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# Association of RNA Polymerase Having Increased $K_m$ for ATP and UTP with Hyperexpression of the *pyrB* and *pyrE* Genes of *Salmonella typhimurium*

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**We investigated the transcription kinetics of RNA polymerase from an *rpoBC* mutant of *Salmonella typhimurium* which showed highly elevated, constitutive expression of the *pyrB* and *pyrE* genes as well as an increased cellular pool of UTP. When bacterial cultures containing an F′ *lac*$^+$ episome were induced for *lac* operon expression, the first active molecules of β-galactosidase were formed with a delay of 73 ± 3 s in *rpo*$^+$ cells. The corresponding time was 104 to 125 s for cells carrying the *rpoBC* allele, indicating that this mutation causes a reduced RNA chain growth rate. In vitro the purified mutant RNA polymerase elongated transcripts of both T7 DNA and synthetic templates more slowly than the parental enzyme at a given concentration of nucleoside triphosphates. This defect was found to result from four- to sixfold-higher $K_m$ values for the saturation of the elongation site by ATP and UTP. The saturation kinetics of the RNA chain initiation step also seemed to be affected. The maximal elongation rate and $K_m$ for GTP and CTP were less influenced by the *rpoBC* mutation. Open complex formation at the promoters of T7 DNA and termination of the 7,100-nucleotide transcript showed no significant difference between the parental and mutant enzymes. Together with the phenotype of the *rpoBC* mutant, these results indicate that expression of *pyrB* and *pyrE* is regulated by the mRNA chain growth rate, which is controlled by the cellular UTP pool. The rate of gene expression is high when the saturation of RNA polymerase with UTP is low and vice versa.**

A mutant, strain KP1475, of *Salmonella typhimurium* was isolated for resistance to 5′-fluorouridine (15). In contrast to other superficially similar mutants, it was found to have increased cellular pools of UTP and other uridine nucleotides; simultaneously it contained highly elevated levels of two pyrimidine biosynthetic enzymes, aspartate transcarbamylase (encoded by *pyrB*) and orotate phosphoribosyltransferase (encoded by *pyrE*). This observation indicated a defect in the mechanism that controls the synthesis of these enzymes (15), since a uridine nucleotide (UTP or UDP) had been identified as a repressing metabolite controlling the expression of the two genes (17, 29). Surprisingly, the mutation mapped in the structural gene for the β or β′ subunit of RNA polymerase, corresponding to either *rpoB* or *rpoC*. This finding suggested that RNA polymerase plays a decisive role in the regulation of *pyrB* and *pyrE* gene expression (15).

To gain insight on the role of RNA polymerase in *pyr* gene regulation, we investigated the transcription kinetics of RNA polymerase from the above *rpoBC* mutant, KP1475, and its parent, KP1469, with templates unrelated to *pyr* gene DNA. We found that the mutant enzyme exhibits a reduced RNA chain elongation rate due to defective binding to the elongation site of UTP and ATP.

## MATERIALS AND METHODS

**Materials.** DNA-cellulose and T7 DNA were prepared as described previously (1, 24). Column materials, fine chemicals, and radioactively labeled nucleotides were obtained from commercial suppliers.

**Nucleoside triphosphates.** Di- or trisodium salts were dissolved individually in distilled water to a concentration of about 60 mM. The pH of the solutions was adjusted to about 7.2 by adding 1 M NaOH. Subsequently, the concentration of the nucleotides was determined spectrophotometrically, and the solutions were stored frozen at −20°C. Before use, the different triphosphates were mixed at appropriate concentrations, and an amount of $MgCl_2$ equal to the total concentration of triphosphate was added.

**Bacterial strains.** The bacterial strains used are listed in Table 1. The isolation and phenotype of the 5′-fluorouracil- and 5′-fluorouridine-resistant RNA polymerase mutant KP1475, which contains a mutation in either *rpoB* or *rpoC*, and its parent KP1469 have been described previously (13, 15). Strains KP1901 and KP1903 carrying an F′ *lac*$^+$ episome and the *rpoBC* region of KP1469 and KP1475, respectively, were constructed as follows. The *zcc*::Tn*10* element of KR45 was used to move the *pyrC1502* mutation of strain KP1468 into KP1494 by means of phage P22(HT) transduction (15), resulting in strain KP1900. This strain contains the *rpoB4* and *rpo-13*(Ts) mutations (4) and is resistant to rifampin and unable to form colonies at 42°C in the presence of uracil. The *rpoB* region of KP1900 was then replaced by transduction with P22 phage grown on either KP1469 or KP1475 and selecting for the ability to form colonies at 42°C in the presence of uracil and picking clones sensitive to rifampin. The resulting strains, KP1901 and KP1903, were then equipped with the F′ *lac*$^+$ episome by mating with *Escherichia coli* CSH23 and selecting for colonies able to grow

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TABLE 1. Strains used

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| *S. typhimurium* LT-2 | | |
| KP1468 | *cdd-9 cod-8 deoD201 udp-11 pyrC1502* | 13 |
| KP1469 | *cdd-9 cod-8 deoD201 udp-11* | 13, 15 |
| KP1475 | *cdd-9 cod-8 deoD201 udp-11 rpoFUR (rpoBC)* | 15 |
| KP1494 | *cdd-9 cod-8 deoD201 udp-11 rpoB4 rpo-13*(Ts) | |
| KP1901 | *cdd-9 cod-8 deoD201 udp-11 pyrC1502 zcc*::Tn*10*/F' *lac*[a] | This work |
| KP1903 | *cdd-9 cod-8 deoD201 udp-11 pyrC1502 zcc*::Tn*10 rpoFUR (rpoBC)* F' *lac* | This work |
| KR45 | *pyrC20 zcc*::Tn*10* | R. Kelln |
| *E. coli* K-12 | | |
| CSH23 | Δ(*lac pro*) *supE spc thi*/F' *lac*$^+$ *proA*$^+$ *proB*$^+$ | Cold Spring Harbor Laboratory |

[a] Called only F' *lac* elsewhere in the text.

with lactose as the carbon source in the presence of tetracycline.

**Growth procedures and enzyme assays.** For physiological studies the cells were grown in the minimal medium of Edlin and Maaløe (11) with the phosphate concentration reduced to 0.3 mM and supplemented with glycerol (0.2%), thiamin (1 μg/ml), and uracil (20 μg/ml). To determine the delay in appearance of β-galactosidase after induction of *lacZ* gene expression by isopropyl-β-D-galactoside (IPTG) (28), this compound was added at 0.1 mM at an $OD_{436}$ of 0.3. Subsequently samples were withdrawn into ice-cold chloramphenicol, toluenized, and assayed for β-galactosidase activity as described previously (16, 28). To determine pools of nucleoside triphosphates, the cells were labeled for 1.5 generations with $^{32}P_i$ as described by Jensen et al. (14). The activity of aspartate transcarbamylase was determined as described previously (15).

**Buffers.** For transcription of synthetic DNA templates, we used the polymix buffer of Jelenc and Kurland (12) but optimized for transcription of T7 DNA with the *E. coli* RNA polymerase (R. Fast, unpublished results). The final concentrations in the reactions were: putrescine hydrochloride, 28 mM; spermidine, 1 mM; potassium phosphate, 5 mM; magnesium acetate, 5 mM; ammonium chloride, 5 mM; and dithioerythritol, 1 mM. The pH was adjusted to 7.2 by addition of 1 M KOH.

For transcription of T7 DNA, the spermidine buffer described by Smith and Chamberlin (30) was used. It contained 40 mM Tris hydrochloride (pH 8), 10 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 120 mM KCl, and 4 mM spermidine.

**Isolation of ternary complexes.** Ternary complexes between RNA polymerase, the poly(dA-dT) template, and a growing RNA transcript were isolated essentially by the procedure of Rhodes and Chamberlin (22). Thus, 2.5 $A_{260}$ units of poly(dA-dT) (alternating) was mixed with 40 to 50 μg of RNA polymerase and 1 mM UTP and incubated at 37°C for 1 min in a buffer containing 50 mM Tris hydrochloride (pH 8), 15 mM $MgCl_2$, and 10 mM 2-mercaptoethanol in a total volume of 0.5 ml. Then 1 mM ATP was added, and incubation was continued for another min. Finally, 50 mM of EDTA was added, and the ternary complexes were separated from excess nucleotides by gel filtration on a 10-ml column of Sephadex G25 with a buffer containing 20 mM Tris hydrochloride (pH 8), 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 5% glycerol while 0.5-ml fractions were collected. The ternary complexes were located under UV light and by ethidium bromide staining after spotting 5 μl of the fractions on DEAE paper. The isolated ternary complexes were found to start incorporation into trichloroacetic acid (TCA)-insoluble material immediately after mixing with radioactive nucleoside triphosphate substrates. The initial rate of incorporation was similar in the presence of rifampin (10 μg/ml) and in its absence. However, in the presence of rifampin incorporation eventually ceased (see Fig. 4).

**Purification of RNA polymerase from KP1469 (*rpo*$^+$) and KP1475 (*rpoBC*).** The two strains were grown exponentially at 37°C in the A+B medium of Clark and Maaløe (7) supplemented with glucose (1%), Casamino Acids (0.5%), and thiamine (5 μg/ml). At an $OD_{436}$ of 7 to 8, the exponentially growing cultures were chilled by adding ice, the cells were harvested by centrifugation, and the pellets were stored frozen at −70°C until use. Since strain KP1475 showed a considerable reversion frequency of its *rpoBC* mutation, the cells were grown in 1-liter portions. Just before harvest, portions were plated on agar and batches containing >1% fast-growing colonies were discarded. To purify RNA polymerase holoenzyme, the procedure of Burgess and Jendrisak (5) was followed, except that the Bio-Rad column was replaced with Sephacryl S-300. RNA polymerase was purified twice from both the mutant KP1475 and the parent strain KP1469. The yield and purity of all purifications were essentially similar: about 10 mg of RNA polymerase holoenzyme from 50 g of wet cell paste. The content of σ-subunit also appeared similar in the preparations as judged from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (not shown). The fraction of active molecules able to initiate transcription of T7 DNA was determined by the assay of Chamberlin et al. (6) (see Fig. 5). In both preparations from the wild-type strain (KP1469) 16 to 18% of the polymerase molecules apparently initiated transcription of T7 DNA, while only 6 to 7% and 9%, respectively, of the enzyme molecules were active in two separate preparations from KP1475.

**Theory.** During elongation the RNA polymerization reaction obeys the kinetics of a Ping-Pong mechanism, according to equation 1 (8, 22):

$$1/v = \Sigma(f_N \cdot K_{NTP}/V_{max} \cdot 1/[NTP]) + 1/V_{max} \quad (1)$$

where $K_{NTP}$ is the $K_m$ for a particular nucleoside triphosphate substrate, $V_{max}$ is the maximal elongation velocity when all substrates are present at saturating concentrations (the catalytic constant, $K_c = V_{max}/[enzyme]$, is considered equal for addition of all types of nucleotide residues to the growing RNA chains [22], and $f_N$ is the fraction of complementary residues in the template strand.

The kinetic constants ($K_m$ and $V_{max}$) were determined by linear regression to data points in double-reciprocal plots ($1/v$ versus 1/[NTP]) with a weighing factor proportional to $v^2$ (9).

## RESULTS

**Induction of *lacZ* expression in vivo.** To study the RNA polymerase defect in KP1475, we constructed two isogenic strains, KP1901 (*rpo*$^+$) and KP1903 (*rpoBC*), containing an

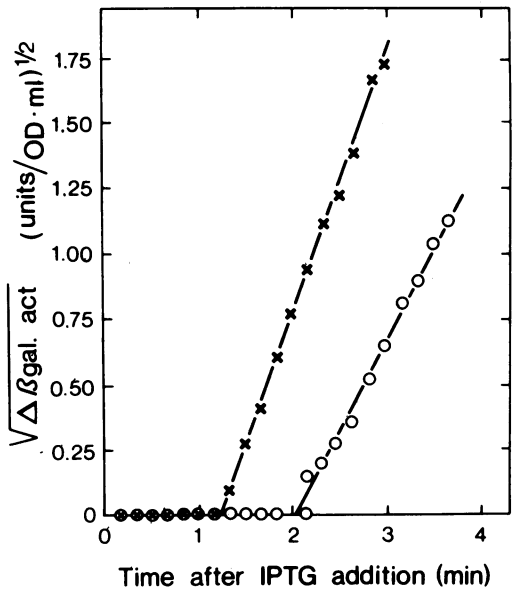


FIG. 1. Initial kinetics of *lacZ* induction in the *rpo*$^+$ strain KP1901 (×) and the *rpoBC* strain KP1903 (○). The strains were grown exponentially at 37°C in a minimal medium supplied with glycerol (0.2%); thiamine (1 μg/ml), and uracil (20 μg/ml). At an $OD_{436}$ of 0.3, IPTG (0.1 mM) was added (time zero) and at 10-s intervals samples (0.5 ml) were withdrawn into 0.5 ml of ice-cold chloramphenicol (0.1 mg/ml) to stop protein chain elongation. After all samples had been taken, the cells were whirled with toluene (16) and assayed for β-galactosidase (βgal) levels (28). The figure shows the initial rise in activity ($\Delta E$) plotted as $(\Delta E)^{1/2}$ as a function of the time of sampling.

F′ *lac*$^+$ episome and the *rpoBC* region from KP1469 and KP1475, respectively (Table 1). Exponential cultures were induced for *lac* operon expression by addition of IPTG at time zero. Subsequently, at short intervals, samples were withdrawn into chloramphenicol to stop protein chain elongation and assayed for β-galactosidase activity. Following a delay in induction which corresponds to the time needed for the coupled transcription and translation of the *lacZ* gene, the amount of β-galactosidase ($\Delta E$) in the culture will increase as a parabolic function of time ($T$) according to equation 2 (28):

$$(\Delta E)^{1/2} = A(T - T_0) \quad (2)$$

This equation is meaningful only for $T \geq T_0$, and it applies only initially, while the rate of mRNA build-up exceeds by far the rate of mRNA inactivation (28).

Figure 1 shows an example of the induction of *lac* operon expression in strains KP1901 (*rpo*$^+$) and KP1903 (*rpoBC*) depicted according to equation 2. The experiment was performed eight times, and Table 2 gives the $T_0$ values together with the pools of nucleoside triphosphates and the level of *pyrB* and *pyrE* expression in the two strains. The induction delay ($T_0$) was 73 ± 3 s for strain KP1901 and between 104 and 125 s (outer limits) for strain KP1903. The observed increase in delay for KP1903 was no doubt the result of a reduced *lacZ* mRNA chain growth rate caused by the *rpoBC* mutation of KP1475, since the two strains differed only by this mutation.

**In vitro studies of RNA polymerization.** The purified RNA polymerase of strain KP1475 (*rpoBC*) and its parent KP1469 (*rpo*$^+$) were assayed with synthetic poly(dA-dT) as the template while incorporation of radioactivity from a labeled substrate ([$^{14}$C]ATP) was measured. In the first assays with only 50 μM concentrations of both substrates (ATP and UTP), the mutant enzyme seemed almost inactive. However, when the triphosphate concentrations were raised, the ability of the mutant polymerase to form an RNA product approached that of the parental enzyme.

We carried out an experiment to determine the $K_m$ for the

TABLE 2. Induction lag in *lacZ* gene expression, nucleoside triphosphate pools, and pyrimidine biosynthetic enzymes in the *rpoBC* mutant and wild-type cells

| Strain | Pool (μmol/g [dry wt] of cells) | | | | Activity[a] (mU/mg) | | Induction lag ($T_0$, s)[b] |
|---|---|---|---|---|---|---|---|
| | GTP | ATP | CTP | UTP | ATCase | OMPppase | |
| KP1901 (*rpo*$^+$) | 1.76 | 4.44 | 1.19 | 1.67 | 12 | 10 | 73 ± 3 |
| KP1903 (*rpoBC*) | 2.28 | 8.23 | 3.00 | 4.17 | 590 | 160 | 115 ± 10 |

[a] ATCase, Aspartate transcarbamylase encoded by *pyrB*; OMPppase, orotate phosphoribosyltransferase encoded by *pyrE*.
[b] Average of eight determinations.

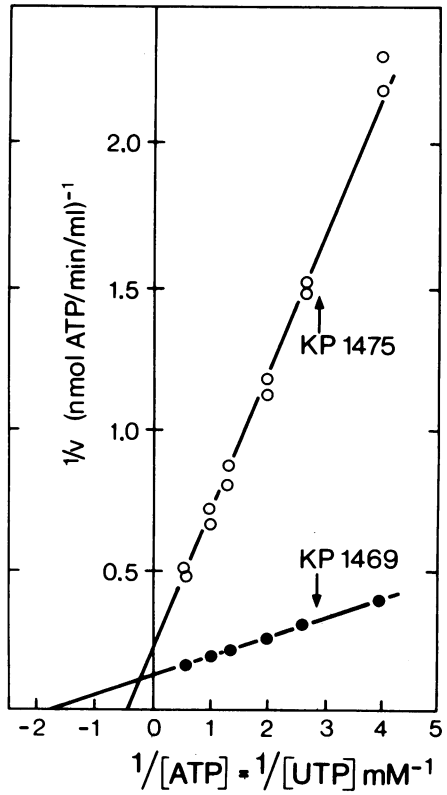


FIG. 2. Effect of varying the concentration of both ATP and UTP on reaction kinetics with poly(dA-dT) as template. The reactions (total volume, 0.5 ml) contained, in addition to the components of polymix buffer, alternating poly(dA-dT) (1.5 $OD_{260}$ units/ml), the indicated, identical concentrations of ATP ([$^{14}$C labeled [1.0 Ci/mol]) and UTP, and RNA polymerase from either (●) KP1469 (16 μg/ml, 18% active) or (○) KP1475 (19 μg/ml, 9% active). The reactions were carried out at 37°C, started by addition of enzyme to the prewarmed mixture of the other components, and monitored by withdrawing 0.1-ml portions for determination of TCA-insoluble radioactivity at 10 s, 2 min, 5 min, and 10 min.

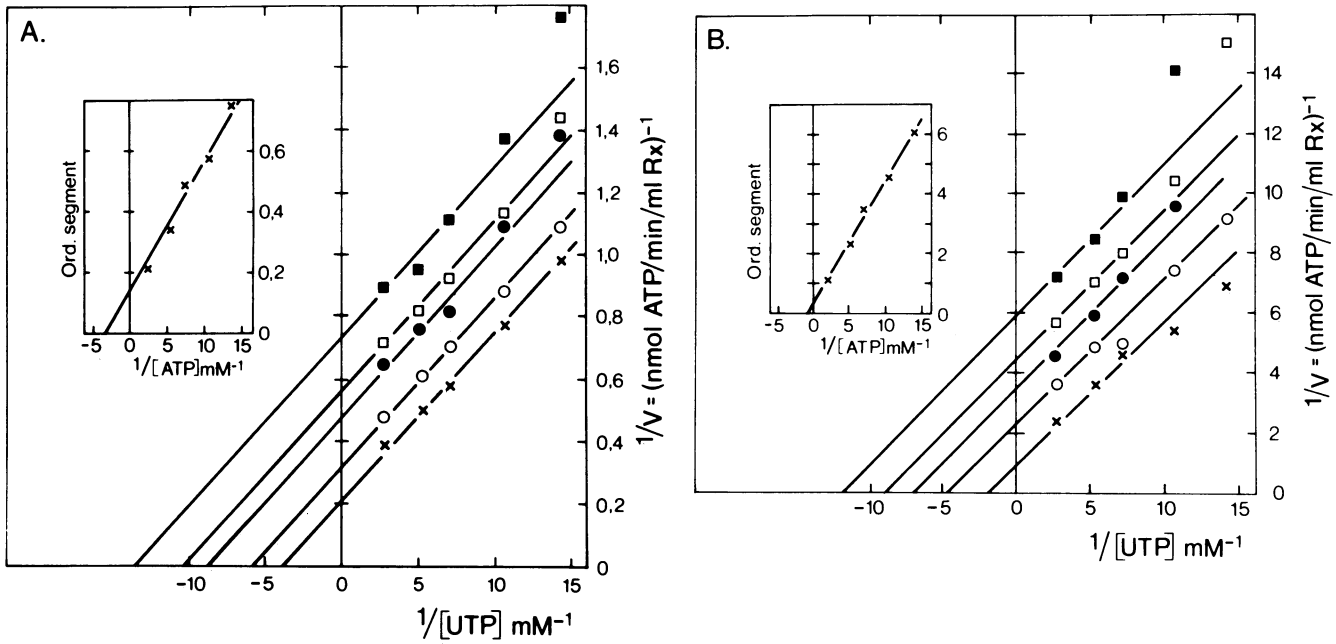


FIG. 3. Effect of varying the UTP concentration on reaction kinetics with poly(dA-dT) as template in the presence of fixed concentrations of ATP. RNA polymerase was from (A) KP1469 and (B) KP1475. The experiments were carried out as described in the legend to Fig. 2, except that the concentrations of UTP was varied as indicated at constant concentrations of 0.4 (×), 0.2 (○), 0.13 (●), 0.10 (□), and 0.07 (■) mM ATP ($^{14}C$ labeled [1 Ci/mol]). Notice that the double-reciprocal plots start to curve upwards at low concentrations of both substrates, particularly with the mutant RNA polymerase (B). In this case the slope was determined from the curves at the highest ATP concentrations, and the lines for the lower ATP concentrations were drawn in parallel through the points at the three highest UTP values. Table 3 summarizes the kinetic constants. (Inset) The ordinate segments have been depicted as a function of the reciprocal concentrations of ATP.

saturation of this reaction, which involves concomitant RNA chain initiation, elongation, and termination (22). The observed reaction velocities as a function of the concentration of ATP plus UTP is shown in Fig. 2. The half-saturation of the reaction required approximately fourfold-higher concentrations of ATP and UTP for the mutant RNA polymerase than for the parental enzyme. Note that the plots did not significantly deviate from straight lines even though the concentrations of the two substrates were varied. This indicates that the assays primarily measured the $K_m$ for the saturation of a reaction which involves only one nucleotide substrate bound to RNA polymerase at a time (8), characteristic for the RNA chain elongation phase (22). Moreover, a pattern of parallel lines was obtained for both enzymes in the double-reciprocal plots when the concentration of UTP was varied at different fixed concentrations of ATP (Fig. 3). These results show that the $K_m$ for both ATP and UTP was four to six times higher for the mutant enzyme than for the parental RNA polymerase in the overall transcription cycle of poly(dA-dT) (Table 3). The initiation of RNA chain synthesis, in contrast, involves bond formation between two dissociable nucleoside triphosphates bound simultaneously to the binary complex between RNA polymerase and the DNA template (24). If this step had been rate limiting for the reaction velocities, the double-reciprocal plots (Fig. 2) should curve up and the lines (Fig. 3) would be expected to intersect (8).

TABLE 3. Kinetic constants for transcription of alternating poly(dA-dT)

| RNA polymerase | $K_m$ (mM) | | Transcription (nmol of residues/min per mg of protein) | |
|---|---|---|---|---|
| | UTP | ATP | $V_{max}$ | Corrected $V_{max}$[a] |
| KP1469 (*rpo*$^+$) | 0.88 ± 0.1 | 0.64 ± 0.1 | 970 ± 60 | 5,700 (5,400) |
| KP1475 (*rpoBC*) | 3.6 ± 0.6 | 3.8 ± 0.6 | 470 ± 70 | 5,200 (6,600) |

[a] Corrected for 100% active molecules in the preparation. Numbers in parentheses were values obtained with a different enzyme preparation.

To test this further we determined the effect of adding rifampin to transcription from poly(dA-dT) by both RNA polymerases at 0.5 mM ATP and UTP (Fig. 4). In the absence of the drug, which prevents initiation without interfering with the elongation of transcripts (23), the incorporation of radioactivity continued for extended periods of time (Fig. 4), but when rifampin was added after polymerization has started, the incorporation of radioactive ATP into RNA gradually leveled off and eventually stopped as the elongating RNA polymerase molecules ran off the template strands. As seen in Fig. 4, this process took a considerably longer time for the mutant enzyme than for the parental RNA polymerase. Because none of the enzymes made any RNA product when rifampin was present before enzyme addition, the simplest explanation of this phenomenon is that the mutant RNA polymerase elongates transcripts of poly(dA-dT) more slowly than the parental enzyme under the conditions (i.e., 0.5 mM ATP and 0.5 mM UTP).

The $K_m$ for ATP and UTP for the elongating RNA polymerases was determined by the procedure of Rhodes and Chamberlin (22). In these assays the RNA polymerases were allowed to initiate chain synthesis in the presence of unlabeled ATP and UTP (1 mM each). After isolation, the ternary complexes were mixed with the experimental con-

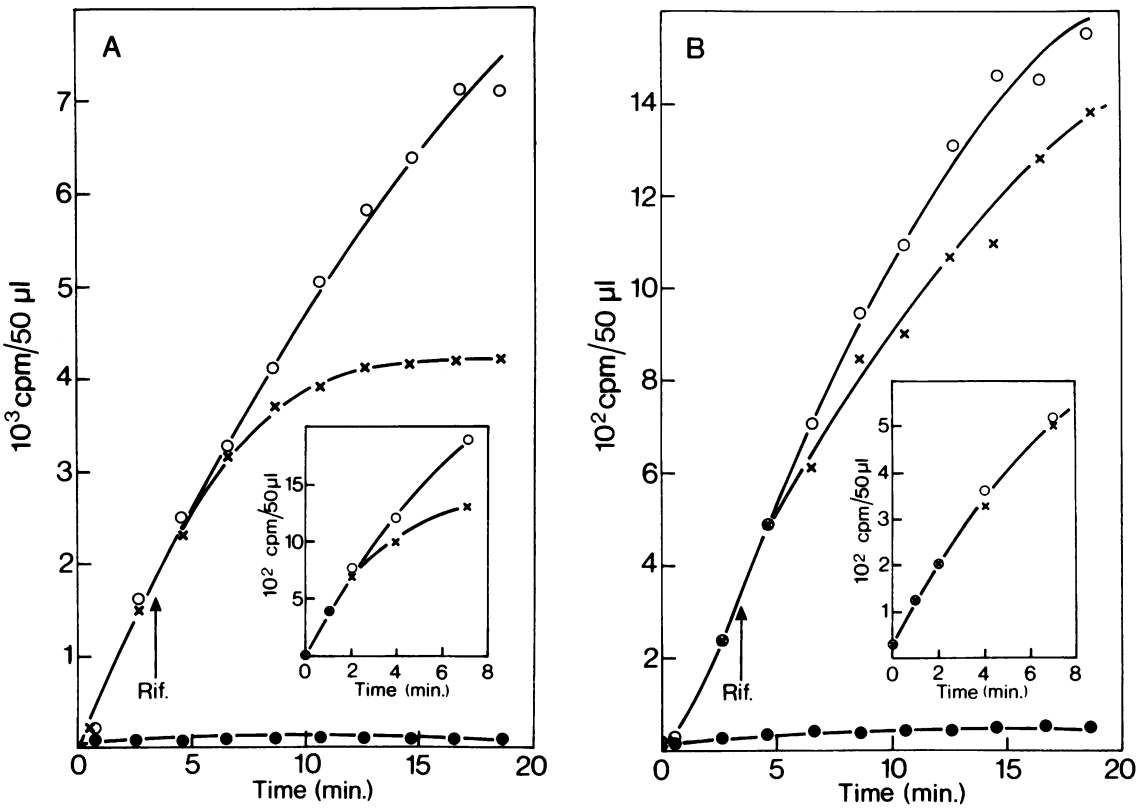


FIG. 4. Runoff transcription from poly(dA-dT) by RNA polymerase from KP1469 (A; 18 µg/ml) and KP1475 (B; 16 µg/ml). For each polymerase, three reactions were mixed which, in addition to the polymix buffer, contained 0.5 mM UTP, 0.5 mM [8-$^{14}$C]ATP (1 Ci/mol), and alternating poly(dA-dT) (2 $OD_{260}$ units/ml). The mixtures were prewarmed at 37°C for 5 min. Reaction was started by addition of RNA polymerase, and samples were withdrawn at the indicated times for determination of TCA-insoluble radioactivity (6). One reaction (○) contained no other additions, another (●) contained rifampin (10 µg/ml) before enzyme was added, and to a third (×) rifampin (10 µg/ml) was added at 3.5 min after enzyme addition. (Insets) In these experiments, isolated ternary complexes were used instead of free poly(dA-dT) and RNA polymerase. The reactions were initiated by addition of these complexes to a prewarmed mixture either without (○) or with (×) rifampin (10 µg/ml).

centrations of the triphosphates, plus rifampin to prevent reinitiation. RNA chain elongation as monitored by measuring the incorporation of $^{14}$C-labeled ATP into acid-insoluble material. The $K_m$ thus determined for ATP and UTP (Table 4) was approximately four times higher for the KP1475 RNA polymerase than for the enzyme of KP1469.

**One-cycle transcription of T7 DNA.** The T7 DNA template contains three strong neighboring promoters for the early genes of the phage that are active with the RNA polymerases from a series of bacteria related to *E. coli*. A transcription terminator is located approximately 7,100 base pairs downstream from the promoters (6).

Figure 5A shows the result of an experiment measuring the transcription kinetics for T7 DNA with the RNA polymerases of KP1469 (*rpo*$^+$) and KP1475 (*rpoBC*). All four nucleoside triphosphates were present at a concentration of 1 mM. The polymerases were allowed to bind to the T7 promoters for 10 min, and then the four triphosphates were added to start polymerization at time zero. Rifampin was added to 1.5 min later to prevent reinitiation, and incorporation of radioactivity in acid-insoluble material was monitored as a function of time. The incorporation curves broke at the point that the transcribing polymerases encountered the terminator (Fig. 5A). The breakpoint was significantly later for the mutant RNA polymerase, 3.7 to 4.2 min, than for the parental enzyme, approximately 2.7 min. From these values and by the rationale of Chamberlin et al. (6) a chain elongation rate of 43 to 50 nucleotide residues per s was calculated for the parenteral enzyme; the corresponding value is 27 to 30 residues per s for the mutant polymerase under the conditions employed. From the ordinate of the breakpoint it can be calculated that about 16% of the protein in the KP1469 preparation was active RNA polymerase

TABLE 4. Kinetic constants for the elongation of poly(dA-dT) transcripts by isolated ternary complexes[a]

| Ternary complex | Apparent $K_m$ (mM) | | |
|---|---|---|---|
| | ATP + UTP | UTP[b] | ATP[c] |
| KP1469 (*rpo*$^+$) | 0.57 ± 0.05 | 0.28 ± 0.02 | 0.16 ± 0.02 |
| KP1475 (*rpoBC*) | 1.9 ± 0.2 | 1.4 ± 0.2 | 0.47 ± 0.11 |

[a] Reaction temperature was 28°C. The reactions contained, in a total volume of 0.2 ml (after addition of ternary complex), the components of polymix buffer, UTP and [8-$^{14}$C]ATP (5 Ci/mol), and rifampin (10 µg/ml). They were started by adding 50 µl of ternary complex to the prewarmed mixture of the other components. At 1, 2, and 3 min, 50-µl samples were withdrawn for determination of TCA-insoluble radioactivity. For determinations of time zero values, similar reactions were mixed without enzyme.

[b] The concentration of [$^{14}$C]ATP was 1 mM, and seven concentrations of UTP between 0.1 and 1.5 mM were used.

[c] The concentration of UTP was 2 mM, and seven concentrations of [$^{14}$C]ATP between 0.1 and 1.5 mM were used.

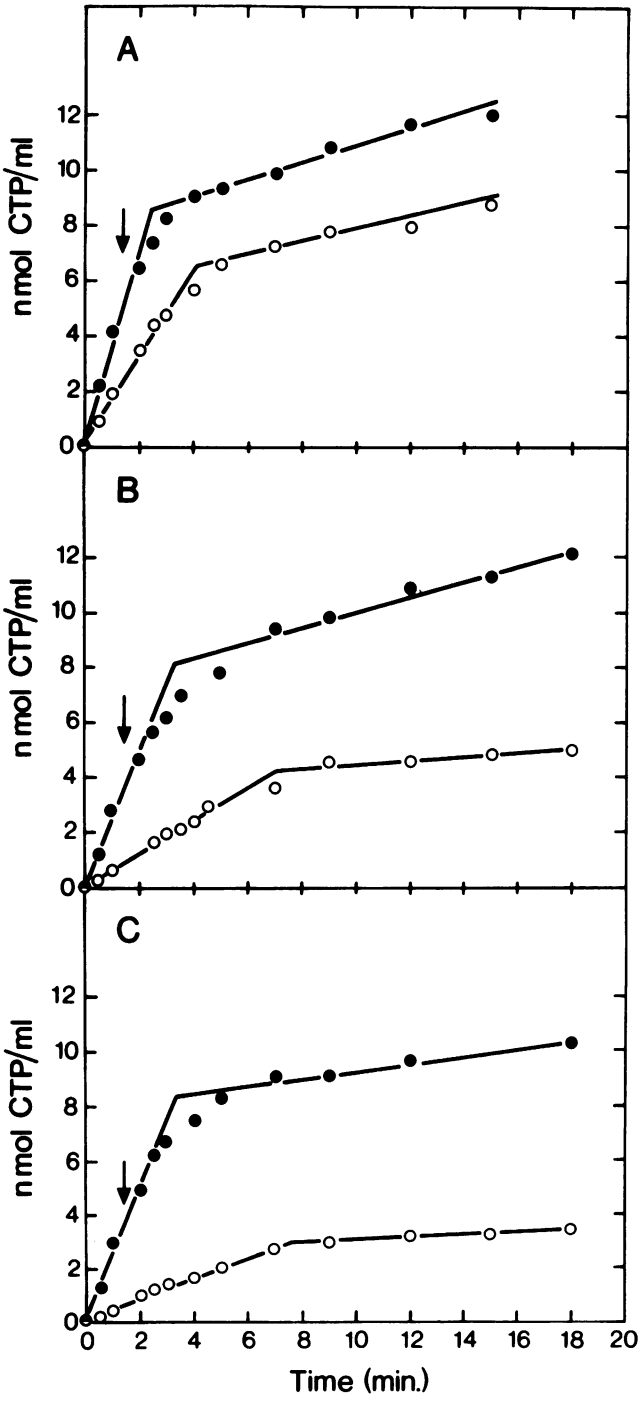


FIG. 5. Transcription cycle on T7 DNA by RNA polymerases from KP1469 (*rpo*$^+$) (●) and KP1475 (*rpoBC*) (○). (A) In addition to the components of the spermidine buffer, the reactions (1.5 ml) contained T7 DNA (90 μg/ml), 1.0 mM each CTP, UTP, GTP, and [$^{14}$C]ATP (1 Ci/mol), and RNA polymerases from KP1469 (36 μg/ml) or KP1475 (32 μg/ml). The reactions were incubated for 10 min at 37°C to allow association before addition of the triphosphates to start polymerization at time zero, and at 1.5 min rifampin (10 μg/ml) (arrow) was added to prevent reinitiation (6). Polymerization was followed by withdrawing 0.1-ml portions for determination of TCA-insoluble radioactivity at the indicated times. A background (about 30 cpm) was determined from a similar reaction without enzyme and was subtracted before calculations. (B) As for panel A, but the concentration of GTP and CTP was reduced to 0.13 mM and [α-$^{32}$P]CTP (2 Ci/mol) was used as a radioactive label. (C) As for panel A, but the concentration of ATP and UTP was reduced to 0.25 mM and [α-$^{32}$P]CTP carried the radioactive label. Similar experiments were carried out in which rifampin was added at time zero together with the nucleoside triphosphates, and similar results were obtained.

TABLE 5. $K_m$ for preinitiated transcription of T7 DNA[a]

| Substrate varied | Fixed substrate | $K_m$ (mM) for RNA polymerase from: | |
|---|---|---|---|
| | | KP1469 | KP1475 |
| ATP | 2 mM UTP, 1 mM CTP, 1 mM GTP | 0.58 | 4.0 |
| UTP | 2 mN ATP, 1 mM CTP, 1 mM GTP | 0.93 | 5.0–6.2 |
| GTP | 2 mM ATP, 1 mM UTP, 1 mM CTP | 0.38 | 0.85 |
| CTP | 2 mM ATP, 1 mM UTP, 1 mM GTP | 0.14 | 0.20 |

[a] RNA polymerases and T7 DNA were mixed in spermidine buffer and incubated at 37°C for 5 min in the presence of 10 μM of all the nucleoside triphosphates (nonradioactive). At time zero, equal volumes of this mixture and a mixture of two times the experimental concentrations of nucleoside triphosphates (containing 5 $C_i$ of [4-$^{14}$C]UTP per mol) and 20 μg of rifampin per ml (also in the spermidine buffer and at 37°C) were mixed, and at 1 and 2 min 100-μl samples were withdrawn for determination of TCA-insoluble radioactivity. To determine the incorporated radioactivity at time zero, a reaction without enzyme was mixed and treated as above.

molecules, while the corresponding value for KP1475 was about 7%. The ratios of slopes before and after the break in the incorporation curves indicate that about 10% of the transcribing polymerase molecules from both preparations were read through the early T7 transcription terminator.

When either the concentration of ATP plus UTP or of GTP plus CTP was lowered relative to the amounts shown in Fig. 5A, the rate of incorporation by the mutant RNA polymerase was strongly reduced; this did not seriously affect the polymerization kinetics of RNA polymerase from strain KP1469 (Fig. 5B and C). Note that both the initial slope and the time needed to reach the transcription terminator were affected by the reduction in the triphosphate concentration. In the presence of only 0.25 mM ATP and UTP, the incorporation continued linearly for at least 7 min. This is consistent with a $K_m$ defect in the KP1475 RNA polymerase for the nucleoside triphosphates at elongation, but initiation of RNA chain synthesis also seems to be affected by the mutation.

**Determination of $K_m$ for individual nucleoside triphosphates.** To determine the $K_m$s for the individual nucleoside triphosphate substrates during RNA chain elongation, we used the experimental protocol of Kingston et al. (18). The RNA polymerases were incubated with T7 DNA in the presence of a low concentration (10 μM) of all four nucleoside triphosphates. This should allow RNA polymerase to initiate the synthesis of a short RNA chain (18). Subsequently, portions of the incubation mixture were mixed with rifampin and the experimental concentrations of the substrates, of which on carried a radioactive label. Elongation was monitored as incorporation into acid-insoluble radioactivity (Table 5). The $K_m$ for ATP and UTP increased five- to sevenfold as a result of the mutation in the KP1475 polymerase, while the $K_m$ for GTP and CTP was less influenced (about twofold).

**Initiation at T7 promoters.** The kinetics of formation of initiation complexes, studied as the formation of RNA

polymerase-DNA complexes able to initiate transcription upon addition of nucleoside triphosphates and rifampin, is shown in Fig. 6. The mutant and the parent enzymes behaved virtually identically, and the pseudo-first-order rate constant for the slow-promoter localization (or open complex formation [27]) was close to 0.018 $s^{-1}$ for both enzymes.

When the open binary complexes between RNA polymerase and the T7 promoters were mixed with nucleoside triphosphates, they rapidly started the synthesis of RNA chains. If rifampin was included together with the triphosphates, fewer chains were initiated, particularly with the mutant RNA polymerase (Table 6). Since rifampin has been shown to prevent RNA chain initiation in competition with the triphosphates (23), the data indicate that the RNA polymerase of strain KP1475 also had an increased $K_m$ for triphosphates at initiation.

## DISCUSSION

We investigated the transcription kinetics of RNA polymerase from a mutant (*rpoBC*) of *Salmonella typhimurium*, which was isolated as being resistant to a combination of 5'-fluorouridine and 5'-fluorouracil, i.e., to 5'-fluorouridine nucleotides (15). This mutation causes a strong overexpression of the *pyrB* and *pyrE* genes as well as a high pool of UTP in the mutant cells. This result is interesting since UTP has been identified as a repressing metabolite for the two genes (17, 29, 31). The experiments in this report were aimed at clarifying the RNA polymerase defect capable of causing such a phenotype.

The results leave little doubt that the elongation of RNA chains [*lacZ* mRNA, transcripts of T7 DNA, and poly(A-U)] occurs more slowly with the mutant RNA polymerase than with the parental enzyme both in vivo and in vitro. Furthermore, the mutant RNA polymerase responded much more dramatically than the parental enzyme to changes in the concentration of nucleoside triphosphates in vitro, demonstrating an increased $K_m$ for saturation by the triphosphate substrates at some stage in the transcription cycle. This deficiency was most pronounced for ATP and UTP, for which the $K_m$ rose four- to sixfold, and less evident for GTP and CTP, for which the $K_m$ was only 1.5- to 2-fold higher than for the wild-type enzyme.

The defect in saturation by triphosphates seems to affect the kinetics of both RNA chain elongation and initiation. For the reasons given below we think that both of these traits are natural consequences of a defective binding of triphosphates to the elongation site caused by the *rpoBC* mutation in KP1475.

TABLE 6. Kinetics of initiation of transcription by binary complexes between RNA polymerases and the T7 promoters: rifampin challenge[a]

| Nucleotides (mM each) | | Incorporation into RNA polymerase (cpm/100 μl at 1.5 min) | | | |
|---|---|---|---|---|---|
| | | KP1469 | | KP1475 | |
| ATP abd UTP | GTP and CTP | Without rifampin | With rifampin | Without rifampin | With rifampin |
| 1 | 1 | 1,530 | 1,242 | 1,193 | 718 |
| 0.25 | 1 | 910 | 740 | 407 | 175 |
| 1 | 0.13 | 1,355 | 1,072 | 625 | 306 |

[a] RNA polymerases in Spermidine buffer (24 μg/ml) (17% active) from KP1469 or 54 μg/ml (7% active) from KP1475 were incubated at 37°C with T7 DNA (0.20 mg/ml) for 7 min. An equal volume of spermidine buffer containing twice the indicated concentrations of the triphosphates (of which CTP carried an α-$^{32}$P radioactive label [5 Ci/mol]) without or with rifampin (40 μg/ml) was added at time zero. Samples were withdrawn for determination of TCA-insoluble radioactivity 1.5 and 3 min after addition of the nucleotide mixture.

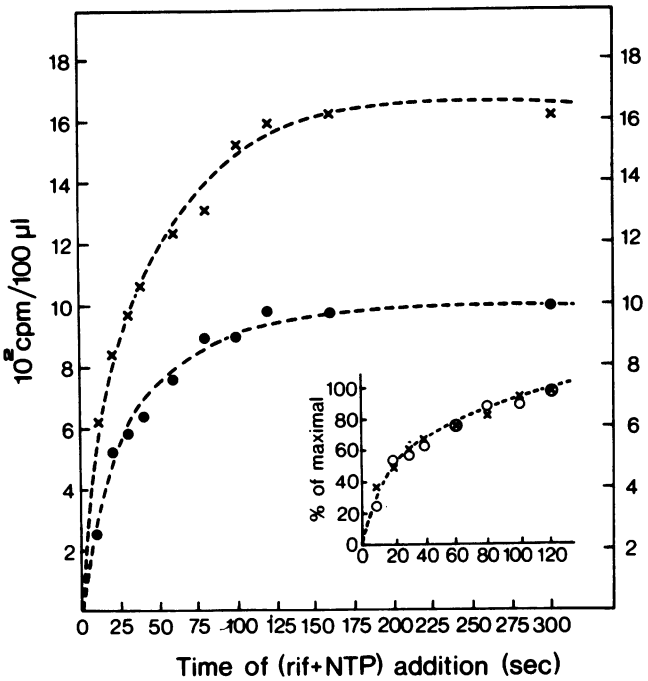


FIG. 6. Kinetics of association to T7 promoters. At time zero T7 DNA (90 μg/ml) was mixed at 37°C with RNA polymerase from (×) KP1469 (12 μg/ml, 18% active) or (○) KP1475 (24 μg/ml, 6% active). At the indicated times, 80-μl portions were mixed with 20 μl of rifampin (rif, 50 μg/ml) and 2 mM of each triphosphate (NTP; [$^{14}$C]ATP [1 Ci/mol]) to allow the promoter-complexed polymerases to initiate and complete transcription of one RNA chain. The reactions continued for 25 min at 37°C before determination of acid-insoluble radioactivity (see the legend to Fig. 5 for the rationale), and the value for a blank reaction to which rifampin was added before the triphosphates was subtracted. The inset shows the same data as percent maximal for each enzyme. The apparent first-order rate constant for the promoter-localization process (27), determined from these and three similar data sets, was 0.018 ± 0.003 $s^{-1}$ for both enzymes.

The initiation of RNA chain synthesis by the open complex between RNA polymerase and DNA is understood as the formation of the very first phosphodiester bond in the transcript (23). The nature of catalysis of this bond formation is the same as for the other phosphodiester bonds in the RNA chain. So although the initiation reaction obeys totally different saturation kinetics than the elongation phase (because both substrates that react are dissociable molecules [8, 23]) the second nucleotides at initiation must be bound to the elongation site. Therefore it seems inevitable that a defect in binding a nucleoside triphosphate at this site on RNA polymerase will also affect the $K_m$ for nucleotides at the initiation step.

No mutation in RNA polymerase has previously been shown to change the affinity of the elongation site for the nucleoside triphosphates (M. Chamberlin, personal communication).

Below we discuss the phenotypic consequences of this *rpoBC* mutation and their implications for understanding *pyr*

gene regulation and control of the coupling between transcription and translation.

Apart from a 30% reduction in growth rate, the *rpoBC* mutation of strain KP1475 also caused a very high UTP pool to accumulate and strong overexpression of the *pyrB* and *pyrE* genes, which code for enzymes active in the biochemical pathway for de novo synthesis of UTP. This compound has been shown to normally cause repression of these genes (17, 29).

The results in this and an earlier paper (15) show that a defect, caused by mutation, in the binding of nucleoside triphosphates at the elongation site of RNA polymerase phenotypically mimics the consequences of a reduced UTP pool in wild-type cells, so that *pyrB* and *pyrE* expression is highly elevated. This is a strong indication that expression of these two genes is controlled not by the UTP pool itself, but rather by the degree of saturation of the elongating RNA polymerase with UTP.

Both genes are regulated by attenuation (3, 19, 20, 26, 32), and the pyrimidine control of mRNA chain termination requires a translation close to the symmetric attenuator from the upstream side (3, 10, 25). Thus, translation of a leader peptide is needed in vivo for transcriptional readthrough past the *pyrB* attenuator (10, 25), while translation of a preceding gene (*orfE*) is needed for transcription past the *pyrE* attenuator located in the intercistronic *orfE-pyrE* region (3, 20, 21). The hypothesis has been made that the coupling between the transcribing RNA polymerase and the leading ribosome determines the frequency of mRNA chain termination at the two attenuators. Thus, a high degree of transcription into the *pyrB* and *pyrE* structural genes occurs when the coupling is very tight, while lower *pyr* gene expression is seen when the RNA polymerase transcribes faster than the ribosomes can follow (3, 32). This hypothesis has been tested by exchanging codons in the *pyrE* leader; when frequently used arginine codons, which correspond to a major $tRNA_{Arg}$ species, are replaced by rarely used arginine codons, the frequency of transcription termination at the *pyrE* attenuator increases (2). This observation provided evidence for a regulatory function of the protein chain elongation rate in *pyrE* expression (2). The behavior of the KP1475 RNA polymerase indicates a regulatory function in *pyr* gene control of the mRNA chain growth rate, dictated by the saturation of the transcribing RNA polymerase by UTP.

Finally it should be mentioned that a reduction in the GTP pool size has also been found to cause increased *pyrB* and *pyrE* gene expression (13). Again, the regulatory effect on *pyrE* expression is exerted at the level of attenuation (P. Poulsen, F. Bonekamp, and K. F. Jensen, unpublished observations). Consequently, a reduction in the cellular concentration of GTP seems to increase the coupling between transcription and translation even though GTP is required for both processes. The observed $K_m$ for saturation of the transcribing RNA polymerase by GTP and UTP in vitro were close to the cellular concentrations of the two compounds (15, 18). Hence it seems likely that the RNA chain growth rate in vivo will respond to physiological variations in the pools of UTP and GTP. In contrast, the concentrations of ATP and CTP are substantially higher than needed for half-saturation of the elongating RNA polymerase.

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