Transcription Start Site Sequence and Spacing between the −10 Region and the Start Site Affect Reiterative Transcription-Mediated Regulation of Gene Expression in *Escherichia coli*

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Reiterative transcription is a reaction catalyzed by RNA polymerase, in which nucleotides are repetitively added to the 3′ end of a nascent transcript due to upstream slippage of the transcript without movement of the DNA template. In *Escherichia coli*, the expression of several operons is regulated through mechanisms in which high intracellular levels of UTP promote reiterative transcription that adds extra U residues to the 3′ end of a nascent transcript during transcription initiation. Immediately following the addition of one or more extra U residues, the nascent transcripts are released from the transcription initiation complex, thereby reducing the level of gene expression. Therefore, gene expression can be regulated by internal UTP levels, which reflect the availability of external pyrimidine sources. The magnitude of gene regulation by these mechanisms varies considerably, even when control mechanisms are analogous. These variations apparently are due to differences in promoter sequences. One of the operons regulated (in part) by UTP-sensitive reiterative transcription in *E. coli* is the *carAB* operon, which encodes the first enzyme in the pyrimidine nucleotide biosynthetic pathway. In this study, we used the *carAB* operon to examine the effects of nucleotide sequence at and near the transcription start site and spacing between the start site and −10 region of the promoter on reiterative transcription and gene regulation. Our results indicate that these variables are important determinants in establishing the extent of reiterative transcription, levels of productive transcription, and range of gene regulation.

Usually during transcription, the nascent RNA transcript and the template strand of DNA move in tandem as an RNA-DNA hybrid. However, during transcription of a homopolymeric tract in the DNA template, the nascent transcript can slip (typically) one base upstream without movement of the DNA template within the active site of RNA polymerase (RNAP) (1, 2). This repositioning allows the same template base to specify an additional nucleotide in the transcript, and when transcript slippage occurs repetitively, the same template nucleotide can specify multiple extra residues. This reaction is called reiterative transcription (also known as RNAP stuttering, transcription slippage, and pseudotemplated transcription) and appears to be catalyzed by all RNAPs (2–5). Reiterative transcription can involve the repetitive addition of any of the four nucleoside triphosphate substrates and occurs during transcription initiation, elongation, and termination (2, 3, 6, 7). During initiation, when the length of the RNA-DNA hybrid can be shorter than that of the 8- to 9-bp hybrid that forms during elongation (8), a homopolymeric tract as short as three residues can enable reiterative transcription (9, 10). In contrast, longer homopolymeric tracts usually are required for reiterative transcription during elongation and termination (6, 7).

The physiological significance of reiterative transcription is that it plays a central role in regulating the expression of numerous prokaryotic, viral, and eukaryotic genes through an assortment of different mechanisms (3, 11). In *Escherichia coli*, expression of at least five operons is regulated by mechanisms involving the reiterative addition of U residues during transcription initiation (4, 12–15). Although these mechanisms can differ fundamentally (11), three examples include analogous mechanisms that regulate, in part, the expression of the *pyrBI* and *carAB* pyrimidine nucleotide biosynthetic operons and the *galETKM* (*gal*) galactose catabolic operon (12–14). The promoters of each of these operons (at least one when there are multiple promoters) include initially transcribed regions that contain a tract of three T·A base pairs located one or two bases downstream from the transcription start site (Fig. 1). These homopolymeric tracts, here referred to as (nontemplate strand) T tracts, enable a fraction of the nascent transcripts to enter the reiterative pathway. Entry into this pathway is enhanced by high intracellular concentrations of UTP (i.e., the reiterative substrate), resulting in a larger fraction of transcripts containing extra U residues. In each case, the addition of extra U residues causes the release of the nascent transcript from the transcription initiation complex, thereby repressing operon expression. This repression allows the cell to use UTP levels to control *pyrBI*, *carAB*, and *gal* expression and synthesize the encoded enzymes at optimal levels for growth. Although these control mechanisms are basically the same, the range of regulation afforded by them varies considerably, specifically, 2-fold, 3-fold, and 7-fold for the *gal*, *carAB*, and *pyrBI* operons, respectively (12–14). These different ranges presumably reflect important differences in promoter sequences, which could include different sequences at or near the transcription start site or the spacing between the start site and the −10 region (Fig. 1).

In this study, we constructed mutant variants of the *carAB* P1 promoter, one of two *carAB* promoters and the only one that participates in reiterative transcription-mediated gene regulation

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                   −10            *
pyrBI    TATAATGCCGGACAATTTGCCG

carAB P1 CAGAATGCCGCCGTTTGCCAGA
                          *

gal P2   TATGCTATGGTTATTTCATACC
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FIG 1 Sequences of the *pyrBI*, *carAB* P1, and *gal* P2 promoter regions from the −10 region through the initially transcribed region. The −10 regions are labeled and underlined, and the transcription start sites are in boldface and marked with an asterisk.

(12). The mutations individually introduce the differing sequence elements of the *gal* and *pyrBI* promoters described above. We examined the effects of each mutation on reiterative transcription *in vitro* and reiterative transcription-mediated control of *carAB* expression *in vivo*. Our results show that both the sequence at or near the transcription start site and the spacing between the start site and −10 region have significant effects on the extent of reiterative and productive transcription and on the range of gene regulation. These effects appear, at least in part, to account for the different ranges of reiterative transcription-mediated regulation of *carAB*, *pyrBI*, and *gal* expression.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* K-12 strain CLT42 [F⁻ *car-94* Δ(*argF-lac*)U169 *rpsL150 thiA1 relA1 deoC1 ptsF25 flbB5301 rbsR*] (16) was used as the parent in the construction of bacteriophage lambda lysogens. Plasmid pDLC126 (12, 17) was used to construct *carAp₁::lacZ* operon fusions. This plasmid contains a functional *E. coli lacZ* gene preceded by a strong ribosome binding site and a more-upstream unique BamHI cloning site. Plasmid pDLC126 does not contain a promoter from which the *lacZ* gene can be transcribed. Operon fusions were made by inserting a BamHI restriction fragment containing either the wild-type or a mutant *carAB* P1 promoter region into the unique BamHI cloning site of plasmid pDLC126. Fusion constructions were confirmed by DNA sequence analysis.

Restriction digests, ligations, and transformations. Conditions for restriction digests, ligations, and transformations were as previously described (16).

DNA preparations and site-directed mutagenesis. Plasmid DNA was isolated by using Qiagen plasmid kits. The *carAB* P1 promoter fragment, which contains nucleotides −58 to +40 of the promoter region (counting from the transcription start site) flanked by BamHI restriction sites, was prepared as previously described (12). Site-directed mutagenesis was used to introduce selected mutations at the transcription start site of the *carAB* P1 promoter fragment. Mutagenesis was performed by using a previously described PCR-based procedure (12). Wild-type and mutant promoter fragments were digested with BamHI prior to insertion into plasmid pDLC126.

***In vitro* transcription.** Purified RNA polymerase holoenzyme containing σ⁷⁰ was prepared as previously described (18, 19). DNA templates were 207-bp or 208-bp fragments containing the *carAB* P1 promoter region (with either a wild-type or mutant promoter) and a downstream segment that included an efficient (~98%) intrinsic transcription terminator, the *pyrBI* attenuator (20). The presence of the *pyrBI* attenuator facilitates RNA polymerase release from the DNA template in our multiple-round transcription assay, thereby increasing the synthesis of long (referred to as full-length) transcripts initiated at the *carAB* P1 promoter (12). All templates were prepared and their concentrations and purity determined as previously described (21). Transcription reaction mixtures (10 μl) contained 10 nM DNA template, 100 nM RNA polymerase, 20 mM Tris-acetate (pH 7.9), 10 mM magnesium acetate, 100 mM potassium glutamate, 0.2 mM Na₂EDTA, 0.1 mM dithiothreitol, 400 μM

(each) ATP, CTP, and GTP, and either 50 or 500 μM UTP. In the reaction mixture, one of the nucleoside triphosphates was ³²P labeled (2 Ci/mmol). Reactions were initiated by addition of RNA polymerase, and the reaction mixtures were incubated at 37°C for 15 min. Heparin (1 μl of a 1 mg/ml solution) then was added to the mixture, and incubation was continued for an additional 10 min. Reactions were terminated by adding 10 μl of stop solution (7 M urea, 2 mM Na₂EDTA, 0.25% [wt/vol] each of bromophenol blue and xylene cyanol) and placing the samples on ice. The samples were heated at 100°C for 3 min, and an equal volume of each sample was removed and run on a 25% (29:1 acrylamide-bisacrylamide)-50 mM Tris-borate (pH 8.3), 1 mM Na₂EDTA sequencing gel containing 7 M urea (21). Transcripts were visualized by autoradiography and quantitated by scanning gels with a Molecular Dynamics PhosphorImager and by densitometry.

Transfer of *carAp₁::lacZ* operon fusions from plasmids to the *E. coli* chromosome. Wild-type and mutant *carAp₁::lacZ* operon fusions carried on derivatives of plasmid pDLC126 were individually transferred to the chromosome of strain CLT42 by using phage lambda RZ5 (22). The presence of a single prophage at the lambda attachment site was confirmed by PCR analysis (23). In this procedure, the concentration of each primer was 500 nM.

Media and culture methods. Cells used for enzyme assays and RNA isolations were grown at 37°C with shaking in N⁻C⁻ medium (24) supplemented with 10 mM NH₄Cl, 0.4% (wt/vol) glucose, 0.015 mM thiamine hydrochloride, 1 mM arginine, and either 1 mM uracil or 0.25 mM UMP. Cell growth was monitored as previously described, and samples were harvested during the exponential phase of growth at the same optical density (25).

Enzyme assays. Cell extracts were prepared by sonic oscillation (22). β-Galactosidase activity was determined as previously described (12).

Isolation of cellular RNA and primer extension mapping. Cellular RNA was isolated quantitatively as described by Wilson et al. (17). Primer extension mapping of the 5′ ends of *carAp₁::lacZ* transcripts was performed as previously described (26), except that 45 μg of RNA from uracil-grown cells and 30 μg of RNA from UMP-grown cells were used for analysis. The different amounts of RNA, which were isolated from the same mass of cells, reflect the different levels of stable RNA in cells growing at different rates. The primer used in these experiments was 5′-CGC GGATCCAGCTGAATCAATGCAAATCTGC, which was labeled with ³²P at the 5′ end. The 29 nucleotides at the 3′ end of this primer hybridize to nucleotides 24 to 52 in the *carAp₁::lacZ* transcript. Each quantitative primer extension mapping experiment described in Results was performed independently two times with essentially the same results.

RESULTS

Effects of *carAB* P1 mutations that change the sequence of the transcription start site on reiterative transcription *in vitro*. To assess the role of transcription start site sequence on reiterative transcription, we constructed two mutant versions of the *carAB* P1 promoter that changed the wild-type G start site to either A or AA (Fig. 2). These changes introduce the start site sequences found in the *gal* P2 and *pyrBI* promoters, respectively (Fig. 1). The wild-type and mutant promoter regions (from −58 to +40 counting from the wild-type transcription start site) were incorporated into 207/208-bp DNA templates that also included a strong (~98% efficient) downstream intrinsic transcription terminator. These fragments do not include the *carAB* P2 promoter, which is located 68 bp downstream from promoter P1 (12). All detectable transcription initiation at the wild-type *carAB* P1 promoter occurs at the G residue (by convention, referring to the nontemplate strand sequence) located 7 bases downstream from the −10 region (Fig. 1) (12). Based on previous studies of preferred sites of transcription initiation in *E. coli* (26), it was expected that essentially all transcription initiation at both mutant promoters would
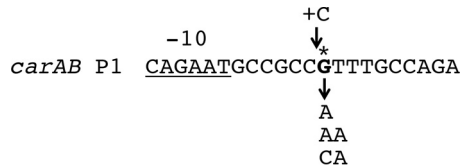
FIG 2 Sequences of *carAB* promoter P1 mutations used to assess the effects of transcription start site sequence and spacing between the start site and −10 region on reiterative transcription and gene regulation. The wild-type *carAB* promoter P1 sequence is shown and marked as described for Fig. 1, and arrows indicate the mutations introduced into this sequence. In two mutant promoters designed to examine start site sequence, the wild-type G start site was changed to either A or AA. In two other mutant promoters designed to examine the effects of spacing, either a C was inserted before the wild-type start site or the wild-type G start site was changed to CA.

also occur 7 bases downstream from the −10 region, at an A residue in this case (Fig. 2). This expectation was confirmed in experiments described below and by primer extension mapping of *in vitro* transcripts (data not shown). To examine effects on reiterative transcription, the DNA templates carrying either the wild-type or a mutant promoter were transcribed *in vitro* in reaction mixtures containing 400 μM each nucleoside triphosphate (NTP) except UTP, which was present at either 500 or 50 μM. The UTP concentrations were chosen to mimic levels found in cells grown under conditions of pyrimidine excess or limitation (27, 28). The reaction mixtures also included either [γ-^{32}P]GTP or [γ-^{32}P]ATP to label the 5′ ends of transcripts initiated at the wild-type and mutant promoters, respectively. Transcripts synthesized in each reaction were separated by gel electrophoresis, and radiolabeled transcripts were visualized by autoradiography (Fig. 3) and quantitated by phosphorimaging and densitometry.

The results with the wild-type promoter were essentially identical to our previously published characterization of *in vitro* transcription from the *carAB* P1 promoter (12). In the presence of 500 μM UTP, nearly all transcripts were included in a regularly spaced and progressively less intense ladder of transcripts with the general sequence GUUU$_n$, where $n = 1$ to >30 (Fig. 3, lane 1). Ladder transcripts longer than 4 nucleotides were the products of reiterative transcription. Several minor transcripts were also detected, and these transcripts, containing 5, 6, 9, 10, and 11 nucleotides, were previously shown to be faithful copies of the DNA template generated by simple abortive initiation (i.e., normal release of short transcripts independent of reiterative transcription) (12). Furthermore, a low level of full-length transcripts, which were terminated at the downstream intrinsic terminator, was detected. These transcripts were previously shown to be faithful copies of the DNA template (12). In comparison, production of the GUUU$_n$ ladder was greatly diminished, while the levels of simple abortive transcripts (especially the 5-mer GUUUC) and full-length transcripts were significantly (≥1.5-fold) increased in the presence of 50 μM UTP (Fig. 3, lane 2). These results clearly demonstrate UTP-mediated control of entry into the nonproductive reiterative mode of transcription at the *carAB* P1 promoter. It also should be noted that the experiments described above illustrate that same-length transcripts with different nucleotide contents migrate at different rates in the gel. Each nucleotide in the transcript contributes differently but consistently to gel mobility (C>A = U>G), which allows the sequence of short (<15-nucleotide-long) transcripts to be determined by the spacing between
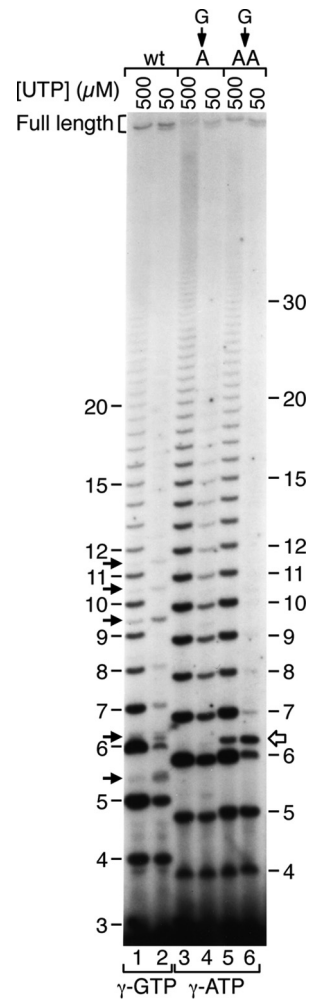

FIG 3 Comparison of reiterative transcription at the wild-type *carAB* P1 promoter and mutant promoters with changes in the sequence at the transcription start site. DNA templates containing the wild-type (wt) *carAB* P1 promoter (lanes 1 and 2) and the G-to-A (lanes 3 and 4) and G-to-AA (lanes 5 and 6) mutant *carAB* P1 promoters were transcribed *in vitro* in reaction mixtures containing 400 μM (each) ATP, CTP, GTP, and either 500 or 50 μM UTP, as indicated. Transcripts were radiolabeled at their 5′ ends with either [γ-^{32}P]GTP or [γ-^{32}P]ATP, as indicated, separated by polyacrylamide gel electrophoresis, and visualized by autoradiography. The lengths (in nucleotides) of transcripts initiated at the wild-type and mutant promoters are indicated at the left and right sides of the autoradiogram, respectively. Arrows indicate transcripts produced by simple abortive initiation: filled arrows indicate transcripts initiated at the wild-type promoter, and the open arrow indicates the AAUUUG transcript initiated at the G-to-AA promoter. Full-length transcripts are enclosed by a bracket and labeled; heterogeneity in the length of these transcripts is due to termination at multiple sites within the downstream intrinsic terminator (12).

adjacent bands in the gel as previously described (4). This technique is used extensively in the experiments described below.

Transcription from the G-to-A mutant promoter at 500 μM UTP also revealed a regularly spaced and progressively less intense ladder of transcripts with gel mobilities consistent with the general sequence AUUU$_n$, where $n ≥ 1$ (Fig. 3, lane 3). This ladder also was shown to comigrate with a known AUUU$_n$ ladder and contain only A and U residues (data not shown). Therefore, ladder transcripts longer than 4 nucleotides were the products of reiterative

transcription. This ladder was more intense and longer (apparently exceeding 100 nucleotides) than that observed with the wild-type promoter under the same reaction conditions. Very low levels of 5-mer and 6-mer transcripts (above the ladder transcripts AUUUU and AUUUUU in Fig. 3) also were detected and evidently were produced by simple abortive initiation. Full-length transcripts also were detected, but the level of these transcripts was much lower than that observed with the wild-type promoter. When the G-to-A promoter was transcribed in the presence of 50 μM UTP, the production of the AUUU$_n$ ladder was reduced roughly 3-fold but remained easily detectable. Additionally, the levels of the two minor aborted transcripts were increased slightly, and the level of full-length transcripts was increased nearly 4-fold (Fig. 3, lane 4). Taken together, these results indicate that reiterative transcription at the G-to-A promoter occurs much more extensively than it does at the wild-type promoter, resulting in a lower level of full-length transcript synthesis.

Transcription from the G-to-AA mutant promoter at 500 μM UTP again produced a regularly spaced ladder of transcripts, indicative of reiterative transcription and with gel mobilities consistent with the general sequence AAUU$_n$, where $n \geq 1$ (Fig. 3, lane 5). This ladder was shown to contain only A and U residues and to comigrate with the AAUUU$_n$ ladder produced *in vitro* by reiterative transcription at the *pyrBI* promoter (data not shown). Only ladder transcripts longer than 5 nucleotides were the products of reiterative transcription in this case. The intensity and length of the ladder produced at the G-to-AA promoter were intermediate between those produced at the wild-type and G-to-A promoters. The only other transcript detected with the G-to-AA promoter was a prominent 6-mer band that migrated slighter slower in the gel than the ladder transcript AAUUUU, indicating that this band was a simple aborted transcript with the sequence AAUUUG. Consistent with this assignment, this transcript was shown to comigrate with the AAUUUG aborted transcript produced at the *pyrBI* promoter (data not shown). When the UTP concentration was reduced to 50 μM, production of the AAUUUU$_n$ reiterative transcription ladder was greatly reduced, with only the 6-mer transcript being readily detectable and very low levels of the 7-mer and 8-mer transcripts observed (Fig. 3, lane 6). On the other hand, the levels of the AAUUUG aborted transcript and full-length transcripts were increased approximately 2-fold compared to the levels of these transcripts produced with 500 μM UTP. Overall, UTP-sensitive reiterative and nonreiterative transcription at the G-to-AA promoter was essentially identical to that at the *pyrBI* promoter (14), perhaps not surprisingly, because the sequences of the first 8 bp of the initially transcribed regions of these two promoters are identical (Fig. 1 and 2). Finally, comparing all three promoters, the extent of reiterative transcription at the G-to-AA promoter appeared to be similar to or slightly greater than that at the wild-type promoter and significantly less than that at the G-to-A promoter. These results suggest that the strength of base pairing between the DNA template and the 5′-end nucleotide(s) of the nascent transcript that precede the U-tract is inversely proportional to the extent of reiterative transcription.

Effects of *carAB* P1 mutations that change the sequence of the transcription start site on gene expression and regulation. To measure the effects of the G-to-A and G-to-AA *carAB* P1 promoter mutations on gene expression and regulation, we constructed a set of *carAp$_1$::lacZ* operon fusions that include either the wild-type or a mutant promoter region. Operon fusions were cre-

TABLE 1 Effects of start site sequence on expression and regulation of *carAp$_1$::lacZ* operon fusions[a]

Strain (*carAp$_1$* genotype)	ß-Galactosidase activity (nmol/min/mg) by pyrimidine level[b]		Regulation (fold)
	Excess	Limitation	
CLT5174 (wild type)	1,024	2,870	2.8
CLT5189 (G to A)	687	3,290	4.8
CLT5190 (G to AA)	1,100	2,780	2.5

[a] Doubling times were 46 ± 2 min for cells grown on uracil and 68 ± 3 min for cells grown on UMP.
[b] Values are the means from at least three independent determinations, with a variation of <8%.

ated by individually inserting a promoter region into plasmid pDLC126 and then transferring the fusion onto phage lambda RZ5 by recombination. The recombinant phage were used to infect strain CLT42 (*car-94 ΔlacZYA*), and lysogens carrying a single prophage at the lambda attachment site in the chromosome were isolated. These strains are pyrimidine (and arginine) auxotrophs, because the *car-94* mutation inactivates the chromosomal *carAB* operon. Each lysogenic strain was grown in glucose-minimal salts (plus arginine) medium containing either uracil or UMP as the pyrimidine source, which provides a condition of pyrimidine excess or limitation, respectively (22). The level of *lacZ*-encoded ß-galactosidase in each culture was assayed as an indicator of expression from the cloned *carAB* P1 promoter (Table 1).

In the wild-type promoter fusion strain (CLT5174), the level of ß-galactosidase in cells grown under conditions of pyrimidine limitation was 2.8-fold higher than that in cells grown with pyrimidine excess, as previously reported (12). In the G-to-A promoter fusion strain (CLT5189), the level of ß-galactosidase in cells grown under conditions of pyrimidine limitation was 4.8-fold higher than that in cells grown with pyrimidine excess, a range of regulation nearly twice that observed with the wild-type promoter fusion strain. This increase in range of regulation was due primarily to a lower level of expression of the mutant fusion operon under conditions of pyrimidine excess. In the case of the G-to-AA promoter fusion strain (CLT5190), the range of regulation (2.5-fold) and the levels of ß-galactosidase in cells grown under conditions of pyrimidine excess or limitation were approximately the same as the range of regulation and ß-galactosidase levels observed with the wild-type promoter fusion strain. A comparison of the results summarized above (Table 1) to the *in vitro* reiterative transcription analyses shown in Fig. 3 suggest that more extensive reiterative transcription, like that exhibited by the G-to-A promoter, permits greater repression of gene expression and a wider range of gene regulation.

Quantitative primer extension mapping of transcripts initiated at the mutant *carAB* P1 promoters with altered transcription start site sequence. To further characterize expression and regulation of the wild-type and G-to-A and G-to-AA mutant *carAp$_1$::lacZ* operon fusions, we used quantitative primer extension mapping to confirm the start sites and measure the levels of productive transcripts encoded by these operon fusions in lysogenic strains CLT5174 (wild type), CLT5189 (G to A), and CLT5190 (G to AA) grown under conditions of pyrimidine excess or limitation. Nonproductive reiterative transcripts synthesized in these strains cannot be detected by primer extension mapping.
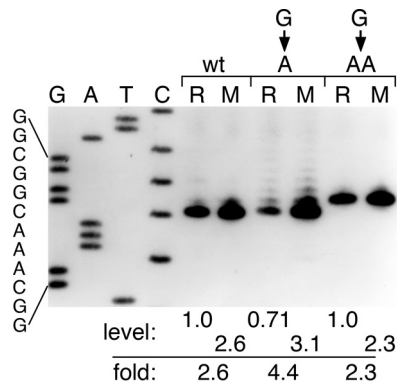
FIG 4 Levels of productive *carAp₁::lacZ* transcripts initiated *in vivo* at the wild-type *carAB* P1 promoter and mutant promoters with changes in the sequence at the transcription start site. Cellular RNA was quantitatively isolated from exponentially growing cells of strains CLT5174 (wild type), CLT5189 (G to A), and CLT5190 (G to AA) grown in the presence of either uracil (R) or UMP (M) (i.e., conditions of pyrimidine excess or limitation, respectively), and transcript levels were measured by primer extension mapping as described in Materials and Methods. The figure shows an autoradiogram of a gel used to separate and analyze primer extension products. A dideoxy sequencing ladder (i.e., the four lanes marked G, A, T, and C) of the wild-type promoter region, which was used to identify both wild-type and mutant transcripts, was produced with the same DNA primer as that used for primer extension mapping. (Note that the sequence shown on the left is that of the template DNA strand, which is complementary to the sequence of the wild-type *carAp₁::lacZ* transcript.) Primer extension product levels, which correspond to transcript levels, were measured with a PhosphorImager. Relative levels of transcripts and fold pyrimidine-mediated regulation are indicated.

Furthermore, transcripts from the resident *carAB* operon of the parent strain CLT42 are eliminated by the *car-94* mutation (12). As expected, our results show that nearly all transcripts initiated at the wild-type and mutant promoters are initiated at a G or A residue located 7 bases downstream from the −10 region (Fig. 4). The G-to-AA transcripts are one residue longer than wild-type (and G-to-A) transcripts due to the insertion of two mutant A residues and initiation at the first. In the case of the G-to-A promoter, we also observed a ladder of minor transcripts that was progressively longer than the dominant transcript. It is likely that these minor transcripts are produced by a small subset of transcripts containing extra U residues that, instead of being released from the initiation complex, are switched into the normal mode of transcript elongation.

With respect to transcript quantitation, our results showed that the relative levels of wild-type, G-to-A, and G-to-AA operon transcripts in cells grown with pyrimidine excess or limitation closely mirrored the operon-encoded ß-galactosidase levels included in Table 1 (Fig. 4). Accordingly, the ranges in pyrimidine-mediated regulation also were similar to those shown in Table 1. The ranges of regulation of transcript synthesis for the wild-type, G-to-A, and G-to-AA operons were 2.6-fold, 4.4-fold, and 2.3-fold, respectively. These results indicate that the observed pyrimidine-mediated regulation of wild-type and mutant *carAp₁::lacZ* operon expression occurs essentially entirely at the level of transcription.

Effects of *carAB* P1 mutations that increase the spacing between the −10 region and transcription start site on reiterative transcription *in vitro*. The *carAB* P1 and *gal* P2 promoters differ from the *pyrBI* promoter in that transcription initiation occurs

either 7 or 8 bases downstream from the −10 region, respectively (Fig. 1). To assess the role of spacing between the −10 region and transcription start site on reiterative transcription, we constructed two additional mutant versions of the *carAB* P1 promoter in which either a C residue was inserted in front of the G start site of the wild-type promoter (+C mutant) or the G start site was changed to CA (G-to-CA mutant) (Fig. 2). Based on the fact that C is an extremely poor initiating nucleotide and other known preferences for transcription start site selection (26), we expected that essentially all transcription initiation at both mutant promoters would occur 8 bases downstream from the −10 region, at a G or A residue at the +C and G-to-CA promoters, respectively. Again, our expectation was confirmed in experiments described below and by primer extension mapping of *in vitro* transcripts (data not shown). Thus, the +C and G-to-CA promoters provided increased-spacing variants of the wild-type and previously described G-to-A promoters. The +C and G-to-CA promoters were analyzed by *in vitro* transcription with either 500 or 50 μM UTP as described above, and the results were compared to transcription from the corresponding shorter-spacing promoter (Fig. 5).

Transcription at the +C promoter produced a set of transcripts (Fig. 5, lanes 3 and 4) with gel mobilities identical to those of the transcripts synthesized with the wild-type promoter (Fig. 5, lanes 1 and 2). Furthermore, we showed that production of the regularly spaced ladder of transcripts initiated at the +C promoter (i.e., numbered transcripts in Fig. 5) required only GTP and UTP as substrates, consistent with the general transcript sequence of GUUU$_n$ (data not shown). These results indicate that the sequences of the transcripts initiated at the wild-type and +C promoters are identical; therefore, the start site at the +C promoters is located 8 nucleotides downstream from the −10 region. Quantitatively, the levels of transcript synthesis and UTP-mediated control of reiterative transcription with the wild-type and +C promoters were similar; however, there were two small but reproducible differences in transcript levels. First, the levels of 4-mer (GUUU) and 5-mer (GUUUU) transcripts initiated at the +C promoter were higher than the levels of these transcripts initiated at the wild-type promoter: 1.5-fold to 2-fold higher at 500 μM UTP and ≤1.5-fold higher at 50 μM UTP. The 4-mer transcripts apparently were produced by simple abortive initiation, while the 5-mer transcripts were the most abundant transcript produced by reiterative transcription. These results suggest that both simple abortive initiation and reiterative transcription were enhanced by a single base increase in the spacing between the −10 region and transcription start site of the wild-type promoter. Second, there was a modest (≤1.5-fold) reduction in the synthesis of full-length transcripts at the +C promoter which was typically slightly greater at 500 μM UTP. This reduction could be the consequence of the observed increases in abortive initiation and reiterative transcription.

Transcription from the G-to-CA promoter produced a set of transcripts (Fig. 5, lanes 5 and 6) that appeared to be identical to those initiated at the G-to-A promoter (Fig. 5, lanes 7 and 8) based on gel mobilities. Consistent with this assignment, we showed that the putative AUUU$_n$ ladder of transcripts initiated at the G-to-CA promoter contain only A and U residues (data not shown). These results demonstrate that the start site at the G-to-CA promoter is located 8 nucleotides downstream from the −10 region. As observed with the G-to-A promoter, UTP-mediated regulation of reiterative transcription at the G-to-CA promoter was readily apparent; however, the levels of transcripts initiated at the two promot-
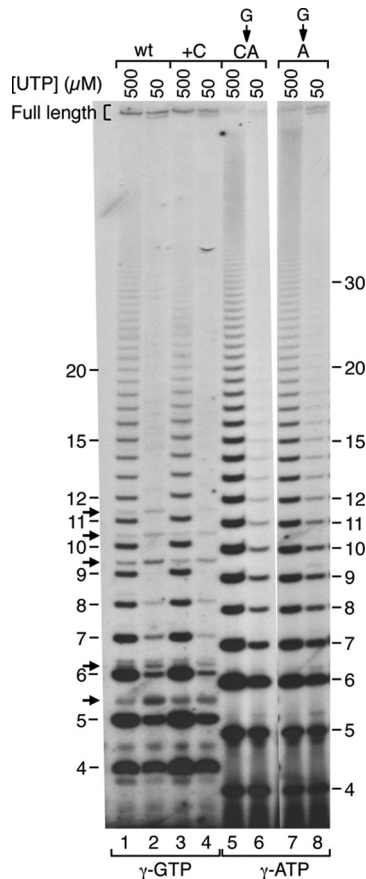
FIG 5 Comparison of reiterative transcription at wild-type and mutant *carAB* P1 promoters with different spacing between the −10 region and the transcription start site. DNA templates containing the wild-type (wt) *carAB* P1 promoter (lanes 1 and 2) and the +C (lanes 3 and 4), G-to-CA (lanes 5 and 6), and G-to-A (lanes 7 and 8) mutant *carAB* P1 promoters were transcribed *in vitro*, ^{32}P labeled at their 5′ ends, and analyzed as described in the legend to Fig. 3. All lanes were from the same autoradiogram, but lanes 7 and 8 were repositioned to facilitate comparison to lanes 5 and 6. The lengths (in nucleotides) of GUUU$_n$ transcripts initiated at the wild-type and +C mutant promoters are indicated on the left, with arrows indicating transcripts produced by simple abortive initiation at the two promoters. The lengths of AUUU$_n$ transcripts initiated on the G-to-CA and G-to-A mutant promoters are indicated at the right. The [γ-^{32}P]NTP used to label the 5′ ends of transcripts is indicated at the bottom, and full-length transcripts are enclosed by a bracket and labeled.

ers were different. At 500 μM UTP, the levels of reiterative transcripts initiated at the G-to-CA promoter were nearly 2-fold higher than those initiated at the G-to-A promoter. This higher level of reiterative transcript synthesis was accompanied by a nearly 2-fold decrease in the synthesis of full-length transcripts at the G-to-CA promoter. At 50 μM UTP, the production of reiterative transcripts at both promoters appeared similar; however, synthesis of full-length transcripts at the G-to-CA promoter was approximately 2-fold less than that at the G-to-A promoter. Therefore, our analysis of spacer-variant promoters, particularly the promoters with an A start site, suggest that the spacing between the −10 region and the transcription start site influences the extent of reiterative transcription and production of full-length transcripts.

Effects of *carAB* P1 mutations that increase the spacing between the −10 region and the transcription start site on gene expression and regulation. To measure the effects of spacing be-

tween the −10 region and transcription start site on gene expression and regulation, we constructed (as described above) two additional variants of strain CLT42 that carry a *carAp1*::*lacZ* operon fusion, including either the +C or G-to-CA *carAB* P1 promoter mutation. The resulting +C (CLT5237) and previously described wild-type promoter strains, as well as the resulting G-to-CA (CLT5238) and previously described G-to-A promoter strains, constitute pairs of strains that differ only in the spacing between the −10 region and transcription start site of the *carAp1*::*lacZ* operon. These four strains were grown in the minimal medium described above containing either uracil or UMP to provide conditions of pyrimidine excess and limitation, respectively. The level of *lacZ*-encoded ß-galactosidase in each culture was assayed as an indicator of expression from the *carAB* P1 promoter, and the ranges of pyrimidine-mediated regulation of *carAp1*::*lacZ* expression were calculated.

The results with each pair of comparable strains indicated that increasing the spacing between the −10 region and transcription start site from 7 to 8 bp resulted in an increase in the range of regulation (Table 2). For the pair of strains with a G start site, regulation occurred over a 2.8-fold range with the wild-type promoter strain compared to a 5.5-fold range with the +C promoter strain. The higher range of regulation with the +C promoter strain was due entirely to a >2-fold lower level of ß-galactosidase in cells grown under conditions of pyrimidine excess. Under conditions of pyrimidine limitation, ß-galactosidase levels were similar in both strains. For the pair of strains with an A start site, regulation occurred over a 4.8-fold range with the G-to-A promoter strain compared to a 7.1-fold range with the G-to-CA promoter strain. In this case, the ß-galactosidase levels in the G-to-CA promoter strain were significantly lower than those in the G-to-A promoter strain under both growth conditions. However, the fold decrease in the ß-galactosidase level was greater under conditions of pyrimidine excess, resulting in the wider range of regulation. It is also noteworthy that, in general, the levels of *carAp1*::*lacZ* expression shown in Table 2 are inversely proportional to the extent of reiterative transcription and directly proportional to the levels of full-length transcripts produced *in vitro* with DNA templates carrying the corresponding *carAB* P1 promoter (Fig. 5).

Quantitative primer extension mapping of transcripts initiated at the mutant *carAB* P1 promoters that increase the spacing between the −10 region and transcription start site. To further characterize the productive transcripts initiated *in vivo* from the +C and G-to-CA promoters, we used quantitative primer exten-

TABLE 2 Effects of spacing between the −10 region and transcription start site on expression and regulation of *carAp1*::*lacZ* operon fusions[a]

Strain (*carAp1* genotype)	ß-Galactosidase activity (nmol/min/mg) by pyrimidine level[b]		Regulation (fold)
	Excess	Limitation	
CLT5174 (wild type)	1,024	2,870	2.8
CLT5237 (+C)	460	2,520	5.5
CLT5189 (G to A)	687	3,290	4.8
CLT5238 (G to CA)	144	1,020	7.1

[a] Doubling times were 46 ± 2 min for cells grown on uracil and 68 ± 3 min for cells grown on UMP.
[b] Values are the means from at least three independent determinations, with a variation of <10%.
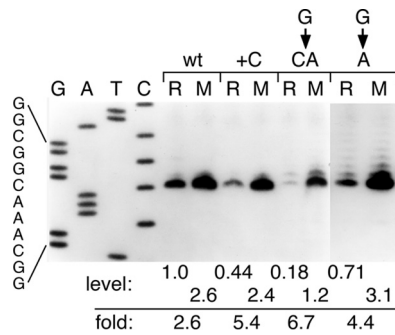
FIG 6 Levels of productive *carAp₁::lacZ* transcripts initiated *in vivo* at wild-type and mutant *carAB* P1 promoters with different spacing between the −10 region and transcription start site. Cellular RNA was quantitatively isolated from exponentially growing cells of strains CLT5174 (wild type), CLT5237 (+C), and CLT5238 (G to CA) grown in the presence of either uracil (R) or UMP (M), and transcript levels were analyzed by primer extension mapping as described above. The primer extension mapping data for strain CLT5189 (G to A) were taken from Fig. 4; the analysis of the data in Fig. 4 and 6 was identical. The dideoxy sequencing ladder (lanes G, A, T, and C) of the wild-type promoter region was generated as described in the legend to Fig. 4. Primer extension product levels, which correspond to transcript levels, were measured with a PhosphorImager, and the relative levels of transcripts and fold pyrimidine-mediated regulation are indicated.

sion mapping to analyze transcripts encoded by the *carAp₁::lacZ* operon fusions in strains CLT5237 (+C) and CLT5238 (G to CA) (and, as a reference, wild-type promoter strain CLT5174) grown under conditions of pyrimidine excess or limitation (Fig. 6). With the exception of one sample, the data revealed that a single major transcript was initiated at both the +C and G-to-CA promoters and that these transcripts were the same length as the major transcript initiated at the wild-type promoter. This result confirmed that the major +C and G-to-CA transcripts were initiated 8 bases downstream from the −10 region (at G and A residues, respectively). In the sample from strain CLT5238 (G to CA) grown under conditions of pyrimidine excess, only low levels of two transcripts were detected, including the transcript described above, which initiates at the A residue located 8 bases from the −10 region. Low levels of this transcript were expected because of the high levels of nonproductive reiterative transcription that occur at the G-to-CA promoter in the presence of high levels of UTP (Fig. 5). The other low-level transcript appeared to be 1 base longer and also was detected in CLT5238 (G to CA) cells grown with pyrimidine limitation. This transcript most likely was initiated at a minor C start site located 7 bases downstream from the −10 region, the preferred start site location for *E. coli* RNA polymerase (26).

To allow comparison of *in vivo* transcription from our two pairs of *carAB* P1 promoters that differ only in the spacing between the −10 region and transcription start site, equivalent primer extension mapping data obtained with strain CLT5189 (G to A) were included in Fig. 6. The data from the four strains showed that the relative levels of productive *carAp₁::lacZ*-specific transcripts closely mirrored the relative levels of *carAp₁::lacZ*-encoded ß-galactosidase shown in Table 2. Consequently, for each strain, the range of pyrimidine-mediated regulation determined from productive transcript levels was nearly the same as the range of regulation calculated from ß-galactosidase levels. Thus, the primer extension mapping data again show that a single-base increase in promoter spacing results in an increase in the range of pyrimidine-mediated regulation of *carAp₁::lacZ* expression.

DISCUSSION

The goal of this study was to determine the effects of promoter sequence on mechanisms of gene regulation in *E. coli* that are based on reiterative transcription at T tracts during transcription initiation. This reaction, which is promoted by high intracellular levels of UTP, causes early transcript release and reduced gene expression. We focused on three analogous control mechanisms involved in regulating *carAB*, *pyrBI*, and *gal* expression through UTP-sensitive reiterative transcription at the *carAB* P1, *pyrBI*, and *gal* P2 promoters, respectively. The range of regulation for these mechanisms varies from 2-fold to 7-fold, and this variation presumably reflects differences in promoter sequences. We used the *carAB* P1 promoter (with an initially transcribed region sequence of 5′-GTTTG) as a platform to introduce sequence elements of the *pyrBI* and *gal* P2 promoters that changed the sequence at or near the transcription start site or the spacing between the promoter −10 region and the start site. Our results showed that both the sequence and spacing of the transcription start site significantly affect the extent of reiterative transcription, level of gene expression, and range of gene regulation.

To examine the effects of start site sequence (or, more accurately, the sequence of the initially transcribed region preceding the T tract), we changed the wild-type G start site to either A or AA, making the sequence resemble that of the *gal* P2 and *pyrBI* promoters, respectively. These changes are particularly relevant because ATP and GTP are the strongly preferred initiating NTPs in *E. coli* (26, 29–31). Our comparisons of the resulting promoters suggest that the importance of start site sequence is, at least in large part, its contribution to the base-pairing strength of the RNA-DNA hybrid formed by the first 4 or 5 residues at the 5′ end of the nascent transcript and the DNA template. Previous studies had, in fact, demonstrated that weak RNA-DNA base pairing facilitates reiterative transcription during transcription initiation and elongation (3, 11, 32, 33). Using different promoter contexts, we showed that an RNA-DNA hybrid containing the 5′-AUUU transcript allowed more extensive reiterative transcription, especially at high UTP concentrations, than that observed with stronger hybrids formed with 5′-GUUU and 5′-AAUUU transcripts. On the other hand, reiterative transcription was similar at the promoters specifying the 5′-GUUU and 5′-AAUUU transcripts, evidently reflecting similar calculated stabilities of the RNA-DNA hybrids formed with these two transcripts. Our results also demonstrated a direct correlation between nonproductive reiterative transcription and reduced gene expression *in vivo* and between the extent of reiterative transcription and range of gene regulation. For instance, changing the start site sequence from G to A increased the range of regulation nearly 2-fold. The wider range of regulation is achieved by increased nonproductive reiterative transcription at high UTP levels, resulting in decreased gene expression, coupled with a smaller effect on reiterative transcription and gene expression at low UTP levels.

The effects of spacing between the promoter −10 region and transcription start site were examined with promoters in which the spacing was either 7 or 8 bases, which corresponds to the spacing in the *carAB* P1 and *gal* P2 promoters or the *pyrBI* promoter, respectively. These two sites are the most preferred locations for transcription initiation in *E. coli*, with position 7 typically the better start site unless it is occupied by a poor initiating nucleotide (26, 29–31). The effect of spacing, observed with promoters

with either GTTT or ATTT as the initially transcribed region, was significant. Increasing the spacing from 7 to 8 bases resulted in increased reiterative transcription *in vitro* (more obvious with A-initiated transcripts), reduced gene expression *in vivo*, and an increase in the range of gene regulation from 1.5-fold to 2-fold with promoters with ATTT and GTTT initially transcribed regions, respectively. Again, the wider range of regulation appeared to be due to a greater increase in the level of nonproductive reiterative transcription (and decreased gene expression) at high UTP concentrations than that observed at low UTP levels.

Taken together, our results show that the level of gene expression and the range of gene regulation established by conditional nonproductive reiterative transcription, like that occurring at the *carAB* P1, *pyrBI*, and *gal* P2 promoters, can be modulated by subtle changes in promoter sequence. We investigated only a limited number of changes in transcription start site sequence and spacing between the −10 region and the start site. There likely are other seemingly modest changes in promoter sequence and architecture that have significant effects on gene expression and regulation. A case in point is the greater than 2-fold different ranges of gene regulation observed with the G-to A mutant *carAB* P1 promoter and the *gal* P2 promoter (13) under identical conditions, even though these two promoters have the same initially transcribed region sequence (i.e., ATTT) and the same 7-base spacing between the −10 region and start site. Presumably, the different ranges of regulation are due to some other difference in the G-to-A and *gal* P2 promoters. The identification of this and other such modulatory promoter features obviously will require additional investigation.

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