*POLYSOME TRANSITIONS AND THE REGULATION OF RIBONUCLEIC ACID SYNTHESIS IN ESCHERICHIA COLI**

By D. W. Morris and J. A. DeMoss

DEPARTMENT OF BIOLOGY, REVELLE COLLEGE, UNIVERSITY OF CALIFORNIA, SAN DIEGO (LA JOLLA)

Communicated by Bruno H. Zimm, May 23, 1966

In a stringent strain (RC^{str}) of *Escherichia coli*, amino acid starvation results in the inhibition of net ribonucleic acid (RNA) synthesis and drastically lowers the rate of incorporation of exogenous precursors into RNA.[1, 2] Mechanisms proposed to explain this control have included (*a*) an inhibition of the RNA polymerase by uncharged transfer ribonucleic acid (tRNA),[2–5] (*b*) an inhibition of RNA synthesis by some component of the protein-synthesizing unit, other than tRNA, which is released during amino acid starvation,[6] and (*c*) an involvement of the translation process in the active removal of nascent RNA from its DNA template.[7, 8] Recent evidence would appear to rule out the inhibition of the RNA polymerase by uncharged tRNA.[6, 9] On the other hand, both of the other theories are compatible with the recent observation that amino acid activation is required for RNA synthesis.[10] From our studies on the *in vivo* level of charged tRNA in cells grown under various conditions, we have concluded that the aminoacyl-tRNA, rather than uncharged tRNA, is responsible for the amino acid regulation of RNA synthesis.[6]

To consider how the availability of charged tRNA might regulate RNA synthesis, we have examined the changes in the polysome level during amino acid starvation and under various conditions where RNA synthesis is uncoupled from protein synthesis.

Materials and Methods.—Chemicals: Bovine pancreatic ribonuclease was 5× crystallized, protease-free, from Sigma Corp. Chloramphenicol was a gift from Parke-Davis and Co.; tetracycline was a gift from Upjohn Co. Uracil-2-C^{14} and the L-amino acids, A grade, were obtained from Calbiochem. All other chemicals were reagent grade and were readily obtainable from commercial suppliers.

Bacteria and culture conditions: The leucine auxotroph is the stringent RNA control (RC^{str}) strain of *Escherichia coli* K12, W2961.[6] The arginine auxotroph is the relaxed RNA control (RC^{rel}) strain of *E. coli* K12 referred to as HAR.[11] These strains were grown in a standard minimal medium[12] with 0.4% glucose and supplemented with 50 μg/ml of the required amino acids and thiamine. For starvation experiments, the cells were grown in 11.5 μg/ml of the required amino acid, which permitted exponential growth to approximately 10^9 cells/ml. At this point the culture abruptly stopped growing due to the exhaustion of the amino acid from the medium. Prior incubation of the same preparation of medium was used to determine the time of amino acid exhaustion. While this method is only accurate to a few minutes, it permits sampling without the necessity of harvesting and washing the cells to initiate amino acid starvation.

Preparation and analysis of extracts: Cells were grown at 37°C in a shaking 1-liter flask and 150-ml samples were withdrawn, poured over crushed ice, and centrifuged for 5 min at 10,000 × *g*. The pellet was suspended in 0.15 ml of TMK buffer [0.005 *M* tris(hydroxymethyl)aminomethane buffer (pH 7.4), 0.01 *M* $MgSO_4$, 0.06 *M* KCl] containing 30% sucrose and 300 μg/ml lysozyme. The suspension was frozen in a dry ice–acetone bath and placed for 2 min in a 37°C water bath. This procedure was repeated and then 3.5 ml of TMK buffer was added rapidly and the resultant extract drawn up and down in a pipette a number of times. The lysate (3 ml) was layered on a 28-ml sucrose gradient tube (15–30% sucrose in TMK) and spun for 3 hr in a Spinco model L at 25,000 rpm. After centrifugation, the bottom of the tube was punctured, the contents were pumped through a Gilford recording spectrophotometer, and the absorption at 260 mμ was monitored continuously. The area under the absorbancy tracing was determined for each region, as defined in Figure 1*A*, with a Filotecnica planimeter.

Determination of RNA synthesis: The culture was incubated in standard medium with 4 μg/ml uracil-2-C^{14} (0.5 mc/mmole). Samples of 0.5 ml were withdrawn at various times and pipetted directly into cold, 6% trichloroacetic acid. The cells were collected by filtration onto Millipore filters, washed with 5% trichloroacetic acid, dried, and placed into scintillation vials containing 10 ml of toluene scintillation fluid.[6] The vials were counted in a Nuclear-Chicago scintillation counter.

Results.—Ribosome distribution in a stringent strain: Ribosomes actively involved in protein synthesis in *E. coli* are bound to messenger ribonucleic acid (mRNA) and sediment in sucrose density gradients as polysomes.[12] By analyzing extracts of *E. coli* on sucrose density gradients according to the procedures in *Methods*, the percentage of ribosomes which are involved in protein synthesis may be defined as: [(area of the polysome region)/(area of the polysome and ribosome regions)] × 100.

In an exponentially growing culture of the leucine auxotroph (strain W2961), 50–60 per cent of the ribosomes sedimented in the polysome region as shown in Figure 1*A* and were converted to free ribosomes by treatment with ribonuclease (Fig. 1*B*). In this stringent strain, leucine starvation resulted in the inhibition of RNA synthesis and, surprisingly, all of the messenger-bound ribosomes in the polysome region were converted to free ribosomes within 15 min after leucine deprivation (Fig. 1*C*).

Kinetics of polysome disappearance and formation: To study the possible relationship between the unexpected loss of the polysomal-bound ribosomes and the inhibition of RNA synthesis, the kinetics of each of these processes were examined at the onset of amino acid starvation. As shown in Figure 2, the rate of RNA synthesis started to decrease immediately upon the exhaustion of leucine, and within 5 min net RNA synthesis was completely inhibited. The disappearance of ribosomes from the polysome region exhibited similar kinetics, and within 8–10 min

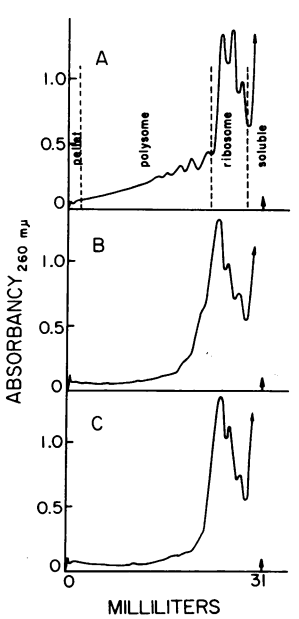

FIG. 1.—Polysomes in exponentially growing and leucine-starved cultures of *E. coli*. The preparation of extracts and the determination from sucrose density gradients of the percentage of ribosomes in the polysome region is described in the text. Part (*A*) is from an exponentially growing culture of strain W2961, and the amount of ribosomes in the polysome region is 53%. Part (*B*) is from the same extract, which had been treated with 2 μg/ml of RNase for 15 min at 4°C before it was layered on the gradient, and the amount of ribosomes in the polysome region is 11%. Part (*C*) is from a culture of strain W2961 which was deprived of leucine for 15 min, and the amount of ribosomes in the polysome region is 9%.

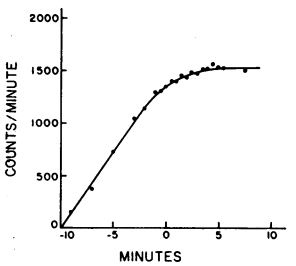

FIG. 2.—RNA synthesis at onset of amino acid starvation. Strain W2961 was grown in minimal supplemented medium with 11.5 μg/ml L-leucine added. Theoretical point of leucine starvation, determined as described in *Methods*, is indicated as zero time. Uracil-2-C^{14} was added 10 min prior to time of leucine exhaustion. 0.5-ml samples were taken at various times. Radioactivity determined as described in *Methods*.

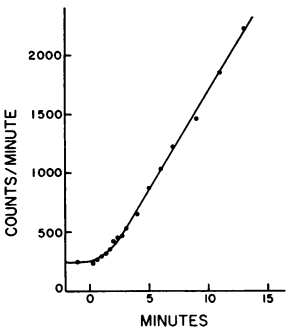

FIG. 4.—Initiation of RNA synthesis upon addition of leucine to starved culture of *E. coli*. Strain W2961 was deprived of leucine for 15 min, uracil-2-C^{14} was added, and culture was incubated for an additional 15 min before addition of 50 μg/ml L-leucine at zero time. Samples of 0.5 ml were taken at various times and radioactivity was determined as described in *Methods*.

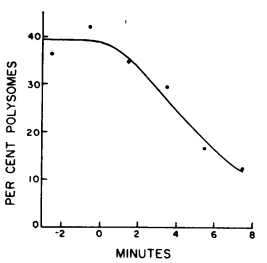

FIG. 3.—Disappearance of polysomes at onset of amino acid exhaustion. Culture conditions as in Fig. 2. Zero time indicates theoretical point of leucine starvation. Percentage of ribosomes in polysome region was calculated from sucrose density gradient profiles (see text).

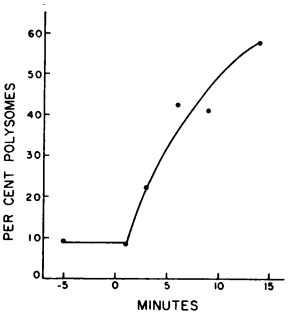

FIG 5.—Formation of polysomes upon addition of leucine to starved culture of *E. coli*. Strain W2961 was deprived of leucine for 30 min, 50 μg/ml of L-leucine was added at zero time, and percentage of ribosomes in polysome region was calculated from sucrose density gradient profiles (see text).

the percentage of polysomal-bound ribosomes had dropped to the steady-state level characteristic of an amino acid-starved culture (Fig. 3). Amino acid starvation, therefore, results in the fairly rapid loss of the polysomes instead of leaving the protein-synthesizing unit in a waiting or fixed state.

When leucine was added to the leucine-starved culture, RNA synthesis resumed immediately and was at its prestarved rate within 3 min (Fig. 4) although more than 10 min were required for the percentage of polysomal-bound ribosomes to

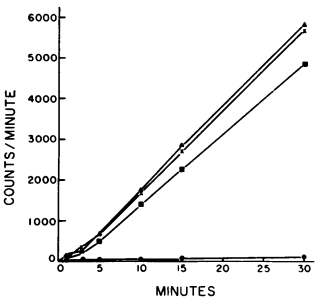

FIG. 6.—Initiation of RNA synthesis upon the addition of protein inhibitors to a leucine-starved culture of *E. coli*. Strain W2961 was deprived of leucine for 25 min, uracil-2-C^{14} was added, and the culture was incubated for an additional 5 min before the various additions were made at zero time. Samples were taken and the incorporation of uracil was determined as described in *Methods*. No additions, ●—●; 50 μg/ml L-leucine, ■—■; 50 μg/ml tetracycline, ×—×; 50 μg/ml chloramphenicol, ▲—▲.

return to the steady-state level of a growing culture (Fig. 5). Protein synthesis, as measured by phenylalanine-C^{14} incorporation, resumed immediately upon the addition of leucine and was at its prestarved rate within 1 min. Thus it appears that both RNA and protein synthesis may proceed at their full rate prior to the formation of a full complement of polysomes.

RNA synthesis in the absence of protein synthesis: Since the change in the rate of RNA synthesis followed closely the change in the rate of protein synthesis both during the onset of amino acid starvation and upon the addition of the amino acid to a starved culture, it is important to examine those cases where RNA synthesis proceeds in the absence of protein synthesis. Addition of chloramphenicol (CAP) or tetracycline to a leucine-starved culture resulted in the resumption of RNA synthesis as shown in Figure 6.

With the addition of CAP there was a rapid conversion of free ribosomes to polysomes even in the absence of protein synthesis (Fig. 7*C*). It has been demonstrated that in the presence of 100 μg/ml of CAP this conversion is complete in less than 1 min, in contrast to the 10–12 min required for this process after the addition of the required amino acid, and that the growth of nascent polypeptide chains is completely inhibited under these conditions.[13]

Tetracycline has been shown to inhibit protein synthesis by preventing the binding of aminoacyl-tRNA to ribosomes.[14] As shown in Figure 7*B*, addition of tetracycline to a leucine-starved culture also resulted in the conversion of free ribosomes to polysomes. Thus the addition of either of these two antibiotics to an amino acid-starved culture leads to the resumption of RNA synthesis and the rapid formation of RNase-sensitive polysomes even though their modes of action for the inhibition of protein synthesis are suspected to be quite dissimilar.

The RC^{rel} strains of *E. coli* differ from the wild type (RC^{str}) in that RNA synthesis continues in such strains even in the absence of a required amino acid.[2, 15] When the relaxed strain (HAR) was starved for arginine, there was no significant loss of ribosomes from the polysome region, although after prolonged periods of starvation there appeared to be a shift in the distribution from the heavier to the lighter polysomes (Fig. 8). The persistence of polysomes in the relaxed strain is not due to some unique property of arginine starvation since it has been shown that in an arginine-starved stringent auxotroph of *E. coli*, the polysomes disappeared in a manner similar to the stringent strain used in these experiments.[13]

Thus, the addition of protein inhibitors to an amino acid-starved RC^{str} culture resulted in the formation of polysomes as well as the stimulation of RNA synthesis. Similarly, amino acid starvation of the RC^{rel} strain resulted in the inhibition of protein synthesis while there was no loss of polysomes and RNA synthesis continued.

Discussion.—The data presented here demonstrate a correlation between the rate of RNA synthesis and the presence of polysomes in *E. coli*. While we have not

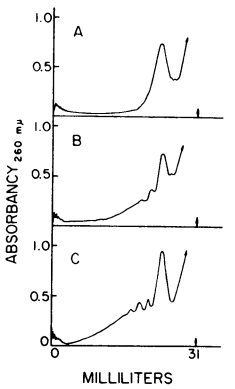

FIG. 7.—Formation of polysomes upon the addition of protein inhibitors to a leucine-starved culture of *E. coli*. Strain W2961 was deprived of leucine for 30 min, divided into three flasks, and treated as described below. The preparation of extracts and the determination from sucrose density gradients of the amount of ribosomes in the polysome region is described in the text. No additions were made in (*A*), and the level of polysomes is 15%. Incubation for 4 min in the presence of 50 μg/ml tetracycline (*B*) and for 1.5 min in the presence of 50 μg/ml chloramphenicol (*C*) increased the level of ribosomes in the polysome region to 56% and 53%, respectively.

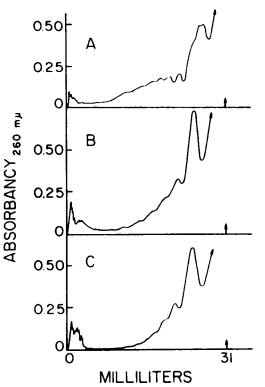

FIG. 8.—Presence of polysomes in an arginine-starved auxotroph of a relaxed strain of *E. coli*. The relaxed strain, HAR, was grown in minimal supplemented medium, extracts were prepared, and the amount of ribosomes in the polysome region was determined from the sucrose density gradient profiles as described in the text. Prior to starvation (*A*), the level of ribosomes in the polysome region is 52%. After deprivation of arginine for 15 min (*B*) and 30 min (*C*), the amount of ribosomes in the polysome region is 47% and 40%, respectively.

determined the precise mechanisms involved in the disappearance and formation of polysomes, this relationship would appear to be compatible with several theories which have been proposed for the regulation of RNA synthesis. Recent experiments seem to indicate that the DNA-dependent RNA polymerase is complexed with both DNA and with ribosomes.[16–18] Thus, if RNA synthesis is dependent on the removal of nascent RNA from its DNA template by ribosomes as proposed by Stent, then the polysomes present during RNA synthesis may be interpreted as a complex of those ribosomes which have combined with and released the nascent RNA from its DNA template. It can be concluded, however, that translation is not required for RNA transcription since treatment with CAP has been shown to inhibit nascent polypeptide chain growth on polysomes under conditions where RNA synthesis has been restored in an amino acid-starved culture.[13] Alternatively, RNA synthesis may be controlled by some mechanism independent of the protein-synthesizing unit, and the formation of polysomes which we observe may be due simply to the resumption of RNA synthesis and the formation of new messenger ribonucleic acid (mRNA).

Both of the above interpretations assume, from the findings that the rate of uracil incorporation is drastically inhibited, that all RNA synthesis is restricted during amino acid starvation. This assumption would appear to be incorrect since

evidence indicates that mRNA is present and continues to turn over during the period of amino acid starvation. The fact that mRNA is present in amino acid-starved cells is shown by the following observations: (*a*) protein turnover occurs in amino acid-starved cultures for extended periods of time,[19] (*b*) re-addition of the required amino acid results in the immediate resumption of protein synthesis prior to the onset of exogenous uracil incorporation,[20] (*c*) a full complement of RNase-sensitive polysomes is restored in less than 1 min when CAP is added to an amino acid-starved culture,[13] although more than 1 min is required before RNA synthesis has resumed at its maximum rate, and (*d*) mRNA, as assayed in an *in vitro* protein-synthesizing system, is present in normal amounts during amino acid starvation.[21] It is also clear that mRNA can be synthesized during the period of amino acid deprivation since specific messengers for either β-galactosidase[22, 23] or T-even phages[24, 25] can be synthesized during this period. Finally, it has been shown that β-galactosidase messenger decays at its normal rate during amino acid starvation.[23, 26] Furthermore, we have found that when cells are pulsed with labeled uracil just prior to amino acid starvation, the amount of labeled RNA in the cell remains constant during the starvation period, even in the presence of an excess of unlabeled uracil. However, the addition of proflavin, or the addition of actinomycin D to EDTA-treated cells, results in a rapid loss of the labeled RNA from these cells.[27] Thus, from all of these observations, it appears likely that mRNA synthesis and breakdown continue during amino acid deprivation.

Although a small amount of radioactive uracil incorporation into mRNA may be demonstrated during amino acid starvation,[21, 22] the failure to observe a significant initial rate of uracil incorporation must then be due to mRNA turnover which generates sufficient pools of nucleotides to preclude much incorporation of exogenous uracil into mRNA. The recent observation that fluorouracil is not incorporated into mRNA during amino acid starvation[28] could also be interpreted in this way.

Two important consequences follow from the deduction that mRNA synthesis continues during amino acid deprivation. First, the disappearance of polysomes upon amino acid starvation must not be due to the disappearance of mRNA, but must result from a decreased rate of attachment of the ribosomes to mRNA. Second, the inhibition of net RNA synthesis must be due either to a block in the transcription of those specific cistrons which code for stable RNA or to an inhibition of one of the steps required for the stabilization of ribosomal and transfer RNA from unstable RNA precursors.

Our results are compatible with the hypothesis that free ribosomes are responsible for the inhibition of stable RNA synthesis in amino acid-starved cultures.[6] When the concentration of any single aminoacyl-tRNA species is lowered sufficiently, the rate of ribosome attachment to mRNA would be significantly decreased even though mRNA continues to be synthesized. In the dissociated state, the free ribosomes, or one of their components, would then inhibit either the synthesis of ribosomal RNA or the conversion of unstable precursor RNA to stable ribonucleoprotein particles. Although the polysomes formed in the presence of tetracycline or CAP are inactive with respect to protein synthesis, the transition from free ribosomes to polysomes now permits the accumulation of stable RNA. This model proposes that the regulation of RNA synthesis by amino acids is not exerted through a control at the RNA polymerase reaction, but that free ribosomes, which are released

from polysomes during amino acid starvation, restrict some step in their own biosynthesis.

Summary.—In a stringent strain of *Escherichia coli*, the polysomes disappeared when net RNA synthesis was inhibited by amino acid starvation. Polysomes reappeared under conditions where net RNA synthesis resumed, both in the presence and absence of protein synthesis. Polysomes did not disappear in a relaxed strain, where RNA synthesis continues during amino acid starvation. Thus, there is a direct correlation between the level of polysomes and the synthesis of net RNA.

Since mRNA synthesis apparently continues during amino acid starvation, the disappearance of polysomes must result from the failure of the ribosomes to reattach to mRNA. A mechanism is discussed whereby the dissociation of polysomes could result in an inhibition of stable RNA formation and prevent the incorporation of exogenous precursors into mRNA.

We should like to thank M. J. Weber for his valuable suggestions and assistance.

* This work was supported by USPHS research grant 1-RO1-GM-13200-01.

[1] Neidhardt, F. C., *Progr. Nucleic Acid Res. Mol. Biol.*, **3**, 145 (1964).
[2] Stent, G. S., and S. Brenner, these PROCEEDINGS, **47**, 2005 (1961).
[3] Kurland, C. G., and O. Maaløe, *J. Mol. Biol.*, **4**, 193 (1962).
[4] Tissières, A., S. Bourgeois, and F. Gros, *J. Mol. Biol.*, **7**, 100 (1963).
[5] Ezekiel, D., *Biochem. Biophys. Res. Commun.*, **14**, 64 (1963).
[6] Morris, D. W., and J. A. DeMoss, *J. Bacteriol.*, **90**, 1624 (1965).
[7] Stent, G. S., *Science*, **144**, 816 (1964).
[8] Stent, G. S., *Lecture for the Mendel Centennial Symposium* (London: The Royal Society, 1965).
[9] Olenisk, J. G., *Federation Proc.*, **25**, 520 (1966).
[10] Eidlic, L., and F. C. Niedhardt, *J. Bacteriol.*, **89**, 706 (1965).
[11] Sypherd, P. S., *J. Bacteriol.*, **90**, 403 (1965).
[12] Kiho, Y., and A. Rich, these PROCEEDINGS, **51**, 111 (1964).
[13] Weber, M., and J. A. DeMoss, these PROCEEDINGS, **55**, 1224 (1966).
[14] Hash, J. H., M. Wishnick, and P. A. Miller, *J. Biol. Chem.*, **239**, 2070 (1964).
[15] Borek, E., A. Ryan, and J. Rockenback, *J. Bacteriol.*, **69**, 480 (1955).
[16] Bremer, H., and M. W. Konrad, these PROCEEDINGS, **51**, 801 (1964).
[17] Byrne, R., J. G. Levine, H. A. Bladen, and M. W. Nirenberg, these PROCEEDINGS, **52**, 140 (1964).
[18] Shin, D. H., and K. Moldave, *Biochem. Biophys. Res. Commun.*, **22**, 232 (1966).
[19] Mandelstam, J., *Biochem. J.*, **69**, 110 (1958).
[20] Morris, D. W., and J. A. DeMoss, unpublished observation.
[21] Forchammer, J., N. O. Kjeldgaard, and K. Moldave, cited in *Control of Macromolecular Synthesis* (New York: W. A. Benjamin, Inc., 1966).
[22] Stern, J. L., M. Sekiguchi, H. D. Barner, and S. S. Cohen, *J. Mol. Biol.*, **8**, 629 (1964).
[23] Nakada, D., and B. Magasanik, *J. Mol. Biol.*, **8**, 105 (1964).
[24] Sekiguchi, M., and S. S. Cohen, *J. Mol. Biol.*, **8**, 638 (1964).
[25] Hayward, W. S., and M. H. Green, unpublished observation.
[26] Képès, A., *Biochim. Biophys. Acta*, **76**, 293 (1963).
[27] Morris, D. W., D. Hopkins, and J. A. DeMoss, unpublished observation.
[28] Edlin, G., and O. Maaløe, *J. Mol. Biol.*, **15**, 428 (1966).