Relationship between Growth Rate and ATP Concentration in *Escherichia coli*

A BIOASSAY FOR AVAILABLE CELLULAR ATP

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Previous studies showed that adenosine triphosphate (ATP) concentrations in *Escherichia coli* changed during certain growth transitions and directly controlled the rate of rRNA transcription initiation at those times. The relationship between ATP concentration and rRNA transcription during steady-state growth is less clear, however. This is because two commonly employed methods for measuring ATP concentrations in bacteria, both of which rely on physical extraction followed by chromatographic separation of small molecules, resulted in dramatically different conclusions about whether ATP concentration changed with steady-state growth rate. Extraction with formic acid indicated that ATP concentration did not change with growth rate, whereas formaldehyde treatment followed by extraction with alkali indicated that ATP concentration increased proportionally to the growth rate. To resolve this discrepancy, we developed a bioassay for ATP based on the expression of a variant of the firefly luciferase enzyme in *vivo* and measurement of luminescence in cells growing in different conditions. We found that the available ATP concentration did not vary with growth rate, either in wild-type cells or in cells lacking guanosine 5'-diphosphate, 3'-diphosphate, providing insight into the regulation of rRNA transcription. More broadly, the luciferase bioassay described here provides a general method for evaluating the ATP concentration available for biochemical processes in *E. coli* and potentially in other organisms.

ATP is the energy currency of the cell, providing energy for enzymatic reactions. ATP also serves as a substrate for RNA synthesis, and it regulates a variety of biological processes. Because of its potential to affect many aspects of cellular regulation, whether cellular ATP concentrations change when growth conditions change is a key question in molecular biology.

The concentration of the initiating nucleoside triphosphate (iNTP) plays a direct role in the regulation of RNA transcription initiation in *Escherichia coli* in response to changes in growth conditions (1–4). For example, when cells outgrow from stationary phase, the levels of ATP (the iNTP for 6 *rrn* P1 promoters) and GTP (the iNTP for the seventh *rrn* P1 promoter) increase dramatically, resulting in a direct and rapid increase in rRNA synthesis (4). Conversely, when cells enter stationary phase, a decrease in ATP and GTP levels (in conjunction with an increase in the concentration of guanosine 5'-diphosphate, 3'-diphosphate, ppGpp) directly inhibits rRNA synthesis. Following nutrient shifts during exponential growth, changes in ppGpp concentration are sufficient to account for regulation of rRNA promoters (4).

It has long been known that when *E. coli* cells are grown on different nutrient sources leading to different steady-state growth rates rRNA synthesis is proportional to the square of the culture’s growth rate (5). The molecular basis for this phenomenon, called “growth rate-dependent control”, still remains unresolved, however. Based on an observed correlation between the concentrations of ATP and GTP and *rrn* P1 promoter activity at different growth rates, we proposed previously that changes in ATP and GTP concentrations were responsible, at least in part, for growth rate-dependent control of rRNA transcription (1). Subsequently, however, another group reported that the concentrations of these NTPs did not change with growth rate (6). There are other examples in the literature supporting both conclusions (e.g. variable NTP concentration with growth rate (7); invariant NTP concentration with growth rate (8)). We show here that the discrepancy in the measurements of NTP concentrations at different growth rates reported by Gaal et al. (1) and by Petersen and Moller (6) resulted from differences in extraction methods.

To resolve this discrepancy, we report the development of a bioassay for ATP. We expressed firefly luciferase in growing *E. coli* cells and controlled for changes in other substrates of the enzyme and for variation in luciferase expression. Because we used a previously characterized luciferase variant with an ~5-fold higher *Km* for ATP than the wild-type enzyme (9), the variant enzyme was able to respond to changes in ATP concentrations in the physiologically relevant range. Using this system, we found that *E. coli* cultures grown at different steady-state growth rates contain the same concentration of available ATP, either in a wild-type strain or in one lacking ppGpp.

**EXPERIMENTAL PROCEDURES**

*Strains and Culture Conditions*—All strains contained a pGEX plasmid derivative (Amersham Biosciences) expressing a variant of *Photinus pyralis* luciferase with a mutation of a histidine residue at position 245 to a phenylalanine (H245F) as a GST fusion protein (a generous gift from B. Branchini) (9). This plasmid was transformed into VH1000 (MG1655; *lacI*–*lacZ*–*pyrE*; RL6238; Ref. 1). All subsequent strains are derivatives of this parent. When indicated, previously described deletion-insertions in the *relA* and *spoT* genes (10) were introduced by transduction with F1vir (11). Cultures were grown with aeration at 30 °C in the media indicated in the figure legends.

*Extraction of ATP*—Cultures used for extraction of ATP were grown for at least three generations in the presence of 20 μCi/ml [32P][KHPO4].
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(900–1100 mCi/mmol, PerkinElmer Life Sciences). Two methods of ATP extraction were compared in each condition. Formic acid extraction was performed as in Ref. 12. Formaldehyde treatment followed by extraction with alkali was performed according to Ref. 13, except that volumes were scaled down 10-fold.

Quantitation of ATP from Extracts—Radiolabeled NTPs were resolved by PEI-cellulose thin layer chromatography (TLC) (12). TLC plates were exposed to phosphorimaging screens and scanned, and relative amounts of ATP were quantified using ImageQuant 5.1 software (Molecular Dynamics).

Measurement of Relative ATP Concentrations Using H245F Luciferase— Cultures identical to those grown for ATP extraction, but without 32P[PH2PO4], were grown for luminescence measurements. Expression of luciferase from the P_F promoter on the pGEX vector was sufficient without induction by isopropyl-1-thio-D-galactopyranoside for detection (>100-fold above luminescence baseline) even at very low cell densities (A600 < 0.1). At the time of the assay, 10 µl was removed from growing cultures and immediately added to a luminescence cuvette (12 mm x 50 mm; Promega) containing 90 µl of a buffer consisting of 10 mM Hepes, pH 6.8, 150 mM NaCl, 0.6 mM KCl, 10 mM MgSO4, 10 mM beetle luciferin (Promega), and polymyxin B sulfate (1 x 10^-8–8 x 10^-10 units/ml as indicated, Sigma). The reaction was vortexed briefly and assayed immediately in a TD2020 luminometer (Turner Designs). Luminescence readings (5/sec) were carried out for 2 min. Data (“in vivo luminescence”) were recorded using Microsoft Excel and Turner Designs software. While the luminescence readings were in progress, a separate aliquot of the culture was removed, and total luciferase activity was quantified after cell lysis (14) using the luciferase assay system (Promega). The entire data set included measurement of the burst of light produced when luciferase uses the ATP—divided by protein. To explore this issue further, we developed a bioassay based on the formic acid method were greater than with the formaldehyde/alkali method. The formic acid method was more reproducible but disagreed (Fig. 1). Because the amounts of ATP recovered with the formic acid method were greater than with the formaldehyde/alkali method (Fig. 1 legend), one potential explanation for the difference was that the former might measure total ATP levels, whereas the latter might measure ATP pools not bound to protein. To explore this issue further, we developed a bioassay for the ATP concentration available to a cytoplasmic enzyme in E. coli.

Bioassay Development—Firefly luciferase catalyzes the ATP-dependent oxidation of luciferin, yielding photons of light as a product (9). Luciferase activity is particularly attractive as a reporter of ATP concentration because, in theory, light output can be measured in intact cells as well as in vitro. Luciferase has been used successfully as a reporter of ATP concentration in mammalian cells (15, 16) by expression of the recombinant enzyme in vivo, diffusion of luciferin into cells, and quantification of the burst of light produced when luciferase uses the endogenous ATP supply to oxidize luciferin. However, previous attempts to use luciferase as a reporter of ATP concentration in prokaryotes (E. coli, Ref. 17; Rhodobacter capsulatus, Ref. 18) have been compromised by several technical problems. We were able to solve these problems as described below.

First, in growing bacteria, wild-type luciferase is saturated with ATP (R. capsulatus, Ref. 18; E. coli, data not shown), rendering light production insensitive to changes in ATP concentration in the physiologically relevant range. By utilizing a previously described variant of firefly luciferase (H245F) that has an ~5-fold higher Km for ATP (9), we made the enzyme responsive to changes in the endogenous ATP concentration (see below).

Second, any potential variation in luciferase enzyme levels caused by differential transcription, translation, or mRNA/enzyme stability would affect interpretation of the results. Therefore, for each measurement of luminescence in vivo, the total active H245F luciferase enzyme concentration was measured in vitro after cell lysis and addition of saturating concentrations of ATP and luciferin. The experimentally determined in vivo values were then normalized to the in vitro values. In practice, this correction for variation in enzyme levels introduced only a small normalization factor (typically 10–20%).

Third, bacterial membranes are relatively impermeable to luciferin. Treatment of E. coli with a permeabilizing drug, polymyxin B, has been utilized to allow diffusion of luciferin into cells (17), but previous studies used low concentrations of the drug for extended periods of time (>30 min). This treatment limits the utility of the assay because cellular metabolism (and therefore ATP concentration) can change very rapidly (4), and such changes would be likely to occur during such a long polymyxin B treatment time. To reduce the potential for adjustments in ATP synthesis or turnover that might occur in response to changes in membrane integrity caused by extended exposure to the permeabilizing agent, we used higher doses of polymyxin B, measured the burst of luminescence occurring immediately, and limited data acquisition to the first 2 min following polymyxin B addition. In addition, we corrected for differences in cell permeability in different media (see below).

The Luciferase Bioassay Responds to Changes in ATP Concentrations in Vivo—Before proceeding with the analysis of ATP concentrations in cells growing at different steady-state growth rates, we tested the H245F luciferase-based bioassay
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**Fig. 2. Luciferase bioassay.** A, kinetics of luminescence recorded from a *purK* strain expressing H245F luciferase (RLG6240) growing exponentially in 0.4% glucose with 20 amino acids (80 μg/ml) as in Fig. 1, supplemented with either 0.1 mM adenine (red) or 0.1 mM guanine (black), and treated with either 4 or 8 × 10⁴ units/ml polymyxin B, as indicated, at time 0. For clarity, only the 8 × 10⁴ units/ml polymyxin B curve is shown for the *purK* culture grown in guanine. Data are shown for two independent cultures, corrected for total luciferase levels determined from lysed cells (“normalized luminescence”; see “Experimental Procedures”). B, kinetics of luminescence recorded from wild-type (RLG6238, black) and *guaB* (RLG6239, red) strains expressing H245F luciferase. Cells were grown as in panel A, except without purine supplements in the growth medium (2), and were treated with either 4 or 8 × 10⁴ units/ml polymyxin B, as indicated, at time 0. C and D, integrated luminescence (area under the peak) at each polymyxin B concentration was plotted as a function of the time required to reach the peak midpoint. The resulting linear regression permits comparison of ATP concentrations for cultures treated with polymyxin B concentrations in *purK* strains grown in adenine versus guanine determined by three methods, as indicated and as described under “Experimental Procedures.” E, relative ATP concentrations in *purK* strains grown in adenine versus guanine determined by three methods, as indicated and as described under “Experimental Procedures.” F, relative ATP concentrations in *guaB* versus wild-type strains grown determined by three methods, as indicated and as described under “Experimental Procedures.” G, relative ATP concentrations measured from wild-type cells expressing H245F luciferase (RLG6238) after chloramphenicol treatment. Chloramphenicol (100 μg/ml, dissolved in 100% ethanol; red lines) or 100% ethanol (1% v/v; black lines) were added at time 0. Relative ATP measurements were made just before and as a function of time after treatment with chloramphenicol by formic acid extraction, formaldehyde/alkali extraction, or H245F luciferase luminescence, as indicated. Each point represents the average of two measurements from two independent cultures with error indicated.

(continued in the rest of this report as the “luciferase bioassay”) in three situations previously shown by both formic acid extraction and formaldehyde/alkali extraction to result in a qualitatively similar change in relative ATP concentration. These included two situations in which strains contained mutations in purine metabolism and one situation in which cells were treated with a protein synthesis inhibitor, the antibiotic chloramphenicol.

Mutation of the *purK* gene leads to complete purine auxotrophy. *purK* strains can grow in minimal medium supplemented with either adenine or guanine, but these conditions result in changes in the absolute concentrations of ATP and GTP and distortion of the ATP:GTP ratio (2, 19). The product of the *guaB* gene participates in the guanine half of the purine biosynthetic pathway. Previous work identified point mutations in *guaB* that led to reduced protein function and increased ATP concentration in *vivo* relative to the wild-type strain (19).

When we measured luminescence in wild-type, *purK*, and *guaB* strains using different concentrations of polymyxin B, we observed that the time required for appearance of luminescence decreased with increasing polymyxin B concentration, consistent with the expected increase in cell permeability to luciferin (Fig. 2, A and B; see also Fig. 3, A and B). Unlike the situation in *vivo* when ATP and luciferin are added in vast excess, luminescence in *vivo* peaked rather than reached a plateau. Although the peak time was fastest at the highest doses of polymyxin B, the amount of luminescence (derived from integration of the peak) was not maximal at the highest doses of polymyxin B (Fig. 2, A and B; see also Fig. 3, A and B). We suggest that the lower amount of luminescence observed at the highest polymyxin B concentrations results from efflux of ATP, reducing the amount of this substrate available to the enzyme (Ref. 17 and data not shown). We further suggest that the peak time is characteristic of a specific ratio of luciferin influx to ATP efflux.

At virtually any polymyxin B concentration (Fig. 2 and data not shown), differences were apparent in the amounts of luminescence from the *purK* mutant grown in adenine versus guanine (Fig. 2A) or in the wild-type strain versus the *guaB* mutant (Fig. 2B), consistent with previous measurements of ATP concentrations from these strains by extraction-based methods (1, 2). Thus, in qualitative terms, the luciferase bioassay successfully reported the expected differences in ATP concentrations. However, at the same polymyxin B concentration, the time of peak luminescence after polymyxin B addition differed for the *purK* strain grown in adenine versus guanine (compare the peak times for cells treated with 8 × 10⁴ units/ml polymyxin B...
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Fig. 3. Kinetics of luminescence recorded from exponentially growing cultures in MOPS medium (see Fig. 1 legend) supplemented with 0.4% glucose and all 20 amino acids (80 μg/ml) (A) or MOPS medium supplemented with 0.4% succinate (B). Data are from cultures treated with different concentrations of polymyxin B (1, 2, 4, 8 refer to 1, 2, 4, and 8 × 10^4 units/ml, respectively) from two independent cultures. C and D, polymyxin B dose-response curves resulting from the data in panels A and B, respectively. E, relative ATP concentrations plotted as a function of growth rate. Cultures (RLG6238) were grown in MOPS minimal medium containing 0.4% carbon source, 10 μg/ml thiamine, and 100 μg/ml ampicillin. Glycerol, galactose, succinate, or glucose were used as carbon sources. These media were also supplemented with 20 amino acids (80 μg/ml), generating 8 different media in all. Relative ATP concentrations were determined using the luciferase bioassay, extraction with formic acid, or formaldehyde treatment followed by extraction with alkali, as indicated. Each point is the average of two measurements from two independent cultures, with error indicated. Circled points in panel E are from the growth media used in Fig. 1 and Ref. 1.

in Fig. 2A) or for the wild-type versus guaB strains in the same medium (compare the peak times for either 4 × 10^4 or 8 × 10^4 units/ml polymyxin B in Fig. 2B). Because this indicates that the cells being compared did not have identical membrane properties, potentially this could introduce bias into the amounts of ATP measured by the luciferase bioassay. To make the comparison of amounts of luminescence between cultures more quantitative, we used a range of concentrations of polymyxin B to generate permeabilizing conditions that resulted in the same luminescence peak time.

Luciferase activity measurements from cultures treated with different polymyxin B concentrations generated plots of integrated luminescence versus peak time that were linear. As a result, simple interpolation allowed identification of amounts of luminescence corresponding to the same peak time (Fig. 2, C and D; see also Fig. 3, C and D). Although in the test cases described in Fig. 2 qualitative differences in amounts of luminescence between cultures were apparent even without this correction method, the correction was employed to make comparisons more quantitative whenever luminescence peak times differed between cultures. This correction for differences in peak time was crucial for comparison of ATP levels from cells growing at different steady-state growth rates (see below and Fig. 3).

The measurements of relative ATP concentration resulting from the luciferase bioassay were compared with measurements using extraction-based methods on the same cultures. The ATP concentration was higher in the purK strain grown in adenine relative to the same strain grown in guanine when measured by formic acid extraction, by formaldehyde/alkali extraction, and by the luciferase bioassay (Fig. 2E). Likewise, the ATP concentration was higher in the guaB mutant versus the wild-type strain when measured by the formic acid and formaldehyde/alkali extraction methods and by the luciferase bioassay (Fig. 2F). The fold increase in ATP concentration observed by luminescence was more similar to that observed by formic acid extraction than by formaldehyde/alkali extraction, but all three methods of detection agreed qualitatively, consist-
ent with the hypothesis that H245F luciferase can detect changes in the concentration of ATP in vivo. The wild-type luciferase showed only a very small change in luminescence under the same conditions (data not shown), consistent with its lower $K_m$ for ATP (9) and confirming its unsuitability for use as a reporter of changes in ATP concentration in vivo.

We also measured changes in ATP concentration in cells treated with 100 µg/ml of the protein synthesis inhibitor, chloramphenicol. Purine NTP pools increase when protein synthesis is inhibited by various antibiotics because translation is a major consumer of both ATP and GTP (2, 20). Again, the two extraction methods and the luciferase bioassay all detected an ~2-fold increase in ATP concentration by 10 min after the addition of chloramphenicol (Fig. 2G). The basis for the delay in detection of the increase in ATP concentration when assayed by formaldehyde/alkali extraction was not examined further. The simplest interpretation of these data is that the luciferase bioassay is responsive to changes in the available ATP concentration.

**ATP Concentration as a Function of Growth Rate**—Having shown that the luciferase bioassay can detect changes in ATP concentration in vivo, we used this assay to measure available ATP concentrations in wild-type cells growing at different steady-state growth rates (generated by varying the carbon source and/or by addition of amino acids). Results from cells grown in two representative media of the eight used to vary the growth rate are presented in Fig. 3, A and B. Cells grown in different media exhibited differential sensitivity to polymyxin B, resulting in a requirement for different concentrations of the drug to produce peaks of luminescence occurring at the same time. The peak time correction method described above was used to make direct comparisons of luciferase activities from cells grown in different media (illustrated in Fig. 3, C and D for the same two media shown in panels A and B). Results from all eight media are plotted in Fig. 3E. The luciferase bioassay indicated that the ATP concentration changed little with growth rate, similar to the conclusion reached from formic acid extraction.

Interestingly, the trend of increasing ATP concentration with growth rate, apparent by formaldehyde/alkali extraction from the partial set of media used in Fig. 1 and in our previous work (1) (Fig. 3E, circled points), was not apparent in the complete media set and was not observed by formic acid extraction or with the luciferase bioassay. We conclude that the available concentration of ATP does not vary appreciably as a function of steady-state growth rate in wild-type E. coli. Furthermore, we conclude that the formaldehyde/alkali procedure is not suitable for comparing ATP extracts obtained from cultures grown in different media (see “Discussion”).

**ATP Concentration Does Not Change with Growth Rate in a ΔrelA ΔspoT Strain**—It was reported previously that the concentration of ppGpp, a negative regulator of rRNA promoters, is inversely proportional to growth rate (21), consistent with the hypothesis that in wild-type strains changing concentrations of ppGpp account for growth rate–dependent control of rRNA promoters. However, rrr P1 promoter activities increase with growth rate even in strains lacking ppGpp (ΔrelA ΔspoT, Refs. 22–24), indicating that ppGpp either is not the regulator responsible for growth rate–dependent control of rRNA synthesis or is not the only potential regulator. In theory, ATP concentrations could change with growth rate in ΔrelA ΔspoT strains, thus accounting for growth rate–dependency of $k_{cat}$ B1 transcription in the absence of ppGpp even though ATP concentration did not change with growth rate in wild-type cells. (In fact, a situation where the ΔrelA ΔspoT mutations led to altered ATP concentrations, which compensated for the loss of the effect of ppGpp on rRNA transcription, was documented recently (25)). Complex media are required to generate a range of growth rates with ΔrelA ΔspoT strains because these mutants cannot grow in media lacking amino acids (10). The formic acid extraction method was unsuitable for detection of ATP in these complex media because components of the media interfered with radiolabeling and co-eluted with ATP in high pressure liquid chromatography.

The development of the luciferase bioassay, which is suitable for use in both rich and defined media, allowed measurement of ATP concentrations at different growth rates in ΔrelA ΔspoT mutants. Fig. 4 demonstrates that, as in wild-type cells, there is no apparent change in ATP concentration in ΔrelA ΔspoT strains as a function of growth rate. Thus, a regulatory system capable of accounting for growth rate–dependent control of rRNA expression in ΔrelA ΔspoT strains (and perhaps in wild-type strains) remains to be identified.

**DISCUSSION**

**Luciferase as a Reporter of ATP Concentration**—Previous attempts to use firefly luciferase as a reporter of ATP concentration in vivo were compromised by saturation of the enzyme with endogenous levels of ATP (18) and by the long polymyxin B treatment times required for facilitating luciferin influx (17). We used a variant of luciferase with a higher $k_{cat}$ (especially the H245F variant; Ref. 9) and variation in luciferase expression over a 100-fold range did not affect cell physiology or growth rate, it is unlikely that the luciferase activity itself affected the ATP pool. The simplest interpretation of these data is that the luciferase bioassay detects changes in ATP concentration in vivo. We then used this bioassay to resolve an issue for which extraction methods did not agree, the relationship between ATP concentration and growth rate.

To allow influx of luciferin, cultures were treated with a high dose of polymyxin B, which disrupts bacterial membranes. Recording of luminescence takes place simultaneously with

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*D. A. Schneider and R. L. Gourse, unpublished results.*

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| Growth Rate (doublings/hour) | Relative [ATP] |
|-----------------------------|----------------|
| 0.8                         | 1.0            |
| 1.2                         | 1.5            |
| 1.6                         | 2.0            |

![Fig. 4. Relative ATP plotted as a function of growth rate, measured using the luciferase bioassay: wild-type (RLG6238) (A), ΔrelA ΔspoT (freshly transduced into RLG6238) (B). Cultures were grown in MOPS medium (see Fig. 1) supplemented with 0.4% glucose (wild-type only), 0.4% glucose and 20 amino acids (80 µg/ml) Luria-Bertani broth, and brain-heart infusion broth. The ΔrelA ΔspoT strain consistently grew more slowly than the wild-type strain in the defined medium but not in the complex media. Each point is the average of four measurements from two independent cultures with error indicated.](image-url)
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drug addition (time 0), and measurements are complete within 2 min (in most cases much less), virtually eliminating the time interval between time of membrane disturbance and recording of the ATP concentration. It should be noted that disruption of the cell membrane (and therefore potentially the ATP gradient) occurs in even the most rapid of extraction methods.

Luciferase uses three substrates to generate light: ATP, luciferin, and oxygen. Variation in the concentrations of any of these substrates could affect luminescence. Our method normalized for potential differences in luciferin influx (see above), and we reduced the potential for variation in oxygen levels by growing cultures with vigorous aeration to equal, low optical densities at all growth rates. Furthermore, we observed no change in luciferase activity when oxygen levels were increased artificially (by sparging the culture in the luminometer cell with oxygen during measurements), suggesting that oxygen levels were not limiting for luminescence. Therefore, under the conditions assayed, we conclude that luciferase activity varied primarily in response to changes in the concentration of only one substrate, ATP.

When luminescence peak times do not vary during the short time courses required in many non-steady-state experiments (e.g. see Fig. 2G), comparisons of luciferase activities can be straightforward without the need for correction for changes in membrane characteristics that would affect luciferin uptake and/or ATP efflux. However, drug dose response curves would be required at each time point in non-steady state experiments if the peak times changed during the time course.

Problems with the Formaldehyde/Alkali Extraction Method—During our development of the luciferase bioassay and comparison with existing methods for detection of ATP, we discovered that the efficiency of formaldehyde treatment followed by alkali extraction of ATP varied with the identity of the growth medium (Fig. 3). These results do not support the hypothesis (2) that the formaldehyde/alkali method might liberate only the ATP pool available for interaction with RNA polymerase (i.e. those molecules not bound to macromolecules) in contrast to the formic acid method that should liberate both bound and available ATP. Because we have not been able to identify the variable in certain media responsible for sequestering or degrading part of the ATP pool during formaldehyde/alkali extraction, it is clear that caution should be used when interpreting ATP concentration measurements obtained using this method.

Available ATP and Total ATP Concentrations—The luciferase bioassay and formic acid extraction methods agreed qualitatively in all situations where both could be utilized. However, we suggest that the size of the ATP pool available as a substrate or regulator is the parameter of interest for regulatory processes rather than the size of the total pool of ATP, the sum of all the ATP molecules in the cell independent of their association with macromolecules. Because luciferase is likely to be subject to the same competition for ATP-binding as other proteins in vivo, it likely is a better reporter of this available ATP pool. However, as shown here, the total ATP pool (as measured by the formic acid extraction method) in many cases parallels the available ATP pool.

The ATP concentration in cells has been estimated at ~3 μM (26). The reported K_m of the H245F luciferase for ATP is 830 μM in vitro (9). We demonstrated that this enzyme is not saturated for ATP in vivo; therefore, the available ATP concentration is either less than 3 μM or cytoplasmic conditions increase the apparent K_m of H245F luciferase for ATP. If the available ATP concentration is significantly lower than the total ATP concentration, the partitioning of the ATP pool by tight interactions with cellular proteins or other macromolecules is apparently constant with growth rate because the results obtained by the formic acid and luciferase assays varied in parallel.

Implications for Control of rRNA Expression—rRNA promoters require higher concentrations of purine NTPs than most other promoters for initiation of transcription because they form unusually short-lived open complexes with RNA polymerase (1, 2, 24). Previously, our laboratory concluded that this “NTP-sensing” by rRNA promoters accounted, at least in part, for changes in rRNA expression with cellular growth rate. We demonstrate here that the measurements of ATP concentration reported in these steady-state growth rate experiments were in error as a result of an extraction procedure not suited for use with different media.

From measurements using the luciferase bioassay, we conclude that ATP concentration changes little, if at all, with growth rate; therefore, NTP sensing is most likely not responsible for growth rate-dependent control of rRNA expression. However, concentrations of NTPs do change under several other physiologically important conditions. For example, NTP concentrations change dramatically during outgrowth of cells from stationary phase and during entry into and maintenance of stationary phase and as a result directly regulate rpn P1 promoter activity (4). Furthermore, transient variation in NTP concentration likely occurs in individual cells as a result of stochastic oscillations in NTP production and consumption, but this variation is undetectable when examining large populations of cells. Because even relatively small variations in cellular purine NTP concentrations can affect rpn P1 promoter activity (3), NTP sensing by rRNA promoters may constantly fine-tune rRNA expression to the demand for protein synthesis even during steady-state growth.

Because ATP concentrations do not change with growth rate in cells unable to make ppGpp (Fig. 4, ΔrelA ΔspoT) and rpn P1 core promoters (lacking binding sites for all other known regulators) continue to display growth rate-dependent regulation under these conditions, we conclude that at least one more regulator of rpn P1 core promoter activity (in addition to changing concentrations of iNTPs and ppGpp) remains to be identified.

Concluding Remarks—The luciferase bioassay reported here allowed resolution of a long-standing question, the relationship between ATP concentration and growth rate in E. coli. In addition, we demonstrated that the luciferase bioassay can be utilized under conditions where it is not feasible to employ extraction-based methods. Our luciferase bioassay overcame the technical problems that compromised previous attempts to use luciferase as a reporter of ATP concentration in growing bacteria. We suggest that our assay reports the ATP concentration available to most enzymes in growing cells, whereas formic acid extraction reports the total pool. Although our results demonstrate that there is a strong correlation between the relative ATP concentrations measured by the luciferase bioassay and those measured by formic acid extraction, there may be cases where results obtained by these two methods would not be the same.

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REFERENCES
1. Gaal, T., Bartlett, M. S., Ross, W., Turnbough, C. L., Jr., and Gourse, R. L. (1997) Science 278, 2092–2097.
2. Schneider, D. A., Gaal, T., and Gourse, R. L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8602–8607.
3. Schneider, D. A., and Gourse, R. L. (2003) J. Bacteriol. 185, 6185–6191.
4. Murray, H. D., Schneider, D. A., and Gourse, R. L. (2003) Mol. Cell 12, 125–134.
5. Schaechter, M., Maaloe, O., and Kjeldgaard, N. O. (1958) J. Gen. Microbiol. 19,
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6. Petersen, C., and Moller, L. B. (2000) *J. Biol. Chem.* **275**, 3931–3935
7. Bagnara, A. S., and Finch, L. R. (1973) *Eur. J. Biochem.* **36**, 422–427
8. Franzen, J. S., and Binkley, S. B. (1961) *J. Biol. Chem.* **236**, 515–519
9. Branchini, B. R., Magyar, R. A., Murtashaw, M. H., Anderson, S. M., Helgerson, L. C., and Zimmer, M. (1999) *Biochemistry* **38**, 13223–13230
10. Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M. (1999) *Biochemistry* **38**, 13223–13230
11. Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp. 201–205, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
12. Jensen, K. F., Houlberg, U., and Nygaard, P. (1979) *Anal. Biochem.* **98**, 254–263
13. Little, R., and Bremer, H. (1982) *Anal. Biochem.* **126**, 381–388
14. Gruber, M. G., and Wood, K. V. (1993) in *Bioluminescence and Chemiluminescence: Status Report* (Staley, A. A., Kricka, L. J., and Stanley, P., eds) pp. 212–216, John Wiley & Sons, Chichester, UK
15. Bowers, K. C., Allshire, A. P., and Cobbold, P. H. (1992) *J. Mol. Cell Cardiol.* **24**, 213–218
16. Koop, A., and Cobbold, P. H. (1993) *Biochem. J.* **295**, 165–170
17. Dement’eva, E. I., Ugarova, N. N., and Cobbold, P. H. (1996) *Biochim Biophys Acta* **1302**, 1285–1293
18. De Tomaso, G., Burghese, R., and Zannoni, D. (2001) *Arch. Microbiol.* **177**, 11–19
19. Jensen, K. F. (1989) *J. Gen. Microbiol.* **135**, 805–815
20. Lund, E., and Kjeldgaard, N. O. (1972) *Eur. J. Biochem.* **28**, 316–326
21. Ryals, J., Little, R., and Bremer, H. (1982) *J. Bacteriol.* **151**, 1261–1268
22. Gaal, T., and Gourse, R. L. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 5533–5537
23. Bartlett, M. S., and Gourse, R. L. (1994) *J. Bacteriol.* **176**, 5560–5564
24. Barker, M. M., Gaal, T., Josaitis, C. A., and Gourse, R. L. (2001) *J. Mol. Biol.* **305**, 673–688
25. Schneider, D. A., and Gourse, R. L. (2003) *J. Bacteriol.* **185**, 6192–6194
26. Neuhard, J., and Nygaard, P. (1987) in *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds) Vol. 1, pp. 445–473, ASM Press, Washington, D. C.
27. Neidhardt, F. C., Bloch, P. L., and Smith, D. F. (1974) *J. Bacteriol.* **119**, 736–747