Protein Synthesis by Long-Lived Messenger Ribonucleic Acid in Bacteria

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1. Experiments were performed to investigate two hypotheses about the function of long-lived messenger RNA in bacteria. After RNA synthesis had been stopped by the addition of actinomycin, continuing protein synthesis was used as a measure of persistent messenger RNA. 2. The hypothesis that messenger RNA responsible for the synthesis of membrane protein is exceptionally long-lived was tested in experiments with protoplasts of *Bacillus megaterium*. However, this messenger RNA proved to be of approximately average stability. 3. The hypothesis that long-lived messenger RNA is responsible for the synthesis of constitutive proteins was tested by comparing the synthesis of penicillinase in an inducible and a constitutive strain of *Bacillus licheniformis*. After the addition of actinomycin, penicillinase synthesis continued for far longer in the constitutive than in the inducible strain. This difference is attributed to a difference in stability of the penicillinase-messenger RNA in the two strains, which does not extend to all messenger RNA indiscriminately. 4. A model is tentatively proposed to account for the altered stability of messenger RNA in the constitutive mutant.

The belief that messenger RNA is unstable in bacteria (Jacob & Monod, 1961) has received widespread experimental support. Nonetheless, reports have sporadically appeared which suggest that a fraction of the total messenger RNA may be relatively long-lived (see, e.g., Pollock, 1963; Yudkin & Davis, 1965; Aronson, 1965). I have begun some investigations of bacterial messenger RNA in an attempt to determine which proteins are specifically synthesized by this long-lived fraction.

The experiments described in this paper were designed to test two hypotheses. The first is derived from the observation that long-lived messenger RNA is associated with the membrane fraction of *Bacillus megaterium* (Yudkin & Davis, 1965): it states that long-lived messenger RNA is responsible for the synthesis of membrane protein. The second is derived from the observation that the proportion of long-lived messenger RNA in *B. megaterium* increases when the synthesis of some proteins is repressed (Yudkin, 1965): it states that unstable messenger RNA is responsible for the synthesis of repressible proteins, and long-lived messenger RNA for the synthesis of non-repressible proteins. (A similar hypothesis has been independently suggested by Moses & Calvin, 1965).

The results of the present experiments disprove the first hypothesis, but provide support for the

second. Studies of penicillinase synthesis in two strains of *Bacillus licheniformis* suggest that inducible synthesis is directed by short-lived messenger RNA, and constitutive synthesis by long-lived messenger RNA.

EXPERIMENTAL

Radioactive precursors. The ^{14}C -labelled algal-protein hydrolysate was supplied by The Radiochemical Centre, Amersham, Bucks., at about $600\ \mu\text{C}/\text{mg}$. It was made up as a solution of $1\ \mu\text{C}/\text{ml}$. For experiments with protoplasts the solution was supplemented with $50\ \mu\text{g}$. each of L-leucine and of L-isoleucine/ml., to prevent the incorporation of radioactivity into the branched-chain fatty acids of the protoplast membrane (Lennarz, 1961). For experiments with whole bacteria the solution of algal-protein hydrolysate was supplemented with (per ml.) $50\ \mu\text{g}$. each of L-leucine and L-isoleucine, and also of DL-alanine, DL-aspartic acid and DL-glutamic acid, to prevent the incorporation of radioactivity into the cell-wall mucopeptide (Salton, 1964).

L-[guanido- ^{14}C]Arginine was supplied at $16\ \mu\text{C}/\mu\text{mole}$ and DL-[1- ^{14}C]phenylalanine at $11\ \mu\text{C}/\mu\text{mole}$; these were used without dilution by non-radioactive amino acid.

Preparation and growth of protoplasts. *Bacillus megaterium* KM was maintained on minimal medium and grown as described by Yudkin & Davis (1965). Bacteria were harvested by centrifuging from an exponential culture at a density of 0.20–0.25 mg. dry wt./ml., and were washed once in protoplast medium, which contained (per l.): sucrose,

75 g.; KCl, 4 g.; $MgCl_2 \cdot 6H_2O$, 4 g.; $(NH_4)_2SO_4$, 1 g.; K_2HPO_4 , 0.1 g.; sodium citrate dihydrate, 0.5 g.; barbituric acid, 2 g.; adjusted with NaOH to pH 7.4. The washed bacteria were resuspended at 10 mg. dry wt./ml. in protoplast medium, and lysozyme (Armour Pharmaceutical Co., Eastbourne, Sussex) was added to give 400 μg /ml. The suspension was incubated at 20° until conversion into protoplasts as judged microscopically was complete (about 20 min.).

The protoplast suspension was diluted tenfold with protoplast medium. A lysate was prepared by diluting the concentrated suspension tenfold with water and shaking vigorously. The diluted suspension of protoplasts and the lysate were then both further diluted tenfold with protoplast medium containing 10 mg. of glucose/ml. and 100 μg . of Evans peptone/ml. These suspensions, whose density corresponded to 0.1 mg. of whole organisms/ml., were used for the incorporation of ^{14}C -labelled amino acids.

Incorporation of amino acids by protoplasts. The diluted protoplast suspension (about 40 ml. in a 500 ml. conical flask) and the protoplast lysate (about 10 ml. in a 125 ml. conical flask) were gently shaken at 20° on a Conway shaker (A. Gallenkamp and Co. Ltd., London, E.C. 2). After 1 hr. incubation, 0.05 μC of ^{14}C -labelled algal-protein hydrolysate or amino acid was added/ml. Incubation at 20° on the Conway shaker continued throughout the experiment.

Estimation of radioactivity incorporated. For the measurement of radioactivity in the acid-precipitable fraction, a 1 ml. sample of the protoplast suspension or the lysate was added to a tube containing 1 ml. of 10% (w/v) trichloroacetic acid. The precipitate was collected on a membrane filter (Oxoid, Oxo Ltd., London, E.C. 4); the tube was washed out twice with 5 ml. of water and the washings were successively passed through the filter. For the measurement of radioactivity in the membrane fraction, a 1 ml. sample of the protoplast suspension was added to a tube with 5 ml. of water containing 1 mg. of Evans peptone and 1 μg . of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.)/ml. The membrane residue from the resulting lysate was collected on a filter, the tube was washed out and the washings were passed through the filter as described above. (These methods are slightly modified from those described by Yudkin, 1963.)

The filter was gummed to a planchet and allowed to dry, and radioactivity was determined with an automatic planchet-counter (Isotope Developments Ltd., Beenham, Berks.): 1 μC gave about 1400 counts/sec. At least 1000 counts were recorded; the rate was corrected for background.

Growth of B. licheniformis. *B. licheniformis* strain 749 (wild-type, inducible for penicillinase synthesis) and strain 749/C (constitutive for penicillinase synthesis) were obtained through the kindness of Professor M. R. Pollock. They were maintained at 30° in tubes of salts medium (Davis & Mingioli, 1950) containing 1% of Difco casein hydrolysate ('casamino acids'). The bacteria were grown overnight at 30° with shaking in flasks of the same medium (with 5 μg . of cephalosporin C/ml. for the induction of penicillinase in strain 749).

Synthesis and estimation of penicillinase. An overnight culture was diluted into a flask of minimal medium containing 1% of Difco casein hydrolysate, and cephalosporin (5 μg /ml.) for strain 749, to give a density of about 0.02 mg. dry wt./ml., and was shaken at 30°. The experiment was

begun when the bacteria were in exponential growth at a density of 0.15–0.25 mg. dry wt./ml.

A 2 ml. sample of the culture was added to a tube containing chloramphenicol (final concn. 100 μg /ml.) and lysozyme (final concn. 40 μg /ml.). Each tube was kept at 30° for 5 min. to allow lysis of the bacteria and was then kept at 0° until assayed. Samples were diluted in triplicate with 0.5% gelatin and assayed by the method of Perret (1954). The standard deviation of replicate samples was $\pm 2\%$.

Incorporation of ^{14}C -labelled amino acids by B. licheniformis. An overnight culture of bacteria was diluted with salts medium containing 0.05% of Difco casein hydrolysate to give a density of about 0.02 mg. dry wt./ml. The culture was shaken at 30°, and the experiment was begun when the bacteria were in exponential growth, at a density of 0.15–0.20 mg. dry wt./ml.

A 1 ml. sample of the culture was rapidly mixed with 0.2 μC of ^{14}C -labelled algal-protein hydrolysate at 30°. After 60 sec., the reaction was stopped by the addition of 1 ml. of 10% trichloroacetic acid. The precipitate was collected on a membrane filter and the incorporation of radioactivity was measured, exactly as described above.

Antibiotics. Merck, Sharp and Dohme, Rahway, N.J., U.S.A., generously supplied a sample of actinomycin D. It was kept at -10° as a solution in 75% ethanol, and used at 1 μg /ml. (final concn.) except where otherwise stated. Cephalosporin C was a gift from Professor E. P. Abraham.

RESULTS

The experiments described in this paper involved the use of the antibiotic actinomycin D in order to investigate the stability of messenger RNA. Actinomycin inhibits the synthesis of RNA (Kirk, 1960), and its addition to a culture of susceptible bacteria leads to a rapid loss of newly synthesized RNA and to a parallel decline in the capacity to synthesize protein (Levinthal, Keynan & Higa, 1962). The decreased rate of protein synthesis presumably reflects the destruction of messenger RNA; thus if the synthesis of one protein ceases soon after the addition of actinomycin while the synthesis of a second protein continues, it is assumed that the messenger RNA responsible for the synthesis of the second protein is more stable than that responsible for the synthesis of the first. The validity of this assumption is considered in more detail in the Discussion section.

The hypothesis that the synthesis of membrane protein is directed by long-lived messenger RNA was investigated by experiments with protoplasts. As previously found (Yudkin, 1963) growing protoplasts rapidly incorporated radioactivity from ^{14}C -labelled algal-protein hydrolysate both into their membrane protein and into acid-insoluble protein. When actinomycin was added to the culture, the rate of incorporation gradually decreased, and by 40 min. incorporation had ended. The membrane fraction ceased to gain radioactivity at about the same time as, or perhaps even earlier

than, the acid-insoluble fraction (Fig. 1). Similar results were obtained when [^{14}C]arginine and [^{14}C]phenylalanine were used as precursors. This observation suggests that the messenger RNA that directs the synthesis of membrane protein is not exceptionally stable.

The hypothesis that constitutive enzyme synthesis is directed by long-lived messenger RNA was investigated by studying penicillinase production. The effect of actinomycin was compared in two strains of *B. licheniformis*: the penicillinase-inducible strain 749 and the penicillinase-constitutive strain 749/C. When actinomycin was added

to a fully induced culture of the inducible strain, the synthesis of penicillinase ceased within 5 min. In contrast, synthesis in the constitutive strain continued for at least 20 min. after the addition of actinomycin (Fig. 2). (This experiment has been repeated several times, and has always yielded the same result.) The observed increase in enzyme after the addition of actinomycin was shown to be due to protein synthesis, since it was inhibited by chloramphenicol.

At first sight, the difference between inducible and constitutive production of penicillinase suggested a difference in the stability of penicillinase-

Fig. 1. Effect of actinomycin on the incorporation of ^{14}C -labelled amino acids by protoplasts of *B. megaterium*. Actinomycin ($1\text{ }\mu\text{g./ml.}$) was added at zero time to growing protoplasts. The incorporation of radioactivity from ^{14}C -labelled algal-protein hydrolysate into the acid-insoluble fraction (O) and into the membrane fraction (●) was measured. Incorporation into the acid-insoluble fraction of lysed protoplasts (Δ) is also shown.

Fig. 2. Effect of actinomycin on the synthesis of penicillinase. Actinomycin ($1\text{ }\mu\text{g./ml.}$) was added at zero time to (a) an inducible and (b) a constitutive strain of *B. licheniformis*. Exactly similar results were obtained when actinomycin was added at $10\text{ }\mu\text{g./ml.}$

messenger RNA in the two strains. Other explanations were nevertheless possible. The experiment with strain 749 was carried out in the presence of cephalosporin, the inducer of penicillinase synthesis, and it was conceivable that the inducer was exerting some unexpected effect. In fact, however, the addition of cephalosporin, even at $100\text{ }\mu\text{g./ml.}$, to a culture of strain 749/C made no difference to the results. Secondly, Pollock (1963) has shown that actinomycin at low concentration has a differential effect on the synthesis of various enzymes. It was possible that strain 749 was more sensitive in general to the antibiotic than strain 749/C, so that even at $1\text{ }\mu\text{g./ml.}$ actinomycin inhibited enzyme production differentially in the two strains. However, this explanation was excluded by the results of an experiment in which the concentration of actinomycin was increased to $10\text{ }\mu\text{g./ml.}$ Once again, penicillinase continued to be synthesized in strain 749/C for much longer than in strain 749. Moreover, actinomycin at $1\text{ }\mu\text{g./ml.}$ caused an immediate cessation of growth in the two strains, as measured by increase of extinction.

These results suggest that messenger RNA is more stable in the penicillinase-constitutive than in the penicillinase-inducible strain, but they provide no evidence to show whether this difference is specific to the penicillinase-messenger RNA. Evidence was obtained from studies of amino acid incorporation in the two strains. After the addition of actinomycin to the culture, the rate of incorporation of amino acids declined sharply at first, and then more slowly (Fig. 3). Similar results have been obtained with *B. megaterium* (Yudkin, 1965). Fig. 3 shows that there was no significant difference

between strain 749 and strain 749/C in the effect of actinomycin on ability to incorporate amino acids. Hence the difference between the two strains in the stability of penicillinase-messenger RNA (Fig. 2) does not extend to messenger RNA in general.

DISCUSSION

The experiments described in this paper have all involved the use of actinomycin, and their interpretation depends on an understanding of the action of this drug. It is generally supposed that messenger RNA rapidly turns over in growing bacteria, and that the decay in protein-synthesizing capacity observed in the presence of actinomycin results from inhibition by the drug of the resynthesis of messenger RNA (Levinthal *et al.* 1962). On the other hand, Acs, Reich & Valanju (1963) suggested that actinomycin caused a direct depolymerization of RNA which did not occur in growing bacteria; however, Chantrenne (1965) has now produced evidence which shows that actinomycin does not provoke any unusual degradation of RNA and which validates the use of the antibiotic in studies of the lifetime of messenger RNA.

Experiments with protoplasts disproved the hypothesis that the synthesis of membrane protein is directed by exceptionally long-lived messenger RNA. The stability of this RNA appears to be approximately the same as that of the average messenger RNA of the cells (Fig. 1).

The fact that induced synthesis of penicillinase continued for only 5 min. after the addition of actinomycin, whereas constitutive synthesis continued for 20 min., suggests that the lifetimes of

Fig. 3. Effect of actinomycin on the incorporation of ^{14}C -labelled amino acids by *B. licheniformis*. Actinomycin ($1\text{ }\mu\text{g./ml.}$) was added at zero time to (a) a penicillinase-inducible and (b) a penicillinase-constitutive strain of *B. licheniformis*. The incorporation of radioactivity from ^{14}C -labelled algal-protein hydrolysate into the acid-insoluble fraction was measured during 60 sec. pulses.

penicillinase-messenger RNA differ in the inducible and the constitutive strains. Pollock (1963), who observed a differential effect of actinomycin on penicillinase synthesis in inducible and constitutive strains of *Bacillus cereus*, proposed an alternative explanation: that at the time of addition of actinomycin the constitutive strain contains an excess of messenger RNA. This explanation now seems rather unlikely. In the first place, there is evidence that the synthesis of messenger RNA is dependent on its translation by ribosomes (Bremer & Konrad, 1964; Byrne, Levin, Bladen & Nirenberg, 1964; Shin & Moldave, 1966). Secondly, the rate of protein synthesis in bacteria depends on the number of ribosomes present (Neidhardt & Magasanik, 1960; Kjeldgaard & Kurland, 1963), and it is improbable that the two strains of *B. licheniformis* growing in the same medium at the same rate will differ in their complement of ribosomes (cf. Schaechter, Maaløe & Kjeldgaard, 1958). These considerations make it unlikely that there is an accumulation of untranslated messenger RNA in the constitutive but not in the inducible strain. Consequently the difference found between the two strains can most probably be ascribed to a difference in the lifetime of messenger RNA, depending on whether the protein synthesized is inducible (i.e. repressible) or constitutive (i.e. non-repressible).

The significance of differing lifetimes of messenger RNA can be considered in terms of two factors: economy of energy and nutrients, and adaptability to new conditions. Instability of messenger RNA forces the organism constantly to synthesize more RNA, but permits a rapid change in the complement of proteins. On the other hand, there are many proteins whose synthesis is essential in all circumstances; if the messenger RNA molecules corresponding to them were unstable, the organism would waste resources in the synthesis of RNA and would gain no compensating advantage. The distinction between short-lived and long-lived messenger RNA is thus reflected in the distinction between repressible and non-repressible protein.

Results reported by Moses & Calvin (1965) are in partial agreement with this idea. These authors found that when cells from a stationary culture of *Escherichia coli* were transferred to fresh medium the synthesis of total protein started almost immediately, but that the synthesis of three inducible enzymes was greatly delayed. There was, however, no apparent difference between an inducible and a constitutive strain in the length of lag before the onset of synthesis of β -galactosidase. Moses & Calvin (1965) concluded that messenger RNA 'for normally inducible enzymes' is more labile than that 'for some normally constitutive proteins', but this formulation implies an absolute difference between inducible and constitutive

proteins that is hard to understand in terms of evolutionary development (see Lerner, Wu & Lin, 1964).

Strain 749/C was derived from strain 749 of *B. licheniformis* by a one-step mutation (Pollock, 1963). The regulatory gene in which the mutation occurred can be distinguished from the structural gene responsible for the amino acid sequence of penicillinase (Dubnau & Pollock, 1965). If my interpretation of the present results is accepted, namely that the penicillinase-messenger RNA in strain 749/C is more stable than that in strain 749, it is necessary to explain how a mutation in the regulatory gene could affect the stability of 'homologous' messenger RNA transcribed from the structural gene. One model that might help to account for a change in stability would endow the repressor produced by the regulatory gene with specificity for the messenger RNA. The repressor substance is believed to interact specifically with a particular sequence of DNA (Jacob & Monod, 1961); it may therefore be able to recognize also the RNA transcribed from that sequence. Since the repressor apparently consists of, or at least contains, protein (Sadler & Novick, 1965), it could perhaps act as an enzyme that specifically inactivates homologous RNA. Thus in the inducible strain of *B. licheniformis* the wild-type repressor would not only prevent the transcription of the penicillinase gene, but would also rapidly inactivate any messenger RNA that was formed. In the constitutive strain the mutant repressor would have lost the ability to prevent transcription, and would also be unable to inactivate the homologous messenger RNA, whose degradation would then be brought about more slowly by non-specific enzymes.

If this suggested explanation of the present results were correct, one might expect that the presence of inducer would alter the rate at which the repressor destroyed messenger RNA. In fact Ben-Hamida & Schlessinger (1965) have shown that β -galactosidase-messenger RNA is degraded more rapidly in the absence than in the presence of inducer.

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