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Messenger Ribonucleic Acid Synthesis and Degradation in *Escherichia coli* During Inhibition of Translation

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Various aspects of the coupling between the movement of ribosomes along messenger ribonucleic acids (mRNA) and the synthesis and degradation of mRNA have been investigated. Decreasing the rate of movement of ribosomes along an mRNA does not affect the rate of movement of some, and possibly most, of the RNA polymerases transcribing the gene coding for that mRNA. Inhibiting translation with antibiotics such as chloramphenicol, tetracycline, or fusidic acid protects extant mRNA from degradation, presumably by immobilizing ribosomes, whereas puromycin exposes mRNA to more rapid degradation than normal. The promoter distal (3') portion of mRNA, synthesized after ribosomes have been immobilized by chloramphenicol on the promoter proximal (5') portion of the mRNA, is subsequently degraded.

The movement of ribosomes along ribonucleic acid (RNA) template has been implicated in both the synthesis and degradation of cellular RNA. Early models (27) postulated that ribosome movement is necessary for transcription to occur, but it is now clear that transcription can take place in the absence of translation. This point has been demonstrated by in vivo experiments in which antibiotics or amino acid starvation were used to inhibit translation (6, 11, 17, 28) without abolishing transcription. The fact that messenger ribonucleic acids (mRNA) can be synthesized in vitro also demonstrates the lack of any obligatory coupling between transcription and translation (5, 7). Nevertheless, it has been suggested (11) that the unimpeded movement of ribosomes along an mRNA is necessary to allow the RNA polymerase to move at its correct velocity. The in vivo peptide chain growth rate of 15 to 20 amino acids per s (8, 16) and the in vivo RNA chain growth rate of 45 to 60 nucleotides per s (2, 21) are consistent with a coupling mechanism between the movement of ribosomes and of RNA polymerase.

Models also have been proposed for coupling between ribosome movement and degradation of mRNA (12, 14, 24). These are based on the findings that (i) degradation of mRNA occurs in the same direction (5' to 3') as its synthesis and translation (23, 24); (ii) inhibition of protein synthesis by chloramphenicol or streptomycin inhibits mRNA breakdown (18, 20); and (iii) a ribosome-bound 5' exonucleolytic activity (RNase V) was reported (15) which is at least partially dependent upon active translation. Though the existence of RNase V has been questioned (1, 9), the notion of exonucleases degrading mRNA behind translating ribosomes is widely accepted.

In an earlier communication (25) we presented a convenient technique for analyzing various aspects of the transcription process. We report here experiments using this technique which bear on the relationship of ribosome movement to RNA synthesis and degradation.

MATERIALS AND METHODS

Bacterial strains. Strain AS19, a derivative of *Escherichia coli* B (26), highly permeable to many antibiotics including rifampin, fusidic acid, and streptolydigin, was used throughout.

Growth conditions. A phosphate minimal medium (4) was used and supplemented with glucose or glycerol at 0.2%. Growth was monitored spectrophotometrically at 450 nm, and cultures were grown exponentially at 37 C for three generations before beginning experiments.

Radioactive labeling. Samples (200 μliters) of

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cultures labeled with [^{3}H] uridine or [^{14}C]proline were lysed with sodium dodecyl sulfate (SDS) at 95 C, precipitated with 5% trichloroacetic acid, and washed on filters, or precipitated with 2 ml of 5% trichloroacetic acid and filtered. Radioactivity was counted in a toluene based scintillation mixture.

β-Galactosidase assays. Strain AS19 was induced for β-galactosidase synthesis with 10^{-3} M isopropyl-β-D-thiogalactopyranoside (IPTG). Samples of 0.5 ml were shaken with 1 drop of toluene and chilled, or added to tubes with chloramphenicol at a final concentration of 200 μg/ml and chilled. Samples were diluted to 1 ml with 0.05 M phosphate buffer, pH 7.0, and the enzyme activity was assayed by addition of 0.2 ml o-nitrophenol-β-D-galactopyranoside (ONPG) (4.2 mg/ml). The reaction was terminated with 0.5 ml of Na_2CO_3 (1 M) and the optical density was read at 420 nm.

Chemicals. Fusidic acid was a gift from W. D. Godtfredsen, Leo Pharmaceutical Products, Ballerup, Denmark. Rifampin was a gift from Ciba-Geigy A/S, Copenhagen. Streptolydigin was a gift from G. Whitfield, The Upjohn Company, Kalamazoo, Mich., Chloramphenicol was from Parke, Davis and Co., London, and IPTG and ONPG were from Sigma Chemical Co.

RESULTS

Assay system. An assay system has been described previously (25) that allows the rapid analysis of several aspects of the transcription process. In brief, the assay system is as follows: Rifampin inhibits RNA synthesis in bacteria by interacting with the RNA polymerase, specifically blocking the initiation of transcription but not interfering with completion of nascent RNA chains. When rifampin and a labeled precursor of RNA are added simultaneously to a culture, the rate of RNA synthesis measured by incorporation of the precursor gradually decreases (Fig. 1) and the incorporation of radioactivity reaches a maximum at 1.5 to 2.0 min (at 37 C). A fraction of the incorporated radioactivity subsequently decays, and after about 10 min a constant plateau of incorporated counts is attained.

The radioactivity represented by the constant plateau after 10 min reflects the amount of stable RNA species completed during the residual synthesis (25). With respect to the unstable

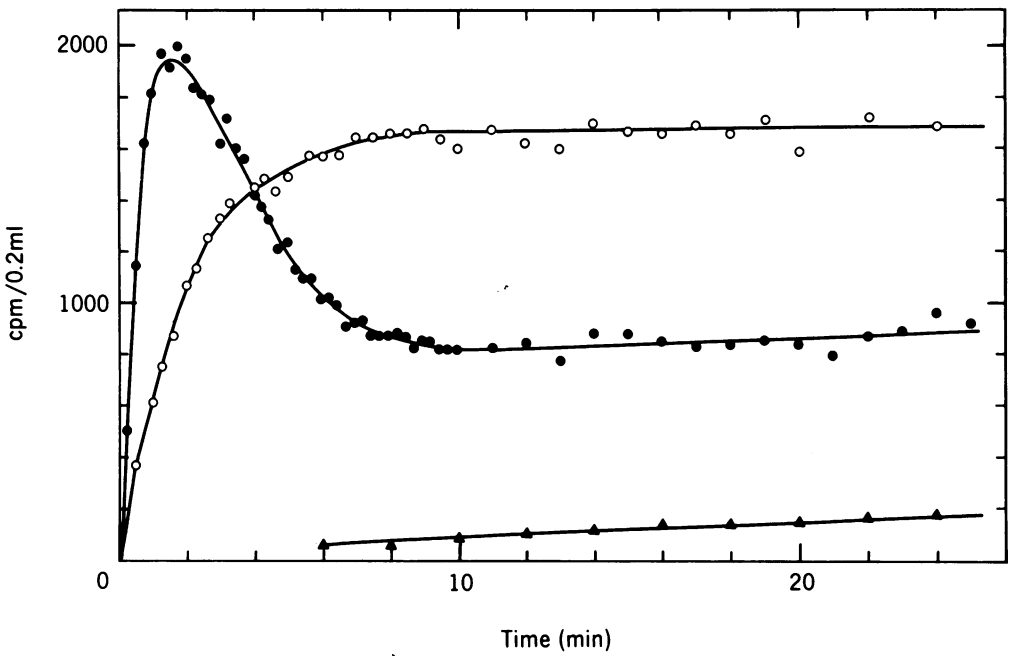

FIG. 1. *Residual RNA and protein synthesis after inhibition of initiation of transcription by rifampin. To 10-ml samples of a culture of strain AS19 growing in glucose minimal medium with a generation time of 42 min were added: (i) rifampin (20 μg/ml), nalidixic acid (20 μg/ml), and [^{3}H]uridine (1 μg/ml; 100 mCi/mmol); (ii) rifampin (20 μg/ml) and [^{14}C]proline (1 μg/ml, 10 mCi/mmol). At intervals, 200-μliter volumes were transferred into trichloroacetic acid for determination of the incorporation of radioactive [^{3}H]uridine into RNA (●) or [^{14}C]proline into protein (○). A control was labeled with [^{3}H]uridine at 5 min (▲) as described previously (25). Nalidixic acid was added to prevent incorporation of [^{3}H]uridine into DNA; identical results can be obtained in the absence of nalidixic acid by determining the amount of incorporation of [^{3}H]uridine that is sensitive to alkaline hydrolysis (25). It is assumed that the use of nalidixic acid does not influence any experiments reported here which use additional antibiotics.*

fraction we find the following results which indicate that it reflects primarily the completion of mRNA chains. (i) The unstable fraction exhibits exponential decay characteristics; it decays with a half-life of 1.3 ± 0.1 min (Fig. 2). (ii) The rate of protein synthesis gradually decreases, as observed by cumulative labeling with [^{14}C]proline after the rifampin treatment (Fig. 1). The determination of the rates of protein synthesis from the curve in Fig. 1 or, more accurately, by pulse labeling with [^{14}C] labeled amino acid at intervals after rifampin addition, reveals exponential decay of this rate also with a half-life of 1.3 min (Fig. 2). (iii) The unstable fraction of incorporated radioactivity is almost completely protected from degradation when translation is blocked by chloramphenicol (see Discussion). Various aspects of this assay system will be used in the following experiments.

If the rate of ribosome motion in some fashion controls the rate of RNA synthesis as well as the rate of mRNA degradation, then retardation of translation should affect both these events. The

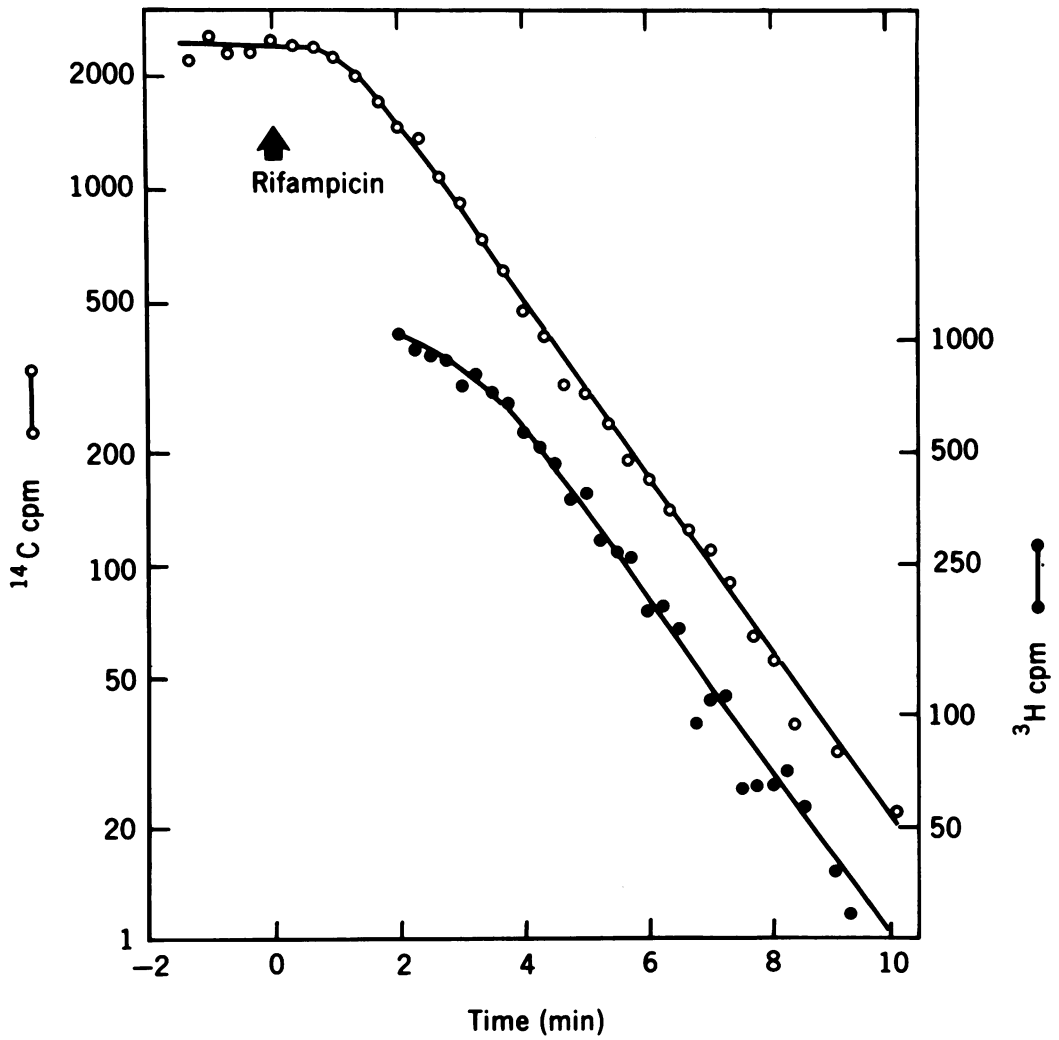

FIG. 2. *Semilogarithmic plot of the rate of protein synthesis and of the amount of unstable RNA after rifampin treatment. The rate of protein synthesis (O) is determined as the amount of [^{14}C]proline (10 mCi/mmol, 0.5 μg/ml) incorporated during 20-s pulses into 0.5-ml portions of a strain AS19 culture (glucose minimal medium, 37 C) before and after rifampin addition (20 μg/ml). The unstable RNA values (●) are taken from the residual RNA curve in Fig. 1 as the difference between the actual counts at any moment and the extrapolated stable plateau value.*

effect of both partial and complete inhibition of translation has been examined.

Effects of complete inhibition of translation on the synthesis and degradation of RNA. When added to strain AS19, fusidic acid and chloramphenicol at concentrations of 20 μg/ml, and oxytetracycline and puromycin at 200 μg/ml, inhibit protein synthesis in less than 10 s by more than 95% (data not shown).

The effects of these antibiotics on the stability of mRNA extant at the time of antibiotic addition were studied. Rifampin and [^{3}H]uridine were added to strain AS19 growing in glucose minimal medium with a 42-min doubling time, as shown in Fig. 1. The antibiotic to be tested was added 2 min later, i.e., after most RNA synthesis was completed (see Fig. 6). In glucose-grown cultures, chloramphenicol or tetracycline stabilizes about 80 to 90% of the normally unstable RNA present at the time of antibiotic addition (Fig. 3A); fusidic acid stabilizes about 70% of this RNA. In the presence of puromycin, the presumptive mRNA fraction remains unstable and decays with a half-life of approximately 0.6 min; i.e., it is only half as stable as in the control culture.

If rifampin and chloramphenicol are added simultaneously to a culture of strain AS19, the portions of mRNA chains synthesized prior to chloramphenicol addition will be stabilized. If ribosomes are immobilized on mRNA by chloramphenicol, hence rendering the extant portion of the mRNA chain insensitive to degradation, and if RNA polymerase can continue to extend the mRNA chains, either partially or to completion, then the portions of mRNA chains synthesized after chloramphenicol addition should be unprotected by ribosomes. The fate of the portions of mRNA chains completed after chloramphenicol addition was examined by adding rifampin, chloramphenicol, and [^{3}H]uridine simultaneously to strain AS19 growing in glucose minimal medium. As shown in Fig. 3B, synthesis of unstable RNA does proceed and degradation of this normally unstable RNA does occur, whereas the amount of stable RNA produced is unchanged.

Effects of partial inhibition of translation on the synthesis and degradation of RNA. The effects of partial inhibition of translation were determined by growing cultures of strain AS19 in the presence of low concentrations of fusidic acid, an antibiotic that specifically inhibits the G factor required for the translocation step of protein synthesis (13). Decreased but constant growth rates, dependent on the fusidic acid concentration, were obtained (Table 1).

The experiment shown in Fig. 4 was performed to demonstrate that the rate of movement of ribosomes is decreased in fusidic acid-grown cultures, whereas the rate of RNA polymerase movement is unaffected. Cultures were induced for β-galactosidase synthesis with 10^{-3} M IPTG and the time of appearance of the first increment of enzyme above the basal level was determined. This lag time, which in essence measures the time required for the first ribosomes to complete a molecule of β-galactosidase

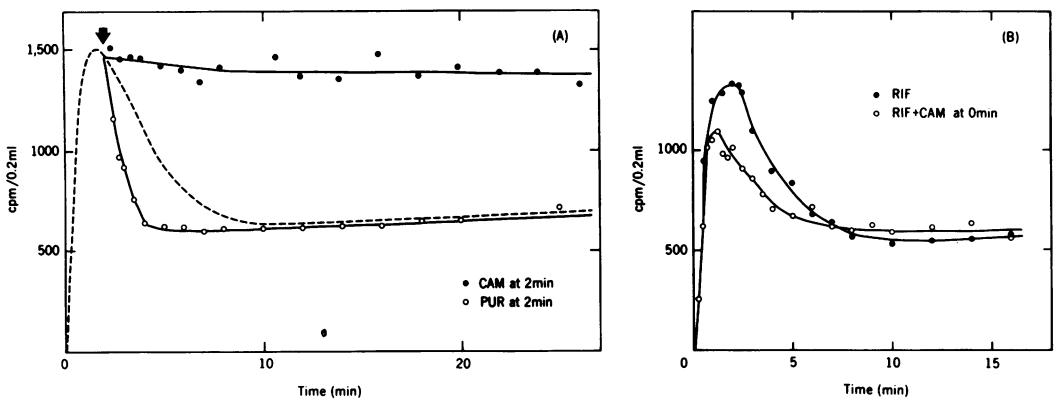

FIG. 3. *Effect of inhibitors of protein synthesis on the stability of mRNA (A) present at the time of the drug addition and (B) synthesized after drug addition. In both experiments rifampin (20 μg/ml), nalidixic acid (20 μg/ml), and [^{3}H]uridine (1 μg/ml; 100 mCi/mmol) were added at time 0 as in Fig. 1. In (A) puromycin (200 μg/ml) (○) or chloramphenicol (100 μg/ml) (●) were added at 2 min (arrow) to samples of the culture. One part was left untreated as a control (---). In (B) chloramphenicol was added at time 0 together with rifampin, nalidixic acid, and [^{3}H]-uridine, i.e., before the residual RNA synthesis had occurred: plus chloramphenicol (○); control (●). At intervals, 200-μliter volumes were transferred into trichloroacetic to determine the amount of incorporation of [^{3}H]uridine into RNA.*

TABLE 1. *Half-life of unstable RNA and of in vivo rate of protein synthesis at various growth rates limited by low concentrations of fusidic acid*[a]

Fusidic acid concn. (μg/ml)	Doubling time (min)	Half-life (min) of	
		Unstable RNA	In vivo capacity
0	43	1.3	1.4
0.05	66	1.8	1.7
0.1	96	2.0	2.1
0.25	125	2.2	2.2
0.5	200	2.6	2.8
1.0	240	3.1	3.5

[a] Estimates of half-lives were obtained as described in Fig. 2.

after induction, is lengthened from about 1.5 min in the control to 2.5 min in the presence of 0.25 μg of fusidic acid per ml (Fig. 4A). Estimation of the time of completion of transcription of the lac Z gene was used to compare the rates of polymerase movement in the absence and presence of fusidic acid. After induction with IPTG, samples were added at intervals to tubes containing streptolydigin at 80 μg/ml to inhibit further RNA synthesis. The samples were then incubated at 37 C for 20 min to express any potential for β-galactosidase synthesis. Any sample taken after the time at which transcription of the Z gene is completed will yield an amount of enzyme greater than the basal level.

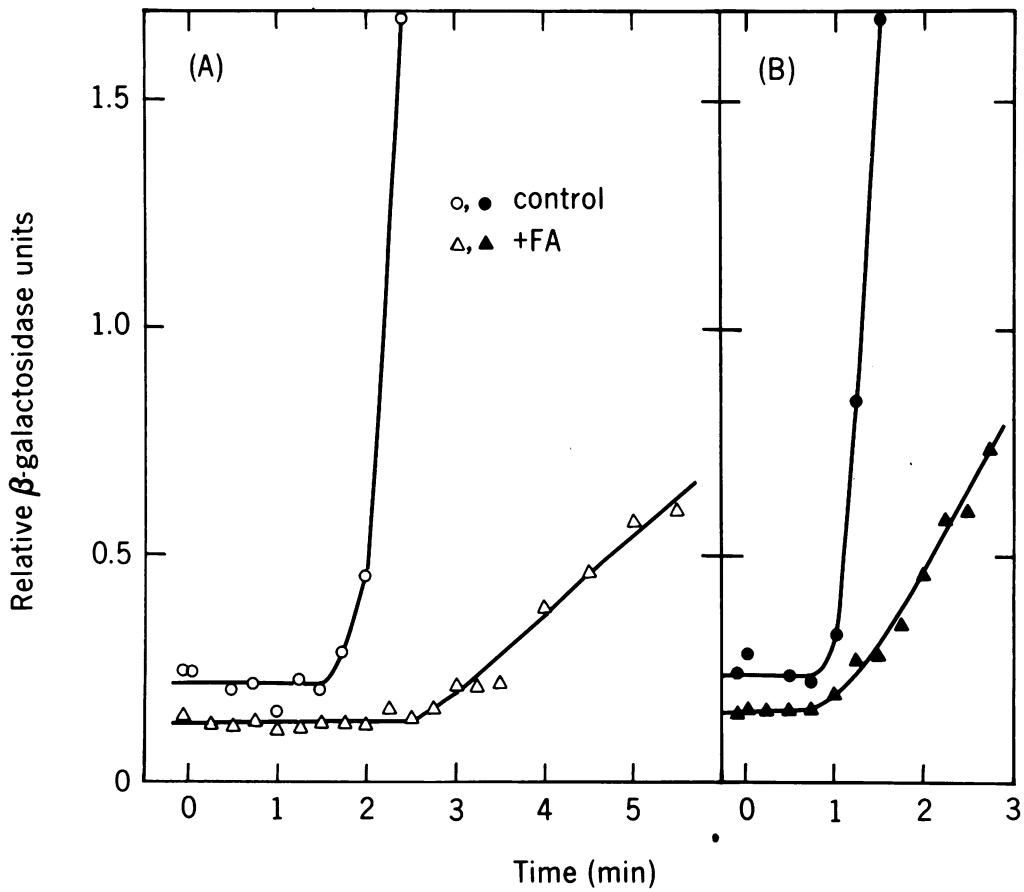

FIG. 4. *Transcription time of the Z gene of the lac-operon and translation time of the Z-mRNA. E. coli B strain AS19 was grown in minimal medium with glucose without* (○) *and with* (△) *a low concentration of fusidic acid (0.2 μg/ml) in steady states at 37 C with doubling times of 43 and 110 min, respectively. At an optical density (450 nm) of 0.40 and 0.48, respectively, IPTG was added to a concentration of 10^{-3} M at time 0. At the indicated times 0.5-ml samples were added to tubes containing: (i) 1 drop of toluene, shaken, and chilled (open symbols); (ii) streptolydigin to give a final concentration of 80 μg/ml and kept for 20 min at 37 C, then chilled and 1 drop of toluene added and shaken (closed symbols). The relative amount of the enzyme β-galactosidase was determined: in (i) representing the actual amount present at any time in the cells, and in (ii) the amount of enzyme which can be translated from completed Z-gene mRNA.*

As shown in Fig. 4B, the time of first increase of enzyme levels above basal level is identical in the absence or presence of fusidic acid, demonstrating that the transcription time of the lac gene is unaltered, for at least some of the RNA polymerases, even though the time required for translation is increased.

The residual RNA synthesis after rifampin addition to cultures growing in the presence of 0.1 and 0.5 μg of fusidic acid per ml, respectively, are shown in Fig. 5A. For comparison, the curves have been arbitrarily normalized to the same peak height, including as a reference the residual synthesis in a culture growing in the absence of fusidic acid. The half-lives of the unstable fractions of counts are increased in the cultures containing fusidic acid to 2.0 and 2.6 min, respectively. The residual protein synthesis (normalized to the same terminal levels) also shows a prolonged half-life of the in vivo capacity for protein synthesis for the cultures growing in presence of low concentrations of fusidic acid (Fig. 5B). In Table 1, half-life estimates are compiled for a series of glucose-grown cultures in which the growth rate was decreased by adding fusidic acid to different concentrations. The half-lives are gradually prolonged, but not to the same extent as the increase in doubling time.

The initial portions of the residual RNA synthesis curves (Fig. 5A) are identical in the presence or absence of fusidic acid. As already noted above, this indicates that the RNA chain growth rate is unchanged, a conclusion that is strengthened by the following experiment. Rifampin was added to cultures growing in the absence or presence of fusidic acid and the time required for engaged polymerases to complete synthesis of nascent RNA chains was determined by measuring the incorporation of [^{3}H]uridine in 20-s pulses. The data in Fig. 6 show that the time course of the decrease in the rate of RNA synthesis after rifampin addition is the same in the presence or absence of fusidic acid.

DISCUSSION

Ribosome movement and mRNA synthesis. The correspondence between the RNA chain growth rate and the peptide chain growth rate can be accounted for by several possible mechanisms: (i) the rate of movement of the ribosomes controls the rate of movement of RNA polymerase (11, 27); (ii) the rate of movement of polymerase controls the rate of movement of the ribosomes; or (iii) the two rates have been evolutionarily matched.

The experiments described above using low concentrations of fusidic acid demonstrate that the first explanation is unlikely. The rate of movement of the ribosomes is decreased by fusidic acid as shown by the increased time required to synthesize a molecule of β-galactosidase in the presence of fusidic acid. The 80 to 85-s lag before appearance of enzyme after induction is consistent with the proposed amino acid step times and the length of the β-galactosidase monomer (1171 amino acids) (29). The rate of movement of some, and possibly most, of

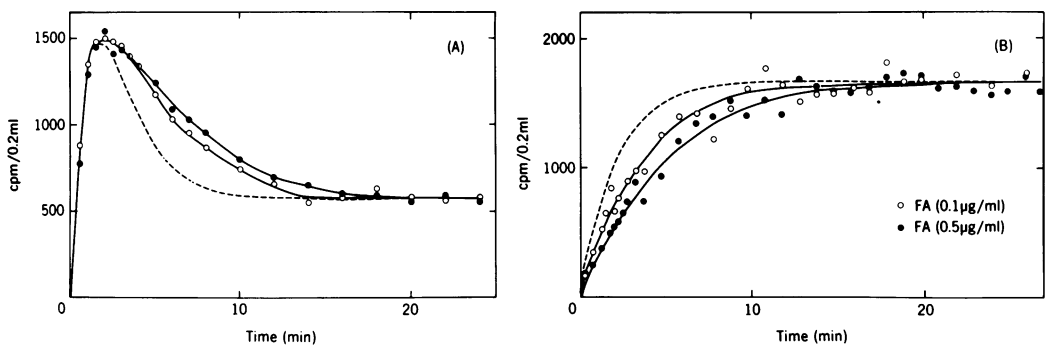

FIG. 5. *Residual RNA and protein synthesis after rifampin addition to fusidic acid-limited cultures. To cultures of E. coli B strain AS19 growing at 37 C in minimal medium with glucose, fusidic acid was added at an optical density (450 nm) of 0.1 to give concentrations of 0.1 (○) and 0.5 μg/ml (●), respectively. At these concentrations a steady state of growth is reached after 2 to 3 mass doublings with generation times of 90 to 100 and 160 to 200 min, respectively. Residual RNA synthesis (A) and protein synthesis (B) after rifampin treatment were recorded by labeling with [^{3}H]uridine (1 μg/ml, 100 mCi/mmol) and [^{14}C]proline (1 μg/ml, 10 mCi/mmol). The curves are normalized to match the peak value in (A) and the final steady state value of the [^{14}C]proline incorporation in (B), respectively. The dashed line represents the standard curves for an untreated glucose minimal culture with a doubling time of 43 min (see Fig. 1).*

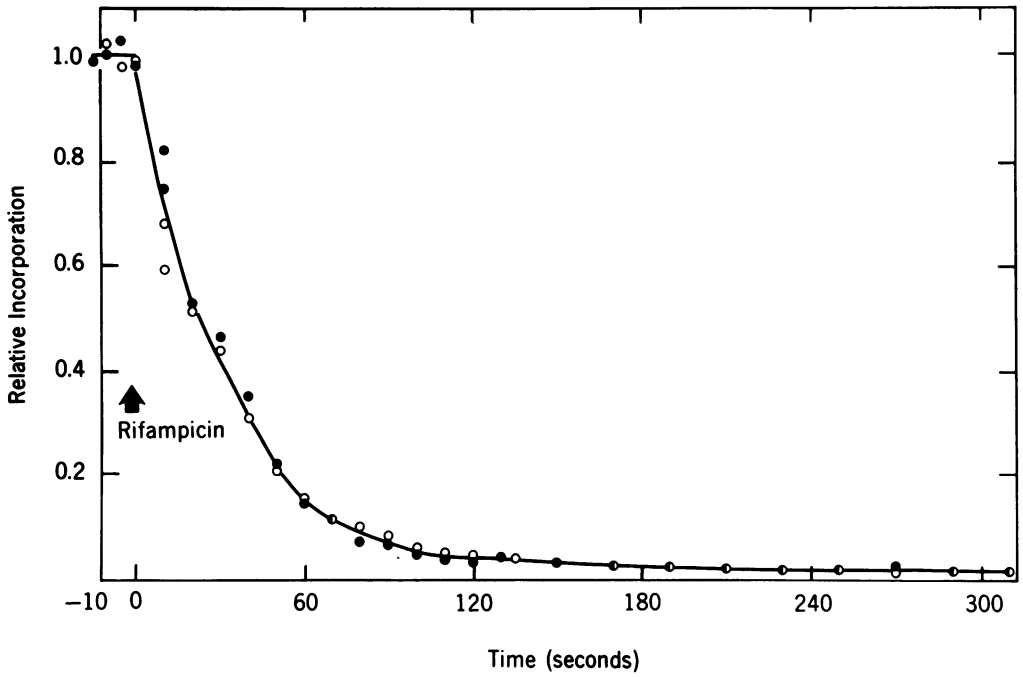

FIG. 6. *Decay of the rate of RNA synthesis after the inhibition of initiation of transcription by rifampin. Cultures of E. coli B strain AS19 were grown in minimal medium with glucose at 37 C without (●) and with 0.25 μg fusidic acid per ml (○). At an optical density (450 nm) of 0.60 rifampin (40 μg/ml) was added and at intervals of 10 or 20 s, 0.5-ml samples were transferred into tubes containing 0.5 μg of [³H]uridine (1 μCi/μg) at the same temperature. After 20 s the labeling was terminated by addition of cold trichloroacetic acid. The relative incorporation of ³H per 20 s is plotted versus time, normalizing the values for the untreated cultures (just before rifampin addition) as 100%. The actual values were 2,175 to 2,335 and 1,600 to 1,650 ³H counts/min without and with fusidic acid, respectively. The values are plotted at the time corresponding to the middle of the labeling period.*

the RNA polymerase is unaffected as shown by the identical times required to transcribe the Z gene in the presence and absence of fusidic acid and by the identical kinetics of RNA synthesis after rifampin addition. The 60 to 70 s required for the transcription of the Z gene may be an underestimate if a measurable lag exists before streptolydigin inhibits RNA synthesis. Quantitation of the fraction of polymerases completing transcription of the Z gene in normal time is difficult. The slopes of the curves in Fig. 4 may be affected by endonucleolytic cleavage of exposed regions of mRNA between the polymerase and slowly moving ribosomes or by premature termination of mRNA chains (10). Either effect will lower the yield of completed β-galactosidase chains per transcription initiation. The kinetic analysis shown in Fig. 6 may not be accurate enough to detect changes in rate of movement of a small fraction of polymerase molecules. These experiments rule out any requirement for normal movement of ribosomes detaching mRNA from its template to allow normal movement of at least some of the RNA polymerases.

An interesting consequence of the second explanation, which suggests that ribosome movement is limited by the rate of polymerase movement, is the possibility that ribosomes could accelerate when nascent mRNA chains are completed and released from the DNA. However, if the ribosome velocity were much greater on "free" mRNAs than on nascent mRNAs, and if a sizeable fraction of the mRNA in the cell is "free" (19), then the average amino acid step time would be greater than that expected from correspondence with the nucleotide step time. It therefore seems likely that the two step times have evolved to their present correspondence, though experimental verification of the equivalence of amino acid step times on "free" and DNA-bound mRNA is necessary. To illustrate the "evolutionary pressure" for matching of the two synthetic rates, one can envision the consequences of the RNA polymerase velocity exceeding the ribosome velocity.

This would lead to exposed regions of mRNA between the polymerase molecule and the first ribosomes which could be attacked by endonucleases as described below.

Ribosome movement and mRNA degradation. The experiments shown in Fig. 3A and B examined the fate of mRNA synthesized before and after addition of chloramphenicol. The stabilization of pre-existing mRNA by addition of chloramphenicol shown here and by others (18, 20, see Fig. 3A) and by addition of tetracycline or fusidic acid is consistent with a coupling between ribosome movement and mRNA degradation as proposed by Morse et al. (24), Kuwano et al. (14), and Kepes (12). Immobilization of ribosomes would prevent 5′ exonucleases such as the putative RNase V (15) from degrading mRNA. The experiment shown in Fig. 3B was performed in such a manner that the initial (5′) portion of the mRNA was covered with ribosomes at the time of chloramphenicol addition. Synthesis of the mRNA chains continues, either to completion or, if premature termination occurs, to a considerable extent. Degradation of this normally unstable RNA proceeds by a mechanism other than a simple 5′ exonucleolytic digestion, as the 5′ portions of the RNA are protected by immobilized ribosomes. Degradation may proceed by endonucleolytic cleavage of exposed regions of the mRNA chains followed by 5′ or 3′ exonucleolytic activity or both, or by premature termination of mRNA chains and subsequent digestion.

Blockage of translation by puromycin does not stabilize mRNA, but rather enhances its degradation (3, 11, see Fig. 3A). In the presence of puromycin, ribosomes may speed along the mRNA without further peptide bond formation or be released from the mRNA exposing it to nucleolytic attack.

The increase of the half-life of mRNA in low concentrations of fusidic acid (Table 1) is readily explained by a decreased rate of 5′ exonucleolytic cleavage resulting from the slowed rate of ribosome movement. However, the half-life of mRNA does not increase in the presence of increasing concentrations of fusidic acid (Table 1) in proportion to the change in growth rate. This may be the result of endonucleolytic attack on unprotected mRNA between the RNA polymerase and the leading ribosome, or of premature release of nascent RNA chains. A consequence of either effect would be a "polarity effect" (24); i.e., appearance of incomplete mRNA and consequently of incomplete, unstable polypeptide fragments. Data demonstrating such an effect is presented elsewhere (Hanson et al., J. Mol. Biol., in press).

The data in Fig. 3A demonstrate that 80 to 90% of normally unstable RNA present at the time of chloramphenicol addition is protected from degradation by the antibiotic. This observation was used to support the contention that most, if not all, the unstable RNA in this experiment is mRNA, and not an unstable form of rRNA or tRNA. Since rRNA, and presumably tRNA, is not translated by ribosomes (most graphically illustrated by the electron micrographic studies of Miller et al.) (22), these RNA species would not be protected against degradation by chloramphenicol. It is possible that a small portion of the unstable RNA under these conditions is not mRNA, and at low growth rates a larger fraction of the unstable RNA may be rRNA (Steen Pederson, personal communication).

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