Accumulation of 30S Preribosomal Ribonucleic Acid in an *Escherichia coli* Mutant Treated with Chloramphenicol†

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ABSTRACT: During growth of strain AB105, an *Escherichia coli* mutant, 0.5% of cellular RNA is present as a 30S precursor of 16S and 23S rRNA. When cultures are treated with chloramphenicol, the large RNA accumulates in bulk quantities. The RNA species is stable in the cultures for more than 2 hr, and after 1.5–2 hr of chloramphenicol treatment it amounts to about 20% of the total cellular RNA. In addition to its size, the indications that the species is identical with the pre-rRNA formed in normal cells include: (1) cleavage in growing cells (when cells are washed free of drug and growth resumed, the 30S pre-rRNA formed in treated cells is cleaved to yield precursors to 16S and 23S rRNA); and (2) the extracted RNA, like that from growing cells, is cleaved *in vitro* by purified RNase III of *Escherichia coli* to yield major 25S and 17.5S RNA products. The extreme stability observed does not extend to all species of cellular RNA; unstable mRNA, for example, decays at the same rate in mutant or parental cells treated with chloramphenicol. The extracted RNA is thus subject to normal cleavage, and other enzymatic steps are the same in mutant and parental strains. One can thus infer that the effective inhibition of RNase III, an enzyme already at low levels in this mutant, is especially extreme in cells treated with chloramphenicol.

Mutant strain AB105 (Keil and Hofschneider, 1973; Kindler *et al.*, 1973) of *Escherichia coli* contains about 0.5% of its total RNA in the form of a large precursor to rRNA ("30S pre-rRNA;" Nikolaev *et al.*, 1973a). The large RNA transcript contains both 16S and 23S rRNA sequences (Nikolaev *et al.*, 1973a; Dunn and Studier, 1973), along with 22% additional sequences (Nikolaev *et al.*, 1974), and survives for 1–2 min in the mutant strain (Nikolaev *et al.*, 1973b); in normal strains, the RNA is cleaved during the course of its synthesis to fragments containing 16S or 23S rRNA sequences (Kossman *et al.*, 1971; Kossman, 1972).

Apparently the prolonged survival of 30S pre-rRNA in AB105 is associated with its lowered levels of RNase III: extracts of the strain show little RNase III activity (Kindler *et al.*, 1973; Nikolaev *et al.*, 1973b), and highly purified RNase III cleaves 30S pre-rRNA to products like those observed in whole cells (Dunn and Studier, 1973; Nikolaev *et al.*, 1973b).

The availability of small amounts of 30S pre-rRNA from growing cells permits a number of studies, but extensive studies of physical properties, and of correlations of function with structure, require larger amounts. Here we report that the cellular content of 30S pre-rRNA in the mutant strain can be specifically increased from 0.5 to 20% of the total cellular RNA by treatment of cultures with chloramphenicol.

Materials and Methods

Buffers and Reagents. The standard buffers for RNA extractions and in sucrose gradients contained Tris-acetate, EDTA, and sodium dodecyl sulfate, at the concentrations listed in the text. Chloramphenicol was the pure product of Parke-Davis. Rifampicin was a gift from Gruppo Lepetit, Milano. Nalidixic acid was obtained from Sigma Chemical Co. [^{3}H]Uridine (30 Ci/mmol), [^{3}H]uracil (25 Ci/mmol), and [^{14}C]uracil (52 Ci/mol) were from Schwarz/Mann. RNase III was purified with the modification by Gotoh *et al.* (1974) of the procedure of Robertson *et al.* (1968).

Growth of Strains. Strains AB105 and its parental A19 (Gesteland, 1966) were grown with aeration at 30° in minimal salts medium fortified with 0.8% technical grade Difco casamino acids, as previously described (Nikolaev *et al.*, 1973a,b). The doubling times in this medium are 70 and 160 min for parental and mutant, respectively.

Preparation of RNA, and Bulk Preparation of 30S Pre-rRNA. The extraction of RNA samples was based on the modified phenol extraction previously described (Nikolaev *et al.*, 1973a,1974). In order to prepare large amounts of the 30S pre-rRNA, 1 l. of culture at 30°, at an optical density of 420 nm of 1.0, was treated with 200 μg/ml of chloramphenicol for 100 min. The cells were poured over crushed ice and harvested in the cold, yielding a pellet of about 1 g wet weight of cells. The pelleted cells were resuspended in 10 ml of 0.2 M Tris-acetate (pH 5.5) containing 0.01 M EDTA and 0.5% sodium dodecyl sulfate. RNA was extracted at 60° by shaking for 15 min with two volumes of redistilled phenol saturated with the same buffer. The aqueous and phenol phases were separated by centrifugation at 10,000*g* for 30 min. Deproteinization of the aqueous phase was repeated three times with phenol, but at 30°. The RNA was then precipitated in two volumes of ethanol at −20° for at least 12 hr. The RNA was resuspended, at about 80 optical density units at 260 nm/ml, in 6 ml of 0.1 M Tris-acetate (pH 7.5)–0.005 M EDTA–0.5% sodium dodecyl sulfate; 1-ml portions were applied to 38-ml linear 10–30% sucrose gradients in the same buffer. After centrifugation at 25,000 rpm for 23 hr at 4° in a Spinco SW 27 rotor, the gradient contents were fractionated from the top. (For some

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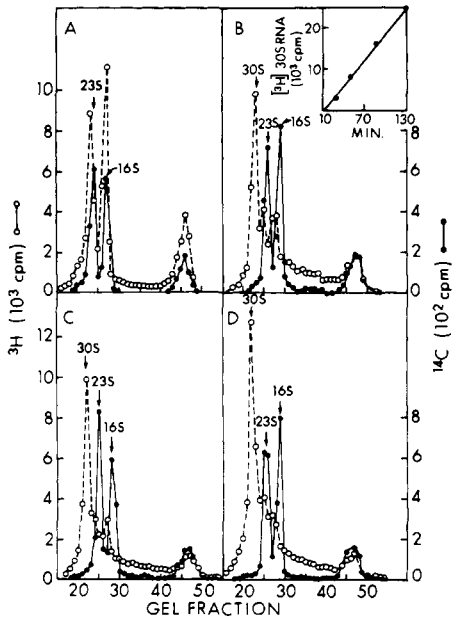

FIGURE 1: Gel electrophoresis of RNA pulse-labeled in strains A19 and AB105 at intervals after addition of chloramphenicol. A 30-ml culture of bacteria was grown at 30° to an optical density at 420 nm of 0.6; 3 ml of a 2 mg/ml of solution of chloramphenicol was then added. For each panel, 10 ml of culture was pulse labeled for 20 min (A) or 10 min (B-D) with 15 μCi of [^{3}H]uridine. The cells were then harvested on ice, centrifuged, and RNA extracted and analyzed by gel electrophoresis in 0.5% agarose-1.8% acrylamide gels (see Materials and Methods). For A, a culture of strain A19 was used, and the pulse labeling was done 10 min after addition of chloramphenicol. For B-D, strain AB105 was pulse labeled at 10, 45, or 95 min after addition of chloramphenicol. In each trial, uniformly labeled [^{14}C]rRNA was added just before the electrophoresis was started to show the mobility of marker RNAs. (- - O - -) ^{3}H-pulse-labeled RNA; (— ● —) ^{14}C-marker RNA. For the inset to panel B: a parallel culture of strain AB105 was labeled continuously for 2 hr, starting 10 min after the addition of chloramphenicol (the 40-ml culture was labeled with 800 μg and 800 μCi of [^{3}H]uracil). At the times indicated, 10-ml portions were harvested and RNA extracted, and gel electrophoresis was carried out on a portion of the RNA. The amount of label in the 30S peak in gels at successive times was then summed up to give the values on the curve.

preparations, cells were labeled with [^{14}C]uracil or [^{3}H]uridine before harvest, as indicated in the text.) The pooled 30S pre-rRNA fractions were then precipitated with ethanol and repurified by an exact repetition of the zonal sedimentation.

Uniformly labeled marker stable RNA was prepared from a 20-ml culture of strain A19 grown for three generations, to an optical density at 420 nm of 0.8, in presence of 1 μCi of [^{14}C]uracil/ml. The cells were harvested and RNA extracted as above.

Gel Electrophoresis. Ten-centimeter gels were poured in 0.8-cm diameter plexiglass tubes. The gel included (Weiss and Schlesinger, 1973) 0.5% agarose and 1.8% acrylamide in Tris, 36 mM; $NaH_2PO_4 \cdot H_2O$, 30 mM; Na_2EDTA, 1 mM; sodium dodecyl sulfate, 0.2%. (The pH is about 7.8.) Each sample was applied in 20-50 μl of the same buffer containing 10% sucrose and some Bromophenol Blue as tracking dye. Gels were electrophoresed 2.5 hr at room temperature, at a constant voltage of 80 V.

Radioactivity Measurements. Bray's (1960) scintillation counting fluid was used in a Packard scintillation spectrometer, with appropriate overlap corrections when both ^{14}C and ^{3}H were counted in the same sample.

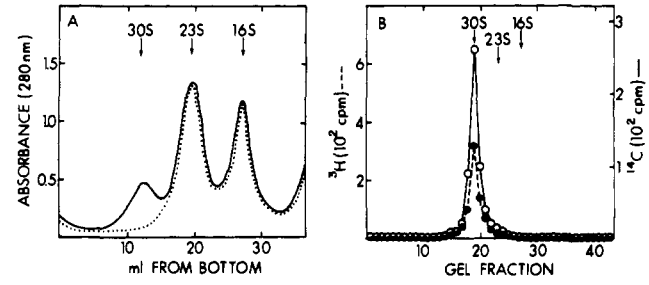

FIGURE 2: Zonal sedimentation and gel electrophoresis of RNA from strain AB105. (A) Zonal sedimentation of bulk RNA from growing (- - -) or chloramphenicol-treated (——) cells. RNA was extracted and fractionated from 1 l. of cells before or after 2 hr of treatment with 200 μg/ml of chloramphenicol (as in Materials and Methods). After sedimentation in initial sucrose gradients the contents of the gradients were pumped through the recording flow cell of a Gilford Model 2000 spectrophotometer and collected. Scans of two gradients are shown superimposed. (B) Coelectrophoresis in acrylamide gels of purified 30S pre-rRNA from cells labeled in presence or in absence of chloramphenicol; 100 ml of cells was labeled with [^{14}C]uracil (0.25 μCi/ml) for 2 hr in chloramphenicol (as in Figure 1); 20 ml of growing culture was labeled with [^{3}H]uridine (25 μCi/ml) for 2 min. The two cultures were then mixed and centrifuged. RNA was extracted, and two successive sucrose gradients were run to purify the 30S pre-rRNA (as in Materials and Methods). The final RNA fraction was precipitated with 95% ethanol, resuspended in electrophoresis buffer, and a portion analyzed. The indicated positions of 16S and 23S rRNA are obtained from an additional parallel gel trial with uniformly labeled [^{14}C]RNA. (- - ● - -), ^{3}H cpm in 30S pre-rRNA from pulse-labeled growing cells; (— O —) ^{14}C cpm in 30S pre-rRNA from chloramphenicol-treated cells labeled for 2 hr.

Results

Accumulation of 30S Pre-rRNA. The accumulation of 30S pre-rRNA in chloramphenicol-treated cells is illustrated in the gel electrophoresis and zonal sedimentation analyses depicted in Figures 1 and 2. In earlier studies it has been established that [^{3}H]uridine added to growing cells rapidly enters 23S and 16S rRNA species; even in strain AB105, where a transient 30S precursor to rRNA is detected, it is saturated with label in 1-2 min (Figure 2, Nikolaev *et al.*, 1973a). The results with cultures treated with chloramphenicol are very different. In normal strains, precursors slightly longer than mature 16S and 23S rRNA accumulate (see review by Pace, 1973, and Figure 1, panel A for strain A19). In AB105 the difference is even more drastic; for most of the label entering rRNA continues to accumulate in 30S pre-rRNA for as much as 2 hr or more (Figure 1). Lesser amounts are seen in 25S and 17.5S species that may correspond to the RNAs formed in the chloramphenicol-treated cells of control strains (see review by Pace, 1973). Figure 1 shows, for example, gel electrophoretic analyses of RNA pulse-labeled for 10 min after 10, 45, or 95 min in the presence of chloramphenicol (panels B-D). At each time, there is a background (which we assume represents mRNA) that increases the relative peak heights of 17.5 S (and possibly 25 S as well). However, even if one assumes that all the RNA in the region of 17.5 S to 30 S is rRNA at any time, the 30S peak contributes at least 57, 62, and 71% of that amount. (If the contribution of mRNA to the region between 17.5 S and 30 S is estimated and subtracted, the fraction of new rRNA in 30S pre-rRNA is even higher.) The distribution of sizes in newly formed rRNA may become slightly enriched for the 30S species with time (Figure 1B-D), but the relative amount synthesized seems to reach a steady-state level very soon after drug addition.

In trials in which label was supplied continuously for up

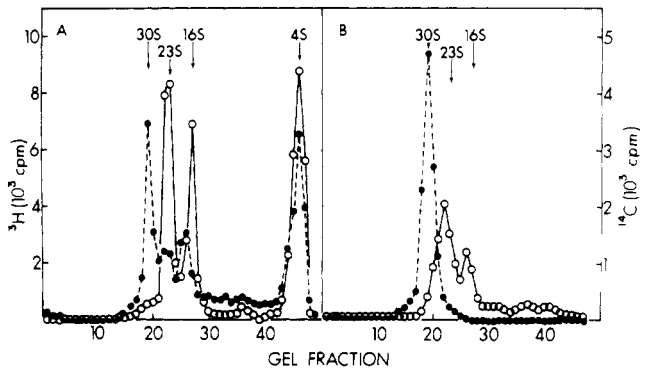

FIGURE 3: Subsequent cleavage of 30S pre-rRNA formed in chloramphenicol-treated cells. (A) Subsequent cleavage *in vivo;* 30 ml of strain AB105 was pulse labeled at 30° with [^{3}H]uridine in presence of chloramphenicol, as in Figure 1B (inset), for 90 min. The culture was then centrifuged and washed twice with minimal salts medium lacking glucose and casamino acids. (The washing procedure was carried out at 30° and took about 25 min.) The cells were then resuspended in 30 ml of fresh growth medium without chloramphenicol, and incubation was resumed at 30°. At 0 min (- - ● - -) and 60 min (— O —), 10-ml samples were harvested and their RNA extracted, mixed with ^{14}C-marker RNA, and fractionated in gel electrophoresis. Patterns of the two gel analyses are shown superimposed. (B) Subsequent cleavage *in vitro.* A portion of ^{14}C-labeled pure 30S pre-rRNA from the experiment of Figure 2B was incubated with or without RNase III. The enzyme reaction was carried out in 50 μl of 20 mM Tris-HCl (pH 7.6) containing 10 mM $MgCl_2$, 150 mM NH_4Cl, 5 mM β-mercaptoethanol, 10% glycerol, 10 μg of enzyme protein, and 10 μg of 30S pre-rRNA. Incubation was for 30 min at 37°. Then 5 μl of 10% sodium dodecyl sulfate and 6 μl of 0.1 M EDTA were added. The mixture was heated at 60° for 5 min and then applied directly for gel electrophoresis. Patterns are superimposed for gel analyses of 30S pre-rRNA before (- - ● - -) and after (— O —) RNase III treatment. The positions of marker RNAs are from the parallel gel trials of panel A.

to 2 hr, the label in 30S pre-rRNA increased progressively and linearly (inset to Figure 1B). The chloramphenicol-treated cultures, like other treated strains of *E. coli* (Osawa, 1965), increased their RNA content about 40–60%, and, as a result, the 30S pre-rRNA increased to a level easily seen in zonal sedimentation analyses of RNA extracted from the cells (Figure 2). Figure 2 includes for comparison the pattern of RNA from a trial with growing cells of strain AB105, where the low steady-state level of 30S pre-rRNA is not resolved. (An identical pattern was observed for RNA from growing or chloramphenicol-treated cells of the parental strain A19.) Since 85% of the cellular RNA is ribosomal, it is evident from Figure 2 that in chloramphenicol-treated cells, the 30S pre-rRNA can increase to at least 20% of the total RNA (23% of the sum of 16S, 23S, and 30S species).

Comparison of Accumulated RNA to 30S Pre-rRNA from Growing Cells. Recentrifugation of the pooled broad 30S pre-rRNA peak (Figure 2A) gave a product indistinguishable in size from the 30S pre-rRNA fraction prepared from growing cells. This was seen both in acrylamide gels (Figure 2B), and by direct length measurement in the electron microscope (reported in the last line of Table II, Nikolaev *et al.*, 1974). The exact protocol for the preparation of 2–3 mg of pure 30S pre-rRNA/l. of culture is given in Materials and Methods.

While the 30S pre-rRNA formed during chloramphenicol treatment was apparently stable as such for hours, it nevertheless appeared to have the intrinsic susceptibility to cleavage observed for the same RNA formed in growing cells. This was observed in two ways, depicted in the electrophoretic analyses of Figure 3. In some trials, the cells containing [^{3}H]RNA labeled during chloramphenicol treatment (Figure 3A, dashed line) were washed free of drug, resuspended in fresh medium without chloramphenicol, and growth resumed. At intervals, RNA was extracted from the growing cells; cleavage of the large RNA was quantitative (Figure 3A, solid line). At least the initial cleavage to 25S and 17.5S RNA species occurred even when the cells were incubated in fresh medium in the presence of rifampicin (data not shown).

In other trials, the extracted RNA was cleaved by purified RNase III (Figure 3B). In still other trials, the identity of the 30S species from chloramphenicol-treated cells and 30S pre-rRNA from growing cells was verified by competition experiments in DNA–RNA hybridization (data not shown).

Selectivity of Chloramphenicol Action. Even in normal strains, addition of chloramphenicol leads to inhibition of some nuclease activities. Accumulation of longer precursors has been reported for tRNA and rRNA (see Discussion), and newly formed mrRNA is partially stabilized (Gurgo *et al.*, 1969; Craig, 1972; Imamoto, 1973). In a number of these respects, strain AB105 is no different from other strains. For example, the region of 8 S to 20 S in electrophoretic analyses, containing most of the unstable RNA, saturates with label even in chloramphenicol-treated cultures of strain AB105 (Figure 1). AB105 itself shows some increased stability of mRNA even in growing cultures (L. Silengo *et al.*, results to be published); but in presence of chloramphenicol, the fraction of unstable RNA shows a slow but progressive breakdown after rifampicin addition that is indistinguishable in rate from that observed in the control strain A19.

In contrast to the case of mRNA, the inhibition of cleavage of 30S pre-rRNA by chloramphenicol is only observed in the mutant. As a result, the relative purity of 30S pre-rRNA from the 30S region of a gradient increases with time after chloramphenicol addition, and becomes almost complete after a brief treatment of cultures with rifampicin before harvest. Only a small contamination with fragments of DNA is then seen in gel electrophoresis or electron micrographs (Figure 1 and unpublished data).

Discussion

The results unequivocally show that chloramphenicol provokes the accumulation in strain AB105 of bulk quantities of an RNA species that comprises up to 20% or more of the cellular RNA. The RNA is apparently identical with the 30S pre-rRNA present in low amounts in growing cells of the strain. Estimating the contribution of 30S pre-rRNA to the gel electrophoretic patterns of Figure 1, one can say that a least 70–80% of the newly formed rRNA transcripts in strain AB105 begin as single units of transcription. The low level of continued production of some 17.5S and 25S rRNA even in the treated cultures may result from some remaining activity of a cleavage enzyme, or from a cellular site at which those species are made independently, rather than from a single promoter. If they were to result from separate sites of synthesis for 16S and 23S precursors, then it seems that not more than one of the five or so rRNA gene copies (Pace, 1973) can exist in that form.

The reason for the effect of chloramphenicol is not known. After addition of the drug there is often an effective inhibition of a number of nucleases *in vivo.* For example, even in an ordinary strain, precursors to tRNA and 5S RNA, as well as p16 and p23S rRNA, accumulate (Pace,

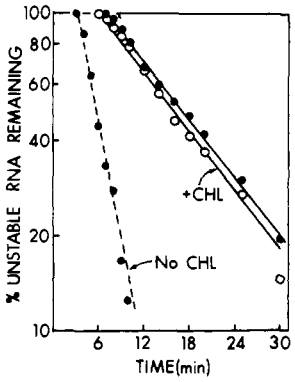

FIGURE 4: Effect of chloramphenicol on chemical decay of pulse-labeled unstable RNA in strains A19 and AB105; 10-ml cultures of cells were grown exponentially at 30° to an absorbance at 420 nm of 0.6. To the indicated cultures, 200 μg/ml of chloramphenicol was added; 5 min later (time zero in the Figure), 5 μCi/ml of [^{3}H]uridine (26 Ci/mmol) was added. One minute thereafter, $\frac{1}{30}$ volume of a solution of rifampicin and nalidixic acid in 50% ethanol was added, to give final concentrations of 300 μg/ml and 10 μg/ml (Pato and von Meyenberg, 1970). At the indicated times, 0.2-ml samples were precipitated in 2 ml of ice-cold 10% CCl_3COOH. After at least 20 min on ice, each sample was filtered onto a glass fiber filter (Reeve Angel Type A3H), and the filter was washed twice with 3 ml of 5% CCl_3COOH in 50% ethanol, and once with 1 ml of 0.1 N HCl. The dry filter was then counted. The total amount of radioactive RNA unstable during a 60-min incubation was set equal to 100%. The fraction of the unstable RNA remaining at each time is plotted against time. The 100% point represents more than 20,000 cpm for each strain. (- - ● - -) and (— ● —) strain A19; (— o —) strain AB105.

1973; Dijk and Singhal, 1974). There is also an inhibition of mRNA decay in chloramphenicol-treated cells; but this effect is very similar in strain AB105 and in its parental strain A19 (Figure 4).

The effect on 30S pre-rRNA accumulation, in contrast, is thus far unique to strain AB105. Since RNase III carries out initial cleavage(s) of the RNA, without any observed requirement for other proteins, the effect is almost certainly related to the deficiency of RNase III (Kindler *et al.*, 1973; Nikolaev *et al.*, 1973b) in the strain *in vivo*. It seems plausible that in an ordinary strain, RNase III is at a level far in excess of that required for the cleavage of 30S pre-rRNA. In strain AB105, a direct or indirect inhibitory effect of chloramphenicol on the low remaining level of RNase III is enough to eliminate nearly all cleavage of 30S pre-rRNA. Presumably the remaining level of enzyme is inhibited rather than destroyed, since the 30S species can be subsequently cleaved when the cells are incubated in absence of chloramphenicol (Figure 3); this occurred even in presence of concentrations of rifampicin that would block renewed synthesis of RNase III (data not shown).

Whatever the mechanism of stabilization, such cultures have yielded, reproducibly and with ease, preparations of 2-3 mg of 30S pre-rRNA/l. of culture, with specific activities of [^{3}H]uridine (or [^{32}P]phosphate from labeling trials in low-phosphate media) essentially as high as those possible for any stable RNA species in ordinary strains.

Availability of large quantities of the 30S pre-rRNA has already permitted a number of studies of structural features that will be reported in detail elsewhere. For example, the state of methylation of bases and ribose has been investigated (J. E. Dahlberg, *et al.*, manuscript in preparation). Also, the position of the 16S sequence 5′-proximal to the 23S sequence in 30S pre-rRNA, suggested from earlier indirect experiments (Kossman *et al.*, 1971; Kossman, 1972; see review by Pace, 1973), has been directly proven (N. Nikolaev and D. Schlessinger, manuscript in preparation).

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