The *Escherichia coli* fis Promoter Is Regulated by Changes in the Levels of Its Transcription Initiation Nucleotide CTP*

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Expression of the *Escherichia coli* nucleoid-associated protein Fis (factor for inversion stimulation) is controlled at the transcriptional level in accordance with the nutritional availability. It is highly expressed during early logarithmic growth phase in cells growing in rich medium but poorly expressed in late logarithmic and stationary phase. However, fis mRNA expression is prolonged at high levels throughout the logarithmic and early stationary phase when the preferred transcription initiation site (+1C) is replaced with A or G, indicating that initiation with CTP is a required component of the regulation pattern. We show that RNA polymerase-fis promoter complexes are short lived and that transcription is stimulated over 20-fold from linear or supercoiled DNA if CTP is present during formation of initiation complexes, which serves to stabilize these complexes. Use of fis promoter fusions to *lacZ* indicated that fis promoter transcription is sensitive to the intracellular pool of the predominant initiating NTP. Growth conditions resulting in increases in CTP pools also result in corresponding increases in fis mRNA levels. Measurements of NTP pools performed throughout the growth of the bacterial culture in rich medium revealed a dramatic increase in all four NTP levels during the transition from stationary to logarithmic growth phase, followed by reproducible oscillations in their levels during logarithmic growth, which later decrease during the transition from logarithmic to stationary phase. In particular, CTP pools fluctuate in a manner consistent with a role in regulating fis expression. These observations support a model whereby fis expression is subject to regulation by the availability of its initiating NTP.

Diverse cellular functions have been ascribed to the *Escherichia coli* Fis (factor for inversion stimulation). In addition to mediating specialized site-specific DNA recombination events (1), this nucleoid-associated protein regulates transcription of ribosomal and tRNAs (2, 3) and a growing number of structural genes (4–16). Fis levels are transcriptionally regulated according to the nutritional conditions and the growth phase (17–19). A dramatic burst in fis mRNA levels is observed when stationary phase cells are outgrown in rich medium, which reach a peak during early logarithmic growth phase, decrease to much lower levels during late logarithmic growth phase, and become undetectable during stationary phase (17, 18), an expression pattern that is closely followed at the protein level (17, 19). The fis mRNA half-life is short (about 2 min) and does not vary appreciably throughout the period of growth phase-dependent expression, indicating that the fis mRNA expression pattern is not attributed to changes in mRNA turnover rates but is primarily attributed to transcriptional control (20). Such drastic changes in intracellular Fis levels are likely to influence its role as a global gene regulator. Prolonged Fis expression well into stationary phase is detrimental to cell viability (21). Hence, cells must maintain the capacity to carefully regulate fis expression in order to transiently benefit from its advantageous contributions under conditions favoring rapid cell growth but then seek protection from its harmful effects during stationary phase or starvation by ensuring timely Fis depletion. The regulation of fis mRNA expression represents an important step in controlling the intracellular Fis levels. Its importance is emphasized by the strict conservation of this expression pattern in other bacterial species (21–23).

A single promoter (fis P)1 is responsible for the transcription and regulation of the fis operon in *E. coli* (17, 18, 23). It is negatively regulated by Fis (17, 18) and by stringent control (18, 23, 24) and stimulated by integration host factor (20). Nevertheless, growth phase-dependent regulation (GPDR)1 continues to be observed at the mRNA level in the absence of Fis, integration host factor, or stringent control (17, 21, 24). Both the GPDR and the response to stringent control only require the minimal promoter sequence from about −38 to +5 (18, 20), suggesting that the precise interactions between RNA polymerase (RNAP) and the minimal promoter DNA sequence plays a determining role in these processes. Mutations within this minimal promoter region were obtained that altered the GPDR but not its response to stringent control, and *vice versa* (24), strongly suggesting that the two processes are controlled by nonredundant mechanisms involving distinct regulatory signals.

Most *E. coli* σ70 promoters initiate transcription with purines (25, 26). However, fis P is unusual in its preferential use of CTP to initiate transcription, which occurs from a site located 8 nucleotides downstream of the −10 promoter region (23, 24, 27). Replacement of +1C with either A or G resulted in prolonged high level expression throughout logarithmic growth phase and early stationary phase (24), indicating that the tight GPDR pattern of fis is strongly linked to a predominant use of CTP at +1C as the transcription initiation nucleotide.

In this work, we present a general characterization of fis

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1 The abbreviations used are: fis P, the fis promoter; GPDR, growth phase-dependent regulation; MOPS, morpholinepropanesulfonic acid; KGlu, potassium glutamate; RNAP, RNA polymerase; ppGpp, guanosine tetraphosphate; nt, nucleotide(s).
transcription in vitro showing that transcription initiation complexes at fis P are short lived at physiologically relevant salt concentrations and that formation of stable transcription initiation complexes requires high concentrations of CTP, its primary initiating NTP. We provide evidence in support of the notion that fis expression is sensitive to the intracellular pools of the initiating NTP. We measured cellular levels of all four NTPs during stationary phase and throughout the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium. We find that CTP pools fluctuate during the logarithmic growth phase and show the entire pattern of expression of NTPs during stationary phase and throughout the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium. We find that CTP pools fluctuate during the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium. We find that CTP pools fluctuate during the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium. We find that CTP pools fluctuate during the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium. We find that CTP pools fluctuate during the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium. We find that CTP pools fluctuate during the logarithmic growth phase and show the entire pattern of expression of these nucleotides when cells are grown in rich medium.

**EXPERIMENTAL PROCEDURES**

**Chemicals, Enzymes, and Growth Media**—Chemicals, enzymes, and growth media were as described (23). Polyethyleneimine-cellulose plates were from Aldrich. o70-saturated E. coli RNAP holoenzyme (EcO70) was from EPICENTRE. Nucleotides and radioisotopes were N,N,N′-trimethyl-N′-t-butyl-urea (TMBU) was from Pierce Chemical Company. DPNH-Tris buffer was from SEIKO. Bacterial strains and plasmids used in this work are described in Table I. The lysogenic strains RO1087 and RO1089 were constructed as described (34). pRTP43 was created by site-directed mutagenesis as described previously for other similarly constructed plasmids (24).

**Primer Extension**—Primer extension reactions were performed as described previously (24, 27) using 10 μg of total cellular RNA and 2 pmol of 32P-end-labeled DNA primers (28). The primers were 5'GATCCACATATGCATCTACTGTTCC) which anneal to the fis P region from -105 to +8 relative to the fis P start site cloned into the EcoRI and BamHI sites of pKK223–3 (Amersham Biosciences), replacing the tac promoter and placing the fis P start site about 340 bp upstream of T1 of the rnb RNA T1, T2 Rho-independent transcription terminator region; AmpR.

**Northern Blots**—Northern blots were performed as described (28) using a 50% formamide hybridization solution. The E. coli fis gene was labeled with [32P]dATP by extension of random primers (28) and used as probe. The fis mRNA signals were detected by autoradiography and quantified using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics, Inc.).

**In Vitro Transcription**—Single-round transcription reactions were performed with either linear or supercoiled templates as described (23), with modifications in salt concentrations as described for each experiment. Supercoiled plasmid DNA (pRTP362) was purified by two passages through a CsCl equilibrium density gradient centrifugation (28). Linear DNA was either pRTP362 linearized with PvuII or a 283-bp fragment containing the fis P region from -168 to +83 flanked by 12 and 20 bp of vector DNA sequence upstream and downstream of this region, respectively. Typically, a 5-fold molar excess of RNA polymerase was incubated with the DNA template in transcription buffer (30 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 1 mM EDTA, 0.1 mM dithiothreitol, and 100 μg/ml bovine serum albumin) and the indicated concentration of KCl or potassium glutamate (KGlu) at 37 °C for 10 min to allow maximum formation of open complexes. Subsequently, 10 μg/ml heparin, 80 μM ATP, 80 μM CTP, 80 μM GTP, 4 μM UTP, and 2.5 μCi of [α-32P]UTP (3,000 Ci/mmol) were added to the mixture and incubated for another 30 min at 37 °C to allow transcript synthesis. When high concentrations of UTP were present in the initial binding mixture, the nucleotide concentrations in the extension mixture were 80 μM CTP, 80 μM GTP, 80 μM UTP, and 2.5 μCi of [α-32P]UTP. For half-life determinations of RNAP-fis P complexes, experiments were similarly conducted, except that the NTP extension mixtures were added at time intervals after heparin addition. The heparin concentration of 10 μg/ml was experimentally tested to be sufficient to completely challenge free RNA polymerase under all conditions used. A 10-fold higher heparin concentration showed no further reduction in either the amount or the half-life of transcription initiation complexes formed at fis P in either

| Strains and plasmids | Description | Source |
|----------------------|-------------|--------|
| Strains | CLT42 | MC4100 araD+ car-94 | Ref. 68 |
| | RO1003 | CSH26  Z(tac pro) ara str' thi' | R. C. Johnson |
| | RO1561 | F- ara139 argF-lac U169 rpsL150 thiA1 relA1 deoC1 ptsF25 ribB530 rbsR | Ref. 69 |
| | RO1617 | MC1000 fis-787 | R. C. Johnson |
| | RO1150 | RJ1561 pTP127 | R. C. Johnson |
| | RO238 | RJ1561 pCA282 | R. C. Johnson |
| | RO790 | RJ1561 pCA324 | R. C. Johnson |
| | RO989 | MC4100 fis-787 | R. C. Johnson |
| | RO981 | CLT42 fis-787 | R. C. Johnson |
| | RO908 | RO890 pTP127 | Ref. 17 |
| | RO910 | RO891 pTP127 | Ref. 20 |
| | RO1087 | RO891 A Rs45 [wild type fis P (-108 to +37):lacZ] lysogen | Ref. 24 |
| | RO1089 | RO891 A Rs45 [-2G→-F fis P (-108 to +37):lacZ] lysogen | Ref. 24 |
| | RO1150 | RJ1561 pTP127 | Ref. 23 |
| Plasmids | pRJ800 | pBR322-derived; pUC18 polylinker region precedes a trp-lac [lac pro] | Ref. 70 |
| | pTP127 | E. coli fis P region from -108 to +105 relative to fis P start site inserted within the KpnI and XbaI sites of pRJ800 | Ref. 70 |
| | pCA232 | Similar to pTP127 but containing the single mutation +1C→A | Ref. 70 |
| | pCA324 | Similar to pTP127 but containing the single mutation +1C→G | Ref. 70 |
| | pTP434 | Similar to pTP127 but containing the single mutation +1C→T | Ref. 70 |
| | pRO362 | E. coli fis P region from -168 to +83 relative to the fis P start site cloned into the EcoRI and BamHI sites of pKK223–3 (Amersham Biosciences), replacing the tac promoter and placing the fis P start site about 340 bp upstream of T1 of the rnb RNA T1, T2 Rho-independent transcription terminator region; AmpR |

* When a source is not indicated, the strain or plasmid was constructed during the course of this work.
linear or supercoiled DNA templates. Multiple-round transcription reactions were performed by incubating 0.5 pmol of RNAP with 0.01 pmol of supercoiled pR062 at 22 °C in transcription buffer containing 100 mM Tris-HCl (pH 8.0), 60 µM GTP, 80 µM UTP, 4 µM ATP, 2.5 mM of [32P]ATP (3,000 Ci/mmol), and either 80 µM or 2 mM CTP. The electrophotochemically separated products were subjected both to autoradiography and quantification by phosporimaging.

α-Galactosidase Assays—Saturated cultures of RO1087 and RO1089 were diluted 75-fold in supplemented C medium containing either 1 mM uracil or 0.25 mM UMP, grown at 37 °C with shaking to an A660 of 0.3, and analyzed for α-galactosidase activity as described (36). Two-dimensional Thin Layer Chromatography—Saturated cultures of the desired strains were diluted into fresh medium, from which 4 ml was transferred to a fresh flask and made 100 µCi/ml [32P]orthophosphate (167 µCi/µmol specific activity). Nucleotides were obtained using the formic acid method of extraction as described (31). Polyethyleneimine-cellulose plates were prewashed as described (37), and two-dimensional TLC was performed as described (38). Guided by the chromatographic behavior of the unlabeled GTP, ATP, CTP, and UTP, we identified the corresponding [32P]-labeled NTPs on the TLC. Spots were quantified by phosporimaging, and the molar quantities of NTP were determined by comparison of individual spot intensities with that of an average of three control spots, each containing 1 µl of the [32P]-labeled growth medium (0.6 nmol of phosphate). Nearly all of the phosphate incorporated was detected only at the NTP γ-position, since we did not detect [32P]-labeled NMPs or NDPs in our TLC plates within the limits of the exposure times used. By combining the information from the relative spot intensities, specific activity of [32P], cfu/ml obtained at each time during growth at which cells were harvested, and the volume of the cell extract spotted onto the TLC plates, we expressed the relative NTP levels as pmol of NTP/10^7 cells.

RESULTS

As shown previously (17, 23, 24, 27), the fis promoter initiates transcription predominantly with CTP from +1C and much less efficiently with GTP from −2G (Fig. 1A). When the fis P +1C was replaced with either A or G, the preference for initiation from this position increased and resulted in a noticeably prolonged high level expression throughout logarithmic growth and into early stationary phase compared with the wild type promoter (24, 27) (Fig. 1B). Replacement of +1C with T resulted in some degree of reiterative transcription (not shown), most likely due to the three consecutive T nucleotides positioned in the region from +1 to +3 that could result in multiple UTP incorporation at the 5’-end (39). Nevertheless, its overall expression pattern more closely resembled that of the wild type promoter than those of either the +1C→A or +1C→G promoters (Fig. 1B). In all cases, transcription increased from undetectable levels in stationary phase to its maximum levels after about 30–60 min, but, in the cases of +1C and +1T, fis mRNA levels dropped to a minimum after about 120 min of growth in these cultures and then exhibited a small peak by about 150 min of growth before dropping again to very low levels during late logarithmic and early stationary phase. Thus, the simple switch from predominant transcription initiation with pyrimidine to purine caused a substantial change in the overall fis mRNA pattern of expression during cell growth. We hypothesized that fis P transcription initiation responds to the availability of its initiating NTP and that use of a very poor choice of initiation NTP, as is CTP (26, 40–42), exacerbates its dependence on this nucleotide to give a narrow GPD pattern in vivo.

Transcription Initiation Complexes at fis P—To begin addressing this hypothesis, we performed in vitro transcription with a 283-bp DNA fragment carrying the fis P region from −168 to +83, such that transcription from fis P was expected to generate a 103-nucleotide (nt) transcript. We found that this transcript could not be detected if 100 mM KCl or K glu was present in the reaction. However, at lower concentrations (e.g., <30 mM K glu), a 103-nt transcript could be detected (Fig. 2A). Confirmation that both the directionality and size of the transcript were of those expected for fis P was obtained from use of a fragment containing additional downstream sequence, which resulted in a correspondingly larger transcript (not shown).

Effect of CTP on fis P Transcription—We relied on in vitro transcription assays to determine if transcription initiation from the fis promoter could be enhanced by the presence of high concentrations of its initiating NTP. RNAp was allowed to bind a 283-bp DNA fragment containing the fis P region from −168 to +83 in buffer containing 25 mM K glu and various concentrations of one of the four NTPs. Subsequently, heparin and a mixture of all four NTPs were added to allow run-off transcription from preformed initiation complexes. We reproducibly observed that fis P transcription increased with increasing CTP concentrations (Fig. 3A). As much as a 20-fold increase in fis P transcripts could be measured in these experiments (Fig. 3C). When the same reactions were performed with increasing con-
centrations of ATP, GTP, or UTP, no appreciable effects were observed (Fig. 3, B and C), demonstrating that the stimulatory effect was specific to CTP. No increase in transcription was observed if high concentrations of CTP were added after the addition of heparin (Fig. 3A, lanes 12 and 13), suggesting that the stimulatory effect occurs during formation of initiation complexes and not during elongation. In fact, a modest decrease in the transcript signal is observed when high concentration of CTP (Fig. 3A, lanes 12 and 13) or any other nucleotide (Fig. 3C) is added to the reaction after the addition of heparin, suggesting that such high concentrations of any NTP cause a slight inhibition of the extension reaction in our in vitro conditions and that the 20-fold stimulatory effect by CTP may actually represent an underestimation. We conclude that CTP plays a distinctive role in facilitating the formation of productive initiation complexes at fisP.

Effect of CTP on the Stability of fisP Initiation Complexes—The previous transcription reactions were performed using only 25 mM KGlu in order to permit detection of fisP transcripts in the absence of CTP enhancement and allow an estimation of a -fold activation effect by CTP. However, we observed that a stimulatory effect by 2 mM CTP was also sustained at salt concentrations in the range from 75 to 150 mM KGlu (Fig. 4A), wherein little or no heparin-resistant fisP transcription product could be detected in the absence of CTP (Fig. 2). Thus, the presence of high concentrations of CTP results in a greater -fold stimulation (which cannot be estimated) at these higher salt concentrations than that measured in the presence of only 25 mM KGlu. This also suggested that the presence of high concentrations of CTP might increase the stability of the initiation complexes at fisP. Therefore, we examined the effect of CTP on the half-life of RNAP-fisP complexes formed on the 283-bp DNA fragment. In the presence of 100 mM KGlu, RNAP-fisP complexes dissociated too quickly to be detected within 15 s of heparin addition. However, the presence of 2 mM CTP during the initial binding reaction resulted in a half-life of about 40 min (Fig. 4B). If the salt concentration is sufficiently lowered so as to accommodate a modest increase in promoter stability (e.g., 25 mM KGlu), a 2-min half-life could be detected in the absence of CTP, whereas the presence of 2 mM CTP increased the half-life to about 4 h (not shown), indicating that the stabilizing effect of CTP is substantial. None of the other three NTPs affected the stability of open complexes (not shown). These results demonstrate that high concentrations of CTP stimulate fisP transcription by substantially increasing the stability of otherwise short-lived initiation complexes.

Effect of DNA Supercoiling—It was previously reported that DNA supercoiling increases the transcription from the fis promoter (43), an effect that we have also observed (Fig. 5A). However, neither the molecular basis for this effect nor its impact on the CTP stimulatory effect has been investigated. We found that optimal fisP transcription activity from supercoiled DNA occurred with about 40 mM KCl or with about 150 mM KGlu (Fig. 5B). Higher salt concentrations significantly lowered the amount of transcripts produced. Thus, whereas...
CTP Levels Regulate the fis Promoter

considerable sensitivity to the salt concentration was exhibited by fis P in supercoiled DNA, its range of optimal KCl and KGl concentration was higher than that of the 283-bp DNA fragment (Fig. 2B) or of linearized pRO362 (not shown). We determined that the half-life of heparin-resistant fis P complexes in supercoiled DNA was about 3 h in the presence of 25 mM KGl, about 9 min in the presence of 150 mM KCl, and undetectably short in the presence of 175 mM KCl (not shown). Thus, whereas the stability of fis P complexes in supercoiled DNA was always greater than that of linear templates, its stability decreased sharply with increasing salt concentration and became undetectably short within physiologically relevant salt concentrations (44).

We wondered whether the effects of CTP could be observed on fis P despite the stabilizing effects contributed by DNA supercoiling or if the two effects were mutually exclusive. We observed, using various salt conditions, that the half-life of heparin-resistant fis P complexes in supercoiled pRO362 was always substantially enhanced in the presence of CTP. Most dramatically, at 175 mM KCl, where the half-life of the fis P initiation complexes in the absence of CTP was too short to allow detection, the presence of 2 mM CTP increased the half-life to about 30 min (Fig. 5C). Thus, CTP substantially extends the stability of initiation complexes at fis P beyond those effects brought about by DNA supercoiling, suggesting that the two stabilizing effects (by supercoiling and CTP) rely on different and nonredundant mechanisms.

Consistent with the stabilizing effects by CTP, single round transcription from heparin-resistant fis P complexes in supercoiled pRO362 in 175 mM KCl were about 20-fold greater in the presence of 2–4 mM CTP (Fig. 5D). None of these effects were observed when high concentrations of the other three NTPs were tested (not shown). To examine whether the increase in initiation complex stability by CTP in supercoiled templates resulted in increased rates of transcription, we performed multiple round transcription reactions in the presence of 80 μM or 2 mM CTP (Fig. 5E). The results showed that the increase in CTP concentration in these reactions caused about a 4-fold increase in the rate of fis P transcription. An increase in any of the other three NTPs did not increase the rate of transcription (not shown). We note that CTP loses its ability to stimulate transcription at salt concentrations closer to those expected in vivo when the DNA is relaxed (e.g. Fig. 4A) but not when it is supercoiled (Fig. 5, D and E). This suggests that DNA supercoiling is necessary to bring about a sufficient level of initiation complex stability to allow substantial CTP stimulatory effects to be observed within physiologically relevant salt concentrations.

Effect of Initiating NTP on fis Transcription In Vivo—To examine the possibility that intracellular levels of NTPs could affect fis P transcription in vivo, we initially made use of an E. coli car fis strain that would allow us to manipulate cellular NTP pools and evaluate the effects on fis P expression without the potential complexities presented by Fis repression. This pyrimidine auxotroph grows normally in the presence of uracil, but, when grown with UMP instead of uracil, pyrimidine levels are said to become limiting, whereas purine levels increase, and the growth rate slows slightly (45). Saturated cultures of RO891 were subcultured in supplemented MOPS containing [32P]orthophosphate (to label NTPs) and either UMP or uracil. Samples were taken at an $A_{600}$ of 0.3, extracted with formic acid, and separated by two-dimensional TLC. We confirmed that growth of this strain with UMP caused about a 2-fold decrease in UTP levels, a 3-fold increase in ATP, and nearly a 2-fold increase in GTP compared with growth with uracil (Fig. 6, A and B). However, the CTP levels did not decrease but rather increased slightly. Results from β-galactosidase assays showed that transcription from fis P in RO1087 was about 2.3-fold higher in cells cultured in UMP than in uracil (Fig. 6C),

FIG. 3. Effect of NTPs on open complex formation. A, effect of CTP on fis P transcription. Run-off transcription reactions were performed with a 283-bp fragment, 25 mM KGl, and the indicated concentrations of CTP. The lane numbers are indicated below the gel. In lanes 1–11, RNAP binding was performed in the presence of the indicated concentrations of CTP prior to the addition of heparin and all four NTPs. In lanes 12 and 13, RNAP binding was performed in the absence of NTPs, and then 50 or 1000 μM CTP was added with the heparin and NTP extension mixture. Nucleotide lengths of denatured DNA concentrations were averaged and plotted relative to the signal intensity in the absence of NTPs, and then 50 or 1000 μM CTP was added with the heparin and NTP extension mixture. Nucleotide lengths of denatured DNA concentrations were averaged and plotted relative to the signal intensity in the absence of NTPs, which was assigned a value of 1. The vertical bars represent S.D. values.

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although the growth rate was slower in UMP ($\mu = 1.0$ for UMP and 0.88 for uracil). The latter is significant given the possibility that Fis levels may be subject to growth rate control (17). Results from primer extension assays revealed that the pre-

| KGlu (mM) | 25 | 50 | 75 | 100 | 125 | 150 |
|-----------|----|----|----|-----|-----|-----|
| fis P     |    |    |    |     |     |     |

FIG. 4. Effect of CTP on the stability of initiation complexes at fis P. A, effect of CTP on the fis P salt sensitivity. Reactions were as in Fig. 3 but were performed in the presence of 2 mM CTP and the indicated concentrations of KGlu. The arrow points to the fis P transcript. B, effect of CTP on the stability of fis P initiation complexes. Kinetics of dissociation of RNAS-fis P heparin-resistant complexes on a 283-bp DNA fragment were performed in the presence of 100 mM KGlu and 2 mM CTP. Results are an average of triplicate reactions, with vertical bars representing S.D. values. In the absence of CTP, dissociation rates were too fast to be detectable within the first 15 s of heparin addition.

Levels were very low in the absence of cytidine but were about 5.6-fold higher in the presence of cytidine (lanes 9–12). In a separate experiment where KP1725 was grown in supplemented glucose-Tris medium, a 4-fold increase in fis mRNA levels was observed during logarithmic growth in the presence of cytidine compared with its absence (Fig. 7C, lanes 13 and 14). Cytidine in this medium resulted in over a 10-fold increase in CTP levels (not shown). Thus, elevated levels of fis mRNA correlated with elevated levels of CTP in these experiments.

Changes in Intracellular NTP Pools during Growth—If the GPDR of fis were influenced by the intracellular pools of the initiating NTP, then the levels of CTP should change in response to an outgrowth from stationary phase in rich medium in a manner that could potentially steer the pattern of fis expression. To examine this possibility, we measured the relative NTP levels during growth of a cell culture in rich medium. We reproducibly observed a substantial increase in the levels of all four NTPs during outgrowth from stationary phase, with ATP levels giving the highest concentrations, followed by GTP, UTP, and CTP in that order (Fig. 5A). During this period, both ATP and GTP levels increased by over 20-fold, whereas UTP and CTP levels increased by about 15-fold. Thereafter, all four NTP levels fluctuated in a reasonably consistent pattern within a 2–3-fold range, with purines maintaining higher levels than pyrimidines throughout the logarithmic growth phase. During late logarithmic growth phase, all four NTP levels decreased until cells entered stationary phase, where substantially lower levels were measured.

fis mRNA levels were determined by Northern blot analysis from parallel cultures identically inoculated with RJ1003 in the same supplemented MOPS medium but lacking $^{32}$Porthophosphate. No difference in the growth rate was observed by the presence of $[^3P]$orthophosphate in the medium (not shown). When plotted together with the relative CTP levels, we observed that the fis mRNA levels oscillated in a pattern that roughly emulated that of the CTP levels (Fig. 5B). The rise in CTP levels measured during the period from 0 to 80 min of outgrowth from stationary phase was accompanied by a rise in fis mRNA levels. CTP levels reached a maximum by about 80 min, and undulated briefly while maintaining rela-
Actively high levels between 80 and 120 min of growth. fis mRNA levels continued to increase between 80 and 120 min of growth when CTP levels are relatively high. Thereafter, both CTP and fis mRNA levels dropped and increased together during mid-logarithmic growth (150–210 min). Finally, both CTP and fis mRNA levels decreased during late logarithmic growth phase to reach substantially lower levels as cells entered stationary phase (≥300 min).

**FIG. 5.** Effect of DNA supercoiling. A, duplicate single round transcription reactions were performed with 0.01 pmol of either linear (lanes 1 and 2) or supercoiled pRO362 (lanes 3 and 4) plasmid DNA and 0.5 pmol of RNAP in transcription buffer containing 25 mM KGlu. The arrow points to the fis P transcript. Positions of denatured DNA used as size standards are indicated on the right. B, effect of salt concentrations on fis P transcription from supercoiled templates. Transcription reactions were performed with supercoiled pRO362 DNA as in A using various concentrations of KGlu (○) or KCl (●). Results are an average of three reactions and are shown as a percentage of the maximum value in each data set. S.D. values were within 10% of the average values. C, effect of CTP on the stability of RNAP-fis P complexes on supercoiled DNA. Reactions were as in Fig. 4B, but using supercoiled pRO362 as the DNA template in the presence of 2 mM CTP and 175 mM KCl. Results are an average of triplicate reactions, with vertical bars representing S.D. values. In the absence of CTP, dissociation rates were too fast to be detectable within the first 15 s of heparin addition. D, effect of CTP on single-round transcription from fis P in supercoiled pRO362 in the presence of 175 mM KCl and the indicated concentrations of CTP. Results are an average of triplicate experiments with S.D. values (vertical bars) and are shown relative to the amount of fis P transcripts obtained in the absence of CTP, which was assigned a value of 1.0. E, effect of CTP on multiple-round transcription from fis P in supercoiled pRO362. Reactions were performed in the absence of heparin at 22 °C and in the presence of 100 mM KCl and either 80 mM (△) or 2 mM CTP (●). Results are an average of three experiments with S.D. values (vertical bars), which were normalized to the amount of fis P transcripts obtained from heparin-resistant complexes formed in the absence of CTP.
**DISCUSSION**

The notion that alterations in the NTP pools could exert control over gene expression was primarily recognized in various nucleotide biosynthetic genes (30, 32, 45, 46, 48, 49). These studies uncovered a variety of fascinating regulatory strategies resulting from changes in intracellular NTP pools, including alterations in the rate of transcription as to control the coupling of translation and transcription attenuation (45), alterations in the predominant transcription start site position so as to regulate the synthesis mRNAs with differential capacity to control translation (30, 49, 50), and stimulation of high rates of reiterative transcription so as to control productive transcript synthesis (39, 51–53). An effect of the transcription initiation NTP concentration on the kinetics of transcription initiation complex formation was demonstrated in the ribosomal RNA promoter rrrB P1 (54). This promoter requires high concentrations of ATP (its initiating NTP) to form stable open complexes with RNA polymerase (54, 55), stimulate the rate of transcription in vitro (54, 56, 57), and increase its expression in vivo (56–59). The fis promoter also emerged as one that required high concentrations of its initiating NTPs to form heparin-stable DNAse I footprints by RNAP (17). However, this promoter relies on the use of CTP as its transcription initiation nucleotide (rather than ATP or GTP) in order to exhibit a narrowly controlled GPD. DNA supercoiling appears to contribute an appropriate amount of stability to the RNAP-fis P complexes as evidenced by a substantial increase in their half-lives (Figs. 4B and 5C) and in their tolerance to higher salt concentrations (Fig. 4A). These observations demonstrate that CTP, the predominant initiating nucleotide, plays a distinctive role in promoting transcription initiation at fis P. CTP is a poor transcription initiation NTP (40), but, when present at sufficient concentrations, it may be recruited within a ternary complex, resulting in an increase in heparin-stable complexes with a consequential increase in the rate of transcription initiation (Fig. 9).

CTP stimulates transcription of both linear and supercoiled DNA. This effect is severely diminished in linear DNA templates at salt concentrations greater than 150 mM K\text{Glu} (Fig. 4A) or greater than 100 mM KCl (not shown). However, DNA supercoiling appears to contribute an appropriate amount of stability to the RNAP-fis P complexes as so as to permit a substantial CTP effect at physiologically relevant salt concentrations. Thus, we envision that DNA supercoiling and high CTP levels are both essential for fis P to be able to overcome the sensitivity to the salt concentrations encountered in vivo and result in productive transcription initiation.

Our observations support the notion that fis P is sensitive to the pools of initiating NTP in vivo. car strains grown in UMP show an increase in the GTP/CTP ratio, a decrease in the UTP pools, a switch in fis P start site from +1C to −2G, and an increase in fis P transcription despite a reduced growth rate. The switch from +1C to −2G under these conditions is probably attributable to the combined effects of (a) an increment in the GTP/CTP ratio, which tends to favor the kinetics of initiation at −2G over +1C and (b) a decrease in UTP pools, which tends to hinder the kinetics of the first phosphodiester bond.
FIG. 7. Effect of CTP pools on fis expression in vivo. Saturated cultures of KP1725 were diluted 60-fold in supplemented MOPS medium containing uracil, with or without cytidine. Four ml of the cultures were transferred to flasks and made 100 μCi/ml [³²P]orthophosphate (for NTP extraction). Cultures were grown at 37 °C and harvested at various time points. A, effect of cytidine on NTP levels. The NTPs were separated by TLC as in Fig. 6A. All four NTPs are labeled on the top pair of TLCs, #, origin. The spots corresponding to CTP in the 90- and 390-min samples are indicated with arrows. B, relative ATP levels. Spots from the TLC results in A and two additional samples (120 and 150 min) were quantified by phosphorimaging. #, with cytidine; #, without cytidine. The numbers above each pair of bars indicate the +cytidine/+cytidine ratio of NTP levels. C, effect of cytidine on fis mRNA levels. Cultures of KP1725 were grown as in A, except that they lacked [³²P]orthophosphate. Northern blot analysis was performed with 10 μg of RNA obtained from cells grown in the absence (−) or presence (+) of cytidine for various times: 45 (lanes 1 and 2), 90 (lanes 3 and 4), 120 (lanes 5 and 6), 150 (lanes 7 and 8), and 390 min (lanes 9–12). Lanes 11 and 12 show a longer exposure of lanes 9 and 10, respectively. Lanes 13 and 14 contain RNA similarly obtained from KP1725 grown for 120 min in supplemented glucose-Tris medium in the absence (−) or presence (+) of cytidine. The fold increase in fis mRNA is indicated below each set of lanes.

formation with +1C and possibly lower the efficiency with which initiation from +1C results in productive transcripts, as has been suggested for the case of the rrnB P1 (55). A diminished effect in the −2G→T promoter supports the notion that most of the stimulation is brought about by a sensitivity to the GTP pool, which serves as the primary initiating NTP under these abnormal conditions. The small increase in transcription from this −2G→T promoter may be attributed to the small increase in CTP levels, which may result in a small increment in initiation from +1C.

Start site selection at fis P is primarily driven by the position preference relative to the −10 promoter region (such that +1, placed 8 bp downstream of the −10 region is the most highly preferred position) and secondarily by a preference for purines over pyrimidines as initiation NTPs (27). However, a shift in the balance of NTP pools, such as what occurs in the case of car strains growing in the presence of UMP, can promote the usage of a good initiating NTP (e.g. GTP) at an otherwise unfavorable position (e.g. −2). Although we did not observe an effect of high concentrations of GTP alone on the activity of fis P in vitro, we have observed that GTP in conjunction with CTP has a greater effect on fis P transcription and stability than CTP alone (data not shown), suggesting that initiation from the unfavorable −2G position may also contribute to formation of stable initiation complexes if sufficient CTP is available to support phosphodiester bond formation with GTP at −2G and −1C.

Growth of the Salmonella strain KP1725 in the presence of cytidine raises the CTP pools (but not those of UTP, GTP, or ATP), and concomitantly raises the fis mRNA levels. Generally, the largest increases in fis mRNA levels correlated with the largest increases in CTP levels, and vice versa. Since the presence of cytidine in the medium had no appreciable effect on the growth rate, potential effects attributed to growth rate control or other global effects that could potentially affect the growth rate, such as generalized changes in RNA decay rates, were deemed unlikely. We did not necessarily expect that these experiments would allow an appreciation of the full range of the CTP effect that could be observed in vitro, since it is not known how the changes in CTP concentrations in these cells relate to its Kₘ for transcription initiation in vitro or how other factors may affect the extent of this response. However, the results are in agreement with the notion that fis P transcription is able to sense and respond to the concentration of CTP.

A standing controversy regarding the reliability of methods of NTP extraction was resolved in favor of the formic acid extraction method on the basis of a newly developed luciferase-based bioassay to measure available ATP pools (60). Using the formic acid extraction method, we observed a substantial increase in all four NTPs during outgrowth of E. coli from static conditions.
Northern blots were performed with 100 μCi/ml [32P]orthophosphate (for RNA extraction). Both cultures were grown in parallel at 37 °C, and samples were withdrawn periodically. Relative CTP levels during bacterial growth. Relative CTP levels from fis expression when CTP pools are short lived, resulting in low fis expression during early logarithmic growth phase. Relative CTP levels from A are plotted relative to its maximum, which was assigned a value of 100. Northern blots were performed with 10 μg of RNA from each sample to quantify the relative fis mRNA levels. Results from three experiments were averaged and shown relative to the maximum, which was assigned a value of 100. fis mRNA levels measured in cultures of RJ1003 oscillated in a pattern that roughly emulated those of CTP, suggesting that changes in CTP levels could play an important role in guiding the synthesis of fis mRNA (Fig. 8). During the first 80 min of outgrowth from stationary phase, both CTP and fis mRNA levels steadily increased. After 80 min, CTP levels slightly undulated while maintaining relatively high levels and while fis mRNA levels continued to increase, reaching a peak by 120 min of growth. The second peak in fis mRNA had been noted previously (17, 20) (Fig. 1B) but had been largely ignored, since it was much less prominent and not always detected due to fewer samplings during this period of growth. Its timing also correlated well with a CTP peak observed during this growth stage. Several factors may act to prevent a greater fis mRNA peak during this stage of growth, such as relatively lower CTP peak levels, greater promoter competition for RNAP holoenzyme during this stage of growth, changes in DNA supercoiling, or involvement of other as yet unknown factors affecting fis expression. For instance, DksA has been recently shown to serve as a factor that is absolutely required for rRNA regulation, since its absence causes rRNA promoters to become unresponsive to growth rate and growth phase control. These effects correlated with a role of DksA in further lowering the sensitivity of fis P to CTP in vivo. The relatively poor interaction between RNAP and CTP as an initiation nucleotide compared with ATP and GTP (40),

FIG. 8. Relative NTP levels during bacterial growth. Saturated cultures of RJ1003 were diluted 60-fold in supplemented MOPS medium (for RNA extraction), from which 4 ml were immediately transferred to another flask and made 100 μCi/ml [32P]orthophosphate (for RNA extraction). Both cultures were grown in parallel at 37 °C, and samples were withdrawn periodically. A, relative NTP levels during growth. NTPs were separated by TLC as in Fig. 6A, and results are shown as an average of three experiments with S.D. (vertical bars). ■, ATP; □, GTP; ▲, UTP; ○, CTP; ●, cfu/ml. NTP levels given for the 0-min time point are from cells grown to saturation (18 h) in the presence of [32P]orthophosphate. B, fis mRNA and CTP levels during bacterial growth. Relative CTP levels from A are plotted relative to its maximum, which was assigned a value of 100. CTP levels were separated by TLC as in Fig. 6A, from which 4 ml were immediately transferred to another flask and made 100 μCi/ml [32P]orthophosphate (for RNA extraction). Both cultures were grown in parallel at 37 °C, and samples were withdrawn periodically.

GROWTH PHASE

STATIONARY & LATE LOGARITHMIC

Low CTP Pool

High CTP Pool

EARLY LOGARITHMIC

Stable Ternary Complex

Short-lived Open Complex

Closed Promoter Complex

FIG. 9. Effect of CTP pools on fis expression. Open complexes at fis P are short lived, resulting in low fis expression when CTP pools are low. During outgrowth from stationary phase in rich medium, high metabolic rates led to high CTP pools that promote stable, transcription-competent, ternary complexes, resulting in high fis mRNA levels during early logarithmic growth phase.
coupled with generally lower CTP pools compared with ATP or GTP, is a good reason to avoid use of CTP as an initiation NTP, and this is what is observed for most promoters (25, 26). However, CTP becomes a valuable regulatory tool for promoters that, like fis P, form short lived initiation complexes and demand rapid expression during early logarithmic growth phase but effective inhibition prior to entering stationary phase. Initiation with UTP could potentially play a role similar to that of CTP for similar reasons. However, reiterative transcription resulting from +1C→T renders UTP a less effective stimulator of fis productive transcripts. We envision that the +1A and +1G mutant fis promoters are also sensitive to the purine levels, because the +1A and +1G promoters are able to efficiently increase transcription during outgrowth from stationary phase. However, the fact that their levels remain high for most of the logarithmic growth suggests that these promoters are not sufficiently sensitive to the purine oscillations that occur at relatively high concentrations throughout logarithmic growth. The very high sensitivity of rrnB P1 to ATP as its initiating NTP may be attributed to an even lower stability of the RNAP-promoter complex at rrnB P1 than at fis P, perhaps caused in part by the less favorable 16-bp spacing between the −10 and −35 promoter sequences in the former (56, 67). The 17-bp spacing between the −10 and −35 sequences in fis P (24) may serve to fine tune the level of stability of initiation complexes at this promoter so as to render it less sensitive to purines as initiating NTPs but still highly sensitive to the concentration of a poor initiating NTP, such as CTP. We suggest that the strong dependence on CTP as its initiating NTP plays an important role in the dramatic expression pattern that is largely confined to the early logarithmic growth phase. The strict conservation of both the use of CTP as an initiating NTP in fis promoters identified in a number of other bacterial species and the growth phase-dependent regulation pattern (22, 23) emphasizes the strong connection between initiation with CTP and GPD. Other promoters, such as the rrnB P2 and rrapA also initiate transcription with CTP, and their transcription is sensitive to the CTP levels in vitro (64, 67). Thus, we anticipate discovery of a growing number of promoters that initiate transcription predominantly with CTP, exhibit a kinetic barrier in their formation of open complexes, and respond to changing levels of CTP prompted by rapid changes in metabolic rates, in accordance to the nutritional availability.

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REFERENCES

1. Finkel, S. E., and Johnson, R. C. (1992) Mol. Microbiol. 6, 3257–3265
2. Ross, W., Thompson, J. F., Newlands, J. T., and Gourse, R. L. (1990) EMBO J. 9, 3723–3742
3. Nilsson, L. and Emilsson, V. (1994) J. Biol. Chem. 269, 9460–9465
4. Gonzalez-Gil, G., Bringmann, P., and Kahmann, R. (1996) J. Bacteriol. 178, 21–29
5. Xu, J., and Johnson, R. C. (1995) J. Bacteriol. 177, 938–947
6. Clarét, L., and Rovière-Yaniv, J. (1996) J. Mol. Bio. 263, 126–139
7. Falconi, M., Brandi, A., La Teana, A., Gualerzi, C. O., and Pon, C. L. (1996) J. Biol. Chem. 271, 595–603
8. Watanabe, Y., and Horiuchi, S. (1996) J. Mol. Biol. 257, 937–950
9. Caramelo, A., and Schnetz, K. (2000) Mol. Microbiol. 36, 85–92
10. Weinstein-Fischer, D., Elgrably-Weiss, M., and Altvitun, S. (2000) Mol. Microbiol. 35, 1413–1420
11. Goldberg, M. D., Johnson, M., Hinton, J. C., and Williams, P. H. (2001) Mol. Microbiol. 41, 549–559
12. Shub, and R. Zitomer for useful discussions during the course of this work.