Regulation of Ribonucleic Acid Accumulation in Vivo by Nucleoside Triphosphates*

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SUMMARY

A continuous and unique variation of stable RNA accumulation with pool levels of nucleoside triphosphates was observed when Escherichia coli (K12 Leu−, rel+) cells were entering into phosphate starvation or recovering spontaneously from inhibition by dinitrophenol. Since the rate of RNA accumulation in these experimental systems surpassed all other known systems exhibiting the same pool levels of nucleoside triphosphates, it is suggested that stable RNA synthesis in these systems was subjected to kinetic regulation by substrate concentrations. This kinetic relationship in vivo between RNA synthesis and nucleoside triphosphate substrates was sigmoidal in character and exhibited a higher half-saturation constant than the similar relationship obtained with RNA polymerase in vitro.

Since the biosynthesis of RNA by RNA polymerase requires ribonucleoside triphosphates as substrates, kinetic regulation by triphosphate levels is expected to represent a simple mechanism regulating RNA synthesis which can readily respond to metabolic changes in the cell. The finding that triphosphates did not mediate the inhibition of RNA synthesis in Escherichia coli during a series of nutrient- and inhibitor-induced shift downs (1) therefore was a surprising one and prompted a search for experimental systems in which kinetic regulation becomes important.

Materials and Methods

Chemicals—Carrier-free [33P]orthophosphoric acid and [14C]-uridine-5'-triphosphate were obtained from New England Nuclear and MN-Polygram CEL-300 polyethyleneimine-impregnated cellulose sheets were from Macherey Nagel and Company, Duren, Germany. Frozen E. coli K12 cells were supplied by General Biochemicals. Whatman microgranular DEAE-cellulose, DE-52 (1.0 meq per g of dry weight), was a product of the H. Reeve Angel Company, Clifton, New Jersey, and N-methyl-N'-nitro-N-nitrosoguanyline was from Aldrich.

Bacteria and Growth Conditions—The E. coli K12 Leu− cells and most of the experimental conditions have been described in the preceding paper (1). Since all growth experiments in this study involved 33P-labeling, the Tris-buffered medium 63 (24 μg per ml of KHP04) was used. When phosphate starvation was to be observed, the KH2PO4 content was reduced further to 15 μg per ml. To observe adenosine deprivation, a leaky purine auxotroph of K12 Leu− (isolated, after mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine, as a small colony on agar plates containing 2.5 μg per ml of adenosine) was grown in the presence of only 7 μg per ml of adenosine. In all instances, cells were allowed to undergo at least three doublings in the experimental medium containing [33P]orthophosphate to about 1 to 2 × 10^8 cells per ml before sampling began. Cell mass was monitored by absorbance at 450 nm in a Gilford spectrophotometer; unit absorbance with a 10-mm light path corresponded to approximately 0.2 mg of dry weight or 4 × 10^9 cells per ml.

Incorporation of [33P]Orthophosphate into Nucleic Acids and Nucleotides—To measure 33P-incorporation into RNA and DNA, one 20 μl cell sample was pipetted into 3 ml of 5% trichloroacetic acid, a duplicate one into 1.5 ml of 0.5 N NaOH, and the two samples were chemically fractionated according to Gallant and Harada (2). To determine nucleoside triphosphates, 100-μl cell samples were pipetted into 150 μl of 1 N acetic acid, extracted by freeze-thawing (3), and 25 μl of each extract were spotted on a polyethyleneimine-impregnated cellulose thin layer sheet. Chromatography was carried out two-dimensionally as previously described (3), or one-dimensionally by developing the thin layer chromatographic sheet twice to 20 cm above the origin in 1 N LiCl and 1 N acetic acid, with an intervening wash in methanol. Scintillation counting in a Nuclear-Chicago...
Mark I counter was employed to determine the 33P content in all cases.

Measurement of RNA Polymerase Activity—RNA polymerase was purified from frozen *E. coli* K12 cells using the procedure of Burgess (4) to the end of chromatography on a DE-52 cellulose column. Peak fractions from the column were pooled and stored in 50% glycerol at -20°. In the kinetic experiments, measurements of uninitiated polymerizations were carried out according to Anthony, Wu, and Goldthwait (5). The reaction mixture (0.5 ml) contained ATP:GTP:UTP (0.58 mCi per mmole):CTP in the ratio of 7:4:3:1, 18 μg of enzyme, and 18 μg of native DNA, which had been isolated from frozen *E. coli* K12 cells using the method of Marmur (6) but omitting treatment with ribonuclease. Polymerizations at 37° were terminated by either precipitation and washing on a Millipore filter (GS, 0.22 μm) with 5% trichloroacetic acid or by the direct filtration technique of Sentenac, Simon, and Fromageot (7). The latter required adding 25 μmoles of ethylenediaminetetraacetate and 1 mg of UTP to the reaction mixture, and washing the mixture through the Millipore filter with the incubation maleate buffer. In either instance radioactivity on the dried filter was determined by scintillation counting.

RESULTS

RNA Accumulation and Nucleotide Levels during Phosphate Starvation—As a culture of *E. coli* K12 Leu- cells progressively depleted the phosphate in a low phosphate medium, cell growth very gradually slowed down. Fig. 1 shows the incorporation of [33P]orthophosphate into RNA during the slow down, and tangents drawn to the smoothed incorporation curve at different times give the rates of RNA accumulation at these times. Simultaneous sampling for ribonucleotide triphosphates yielded the results of Fig. 2. Over a period of 3 hours, the levels of ATP, GTP, and CTP dropped to 25% of their normal levels, and UTP to less than 15%.  

RNA Accumulation and Nucleotide Levels during Spontaneous Recovery from Dinitrophenol—As recorded earlier (1), RNA accumulation and growth of K12 Leu- cells were severely inhibited when first exposed to 0.5 mM dinitrophenol, but later exhibited a spontaneous recovery to close to 90% control rates. This pattern of spontaneous recovery persisted with higher dinitrophenol concentrations, but the recovered rate of RNA accumulation as well as triphosphate levels varied with dinitrophenol concentration (Fig. 3).  

RNA Accumulation and Nucleotide Levels during Adenosine Starvation—When mutant cells of K12 Leu- which required purines for fast growth were cultured in the presence of limited adenosine, the exhaustion of adenosine was marked by a smooth transition of RNA and DNA synthesis to a slower, steady rate. The nucleoside triphosphates, however, exhibited a complex pattern of changes (Fig. 4).  

Kinetics of RNA Polymerase in Vitro—The substrate dependence of RNA polymerase was analyzed in order to compare substrate regulation of RNA synthesis in vivo and in vitro. In Fig.

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**Fig. 1.** Incorporation of [33P]orthophosphate into RNA during phosphate depletion. The dotted tangential lines drawn to the incorporation curve at the indicated times give the relative incorporation rate at these times. Different symbols represent data from different experimental cultures.
5, ATP, GTP, UTP, and CTP were used in the ratio of 7:4:3:1, which approximated the spread of triphosphate concentrations in their decreases during phosphate starvation. As described under "Materials and Methods," two different procedures were used to terminate RNA polymerization and to collect the newly synthesized RNA. The progress curves employing acid precipitation showed a significant lag, but the curves employing direct filtration did not, likely reflecting the failure of the acid to precipitate very short nascent RNA chains. The rate curves derived from the two procedures were also significantly different (Fig. 6). The acid precipitation curve was slightly sigmoidal and gave a half-saturation constant, or $K_m$, about 0.29 mM in terms of ATP, whereas the direct filtration curve was hyperbolic with a $K_m$ about 0.08 mM in terms of ATP.

**DISCUSSION**

**Regulation of RNA Accumulation by Nucleoside Triphosphates**

For the rate of stable RNA accumulation to be controlled by the nucleoside triphosphate substrates, there should be a continuous, monotonic correlation between variations in RNA accumulation and in triphosphates. Moreover, given any triphosphate level, the rate of RNA accumulation in systems under substrate control should not be exceeded by systems under other means of regulation. On the basis of these two criteria, sub-
Fig. 3. RNA accumulation and nucleoside triphosphates in cells recovering from different concentrations of dinitrophenol. Cells treated with dinitrophenol showed no net RNA synthesis initially but later recovered to give a steady rate of RNA accumulation (1), the magnitude of which depended on the dinitrophenol concentration. The rate of RNA accumulation and triphosphate levels indicated were measured 30 min after such a steady recovery was achieved. Symbols are: ●, ATP; ○, GTP; ▲, CTP; △, UTP; and □, rate of RNA accumulation.

substrate control was demonstrated to be of limited importance for cells undergoing a number of nutrient- and inhibitor-induced shift downs (1). In contrast, the measurements on phosphate starvation represented in Figs. 1 and 2 satisfied both criteria. First, there existed a continuous, monotonic correlation between RNA accumulation and triphosphate levels, in the form of either Curve I of Fig. 7 computed for the average values for all four triphosphates, or Curve II computed for UTP alone (which underwent greater per cent decreases than the others). This correlation was also adhered to by measurements on cells recovering from varying concentrations of dinitrophenol (Fig. 7), even though the detailed cellular changes underlying inhibition and recovery in dinitrophenol are as yet undefined. Secondly, although a number of shift downs (1) supplied examples of experimental systems lying to the right of Curve I and II, showing extensive inhibition of RNA accumulation even though the triphosphates were above the 50 to 60% mark, so far no system has been encountered lying to the left of Curves I and II and accumulating stable RNA faster than allowed by these curves.

Kinetic Regulation of RNA Synthesis—A correlation between RNA accumulation and nucleoside triphosphate levels suggests that the nucleoside triphosphates serve as metabolite signals for regulating stable RNA accumulation, but the precise mechanism of regulation remains to be defined. Since the nucleoside triphosphates are the substrates for RNA polymerization, if their concentrations are sufficiently reduced sooner or later a rate limitation of RNA synthesis by substrate concentrations must occur. Before the triphosphate reductions were sufficient to bring about this kinetic mechanism of transcriptional control, however, other mechanisms regulating at the transcriptional or degradative stage of stable RNA metabolism already might be set in motion by the changing triphosphate concentrations.

In a recent study, Maruyama and Mizuno (8) reported a degradation of preformed RNA during complete phosphate starvation of the extent of 2.5% per 10 min during the first 2 hours of incubation. In the experiment in Fig. 1, the culture had not yet reached the point of complete phosphate exhaustion, and the turn off of RNA accumulation between zero time and the 40-min mark was close to an average of 20% per 10 min. Consequently the contribution by degradative processes to the turn off of RNA accumulation might be limited. It is important, nevertheless, to define the consequences of any degradative control, as well as nonkinetic types of transcriptional control, operating in the phosphate-depletion system of Fig. 1.

If the turn off of RNA accumulation shown in Figs. 1 and 7 resulted strictly from a kinetic regulation of synthesis, the calibration Curves I and II would define the exact rate dependence in vivo of stable RNA synthesis on substrate concentrations.
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FIG. 5. Progress curves for the RNA polymerase reactions with the substrate ratios of ATP:GTP:UTP:CTP (7:4:3:1). After various times of incubation, the reaction using E. coli K12 DNA as template was terminated by either precipitation with 5% trichloroacetic acid (i) or direct filtration on to Millipore filter (ii). [³⁵S]UTP incorporation is expressed in terms of micromoles per min. The ATP concentration in millimolar used in any reaction mixture is marked opposite to the corresponding progress curve.

On the other hand, if any degradative control and nonkinetic transcriptional control should be operating instead of or jointly with the kinetic mechanism, the calibration Curve I and II would set an upper limit to the in vivo rate curve. The actual rate curve would have to lie to the left of these curves in Fig. 7.

At present, because no experimental system has been encountered which give measurements lying to the left of Curve I and II, the kinetic model of biosynthetic regulation shown in Fig. 8 can be advanced as a working hypothesis to explain the regulation of RNA accumulation during the entry of K12 Leu cells into phosphate starvation. The mechanism actually includes two successive kinetic controls by substrate level, first in the phosphorylation of nucleosides and nucleotides, and secondly in the polymerization of RNA. In this study no enquiry has been made into the quantitative control of phosphorylation, but the preservation of moderately constant ratios among ATP, GTP, UTP, and CTP during their decreases in Fig. 2 is consistent with a tightly controlled partition of phosphate by the phosphorylating and kinase systems. Because phosphate and nucleosides are both consumed in RNA synthesis, the two-stage mechanism given in Fig. 8 might be expected to operate equally during nucleoside starvations. Indications, however, are that nucleoside starvations may activate additional regulatory complexities.

Nucleoside Starvations—Gallant and Harada (2) first quantitated the relationships between triphosphates and RNA synthesis under nucleoside starvations. During adenine exhaustion of their adenine-less E. coli strains DB429 and 156-2a, cessation of...
RNA synthesis was accompanied only by moderate decreases in ATP and GTP down to 60% of their glucose-grown levels. During guanine exhaustion of the glucose-grown strains R257 and AT2465, however, GTP dropped to below 20%. Uracil exhaustion of strain BD1 similarly lowered UTP to the neighborhood of 20%. The findings with R257, AT2465, and BD1 cells, showing a severe inhibition of RNA synthesis at 20% triphosphates, are therefore entirely in accord with the kinetic calibration given by Curves I and II in Fig. 7. Measurements from the DB429 and 156-2a systems, however, lie far to the right of Curves I and II, and suggest the intervention of regulatory mechanisms other than straightforward substrate control. This type of aberrant behavior was not restricted to these cells, and the addition of uracil to uracil-starved 15-T cells was found by Lazzarini, Nakata, and Winslow (9) to stimulate RNA accumulation with only a small rise in UTP. In the present study a leaky adenine-requiring mutant of K12 Leu- gave a similarly complex response when placed under nucleoside restriction short of complete starvation. As seen from Fig. 4, even though RNA synthesis proceeded at 25% the normal rate after adenosine exhaustion, ATP dipped to 40% only briefly and climbed back to about 68%. CTP stayed above 50%, GTP showed only a small decrease, and UTP actually accumulated in excess. Recently, Thomas, Varney, and Burton (10) have begun to unravel some of these difficult relationships between RNA synthesis and triphosphates during nucleoside starvations in terms of the multitude of feedback regulations encountered in the biosynthetic pathways for nucleotides.

Comparison of in Vivo and in Vitro Rate Curves—During progressive phosphate exhaustion, the concentration of UTP dropped more severely than the other three triphosphates, and there is at present no basis to decide which of Curves I and II value for UTP, which underwent greater per cent decreases in Fig. 2 than the other triphosphates. The open circles represent points derived from the dinitrophenol-treated cells in Fig. 3, and the closed circles (Curve III) reproduce the in vitro results of acid-precipitated polymerizations from Fig. 6.

In Fig. 7 more closely defines the in vivo rate curve for RNA transcription in phosphate starvation. Indeed, the exact form and position of the rate curve might vary somewhat with the prevalent ratios between the four substrates, and the area bounded by Curves I and II in Fig. 7 can be more usefully regarded as a regulatory zone where a simple substrate control of RNA synthesis appears to operate.

Curve III in Fig. 7 provides an in vitro rate curve for comparison with the in vivo rate dependence. The control levels of triphosphates in the K12 Leu- cells were 6.3 µmoles of ATP, 3.3 µmoles of GTP, 2.9 of µmoles UTP, and 1.7 µmoles of CTP per gram dry weight. The 100% scale used in Fig. 7 therefore corresponds to 1.6 mm in terms of ATP, assuming a 4:1 ratio between wet and dry cell weights. Again using ATP concentration as a basis of calculation, the in vitro rate curve for RNA polymerase from Fig. 6, obtained by acid precipitation, therefore assumes the form of Curve III in Fig. 7 lying to the left of the I-II region. It would move even further left if the rate curve from Fig. 6 obtained by direct filtration were used. Because of the vast differences between in vivo and in vitro conditions, a discrepancy between III and I-II is not entirely unexpected. Beside the possible activities of degradative and nonkinetic transcriptional
controls as already discussed, and the multitude of metabolites and protein factors which might modulate RNA polymerase kinetics in vivo, at least three factors can be recognized as possibly contributing to the discrepancy. First, although a survey (11) indicated that the $K_m$ for RNA polymerase in vitro was not extensively altered using denatured compared to native E. coli DNA as template, or using the four triphosphates in the ratio of 1:1:1:1 compared to 7:4:3:1, the two rate curves recorded in Fig. 6 suggest that the in vitro $K_m$ may not be a fixed value but vary with the length of nascent RNA chains selected for measurement, acid precipitation being less efficient than direct filtration in isolating the very short RNA chains polymerized (7). Secondly, part of the substrate triphosphates inside the cells might be bound to membrane structures and therefore not freely available for RNA synthesis (10). Finally, if oxidative phosphorylation occurs at the cell membrane and RNA synthesis at the cell center, the triphosphates have to travel down a diffusion gradient to reach the site of RNA synthesis. From the calculations recorded in Fig. 9, it is apparent that the concentration gradient $\Delta C_{II}$ likely will be small in the cytoplasmic region. The gradient $\Delta C_I$ in the DNA region, however, may become quantitatively important on account of high viscosity seriously impeding the diffusion of triphosphates. Because of the packaging of DNA at a density of 0.4 g per cm$^3$ (12), a relative viscosity of $10^6$ or $10^8$ is not excluded for the DNA region, extrapolating from its known variation with lower DNA concentrations (13). If a large $\Delta C_I$ indeed exists, the effective triphosphate concentrations at the site of RNA synthesis will be lower than the average cellular concentrations, and the regulatory $I-II$ zone in Fig. 7 will have to be moved left. Physical factors which affect the organization and viscosity of the DNA also unavoidably will exert an important influence on the rate of RNA synthesis. Whether or not they will be utilized by the cells for regulatory purposes remains to be considered.

Acknowledgment—We are indebted to Mrs. G. Koper for her excellent assistance.

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Regulation of Ribonucleic Acid Accumulation in Vivo by Nucleoside Triphosphates
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J. Biol. Chem. 1972, 247:798-804.

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