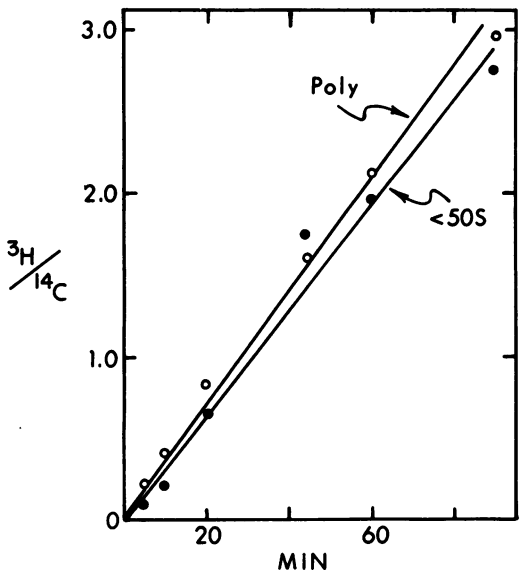

FIG. 6. *New ^3H-RNA free of polyribosomes and in polyribosomes of starving cells. A 60-ml amount of S31 cells was grown in fragile form in minimal medium and labeled uniformly during growth with 6 μCi of ^{14}C-uracil; 1.4 ml of L-valine (20 mg/ml) was added, and 60 min later 750 μCi of ^3H-uracil was added without carrier uracil. At the intervals shown, 10-ml portions of culture were poured over ice, harvested, and lysed, and lysates were centrifuged through sucrose gradients as in Fig. 1. The total ^{14}C and ^3H counts/min were summed up for each gradient in two fractions: polyribosomes, including monoribosomes (○); material sedimenting more slowly (50S and less) (●). For each fraction, the ratio of new to old RNA ($^3H/^{14}C$) is plotted as a function of time.*

long labeling times, and (ii) the ratio of label in polyribosomes and free of polyribosomes remains constant with time during a continuous label.

The primary phenotype of the relaxed mutation seems to be the continued synthesis of rRNA during starvation. Therefore it is likely that this new class of strains contains the relaxed allele, because they do continue to make rRNA. In other trials, the strain was uninducible for β-galactosidase when starved of methionine, behavior characteristic of the standard relaxed strain (4). Other genes besides the *rel* locus may be responsible for the degradation of the nascent rRNA. These strains may have an altered nuclease system which, although inactive in growing cells, degrades rRNA during starvation. This system would remain undetected in stringent cells, because no rRNA is synthesized in the first place.

Although available results can thus be interpreted in a self-consistent way, the intermediate class of *rel* strains could instead indicate that both *rel* and *rel*$^+$ strains make ribosomal RNA as well as mRNA, but that in stringent strains, the ribosomal RNA is degraded as rapidly as it is formed, whereas in relaxed strains the rRNA accumulates. In that case, strains like K-12 3000 would be *rel*$^+$, but with less active nucleases, rather than *rel* strains with *more* active nucleases. The alternative of primary control by selective arrest of synthesis seems more likely, because it is hard to imagine nuclease activity selective for otherwise stable RNA and inactive against mRNA. Nevertheless, only further information about the *rel* gene product can permit an unequivocal choice between these alternatives.

The relation of these observations to other data on relaxed and stringent strains is complex and as yet not clear. For example, during amino acid starvation an unusual guanine tetraphosphate, ppGpp, accumulates in a standard stringent strain, but not in the isogenic relaxed strain. However, the reisolated K-12 3000 strain examined here accumulates ppGpp even though rRNA is being made (M. Cashel, *personal communication*). As another example, AB301, which is classified as a relaxed strain and shows no ppGpp formation, nevertheless shows only limited RNA accumulation and then exhibits synthesis and breakdown of RNA similar to that found in K-12 3000. Perhaps these observations can help to explain some of the conflicting interpretations

FIG. 5. *Zonal sedimentation of RNA labeled with ^3H-uracil in growing and starving cells. RNA in growing cells (A) or cells starved for an amino acid (B–D) was labeled, extracted, and fractionated on sucrose gradients as described in Materials and Methods. The position of marker 16S and 23S RNA species was determined by the optical density at 260 nm of unlabeled carrier RNA (A, B, D) or by its content of ^{14}C-RNA extracted from cells labeled during five generations of growth (panel C). Unlabeled carrier RNA was used at 3 mg/gradient, yielding an optical density at the 23S RNA peak of 1.2 in a 1-cm cell. Gradients in panels A and B were centrifuged for 14 hr at 4 C; those in C and D were run for 20 hr to resolve the 16S region better. (A) RNA from growing cells; ●, 1-min label; ○, 3-min label. (B) Starving stringent cells labeled for 1 (●), 3 (○), or 20 (▲) min. (C) Starving cells labeled for 3 min; ●, rel$^+$; ○, rel; ▲, S31; □, ^{14}C-stable RNA. (D) Starving cells of S31 were labeled for 1 (●), 3 (○), or 20 (▲) min.*

of the effect of amino acid starvation on RNA accumulation. Conflicts between hybridization results which suggest that pulse-labeled stable RNA is being made (5, 6) and pool studies that showed a selective fall in stable RNA synthesis (12) may be due to strain differences, with differences in stability as well as synthesis of RNA.

ACKNOWLEDGMENTS

These studies were funded by Public Health Service grant CA-12021 from the National Cancer Institute; National Science Foundation grant GB-23052; American Cancer Society grant P-477B; and Public Health Service training grant AI-00257 from the National Institute of Allergy and Infectious Diseases. One of us (D.S.) is supported by Public Health Service Research Career Development Award GM-11710 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Alföldi. L., G. S. Stent, M. Hoogs. and R. Hill. 1963. Physiological effects of the RNA control (RC) gene in *E. coli*. Z. Vererbungsl. **94**:285–302.
2. Borek, E., J. Rochenback, and A. Ryan, 1956. Studies on a mutant of *Escherichia coli* with unbalanced ribonucleic acid synthesis. J. Bacteriol. **69**:460–467.
3. Dische, Z., G. Ehrlich, Z. Munoz, and L. von Sallmann. 1953. The content of glutathione and nucleotides of the lens capsule and its relation to lens permeability. J. Gen. Opthal. **54**:157–165.
4. Fiil, N., and J. D. Freisen. 1968. Isolation of "relaxed" mutants of *Escherichia coli*. J. Bacteriol. **95**:729–731.
5. Friesen, J. D. 1966. Control of messenger RNA synthesis and decay in *Escherichia coli*. J. Mol. Biol. **20**:559–573.
6. Friesen, J. D. 1968. A study of the relationship between polyribosomes and messenger RNA in *Escherichia coli*. J. Mol. Biol. **32**:183–200.
7. Kennell, D. 1968. Titrations of the gene sites on DNA by DNA-RNA hybridization. II. The *Escherichia coli* chromosome. J. Mol. Biol. **34**:85–103.
8. Kennell, D., and A. Kotoulas. 1968. Titration of gene sites on DNA by DNA-RNA hybridization. I. Problems of measurement. J. Mol. Biol. **34**:71–84.
9. Lazzarini, R. A., and A. E. Dahlberg. 1971. The control of ribonucleic acid synthesis during amino acid deprivation in *Escherichia coli*. J. Biol. Chem. **246**:420–429.
10. Luzzatto, L., D. Apirion, and D. Schlessinger. 1968. Mechanism of action of streptomycin in *E. coli*: interruption of the ribosome cycle at the initiation of protein synthesis. Proc. Nat. Acad. Sci. U.S.A. **60**:873–880.
11. Mangiarotti, G., D. Apirion, and D. Schlessinger. 1966. Selection of sucrose-dependent *Escherichia coli* to obtain envelope mutants and fragile cultures. Science **153**:892–893.
12. Nierlich, D. P. 1968. Amino acid control over RNA synthesis: a re-evaluation. Proc. Nat. Acad. Sci. U.S.A. **60**:1345–1352.
13. Ramakrishna, T., and E. A. Adelberg. 1965. Regulatory mechanism in the biosynthesis of isoleucine and valine. II. Identification of two operator genes. J. Bacteriol. **89**:654–660.
14. Sands, M. K., and R. B. Roberts. 1952. The effects of a tryptophan-histidine deficiency on a mutant of Escherichia coli. J. Bacteriol. **63**:505–511.
15. Sarkar, S., and K. Moldave. 1968. Characterization of the ribonucleic acid synthesized during amino acid-deprivation of a stringent auxotroph of *Escherichia coli*. J. Mol. Biol. **33**:213–224.