The Upstream-activating Sequences of the $\sigma^{54}$ Promoter $Pu$ of *Pseudomonas putida* Filter Transcription Readthrough from Upstream Genes*

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Although the $m$-xylene-responsive $\sigma^{54}$ promoter $Pu$ of *Pseudomonas putida* mt-2, borne by the TOL plasmid pWW0, is one of the strongest known promoters in vivo, its base-line level in the absence of its aromatic inducer is below the limit of any detection procedure. This is unusual because regulatory networks (such as the one to which $Pu$ belongs) can hardly escape the noise caused by intrinsic fluctuations in background transcription, including that transmitted from upstream promoters. This study provides genetic evidence that the upstream-activating sequences (UAS), which serve as the binding sites for the pWW0-encoded XylR protein (the $m$-xylene-responsive $\sigma^{54}$-dependent activator of $Pu$), isolate expression of the upper TOL genes from any adventitious transcriptional flow originating further upstream. An in vivo test system was developed in which different segments of the $Pu$ promoter were examined for the inhibition of incoming transcription products from an upstream promoter in *P. putida* and *Escherichia coli*. Minimal transcription filter ability was located within a 105-bp fragment encompassing the UAS of $Pu$. Although S1 nuclease assays showed that the UAS prevented the buildup of downstream transcripts, the mechanism seems to diverge from a typical termination system. This was shown by the fact that the UAS did not halt transcription in vitro and that the filter effect could not be relieved by the anti-termination system of $\lambda$ phage. Because the $Pu$ promoter lies adjacent to the edge of a transposon in pWW0, the preset transcriptional filter in the UAS may isolate the upper TOL operon from undue expression after random insertion of the mobile genetic element in a new replicon.

Bacterial promoters form part of regulatory networks through which signals are propagated faithfully from one member to the next (1, 2). This course of events is frequently affected by fluctuations brought about by variations in the pool of housekeeping regulatory proteins (the most common of which is RNAP$^D$ and its $\sigma$ factors) as well as by changes in environmental conditions (3–5). Although noise is intrinsically associated with molecular events involving few components, how cells keep regulatory noise within limits is still unknown (4). Although cells may occasionally gain from the biological consequences of random fluctuations in gene expression, noise may end up destroying biological circuits. However, bacteria appear to control noise in natural gene networks and thus avoid regulatory and metabolic chaos (1, 2).

Although some cellular mechanisms can tolerate transcriptional noise, the same may be detrimental in scenarios in which carefully coordinated gene community behavior is necessary. For example, regulatory circuits that control the expression of metabolic programs for the biodegradation of pollutants in soil bacteria require the suppression of transcriptional noise if these organisms are to survive initial exposure (6). How cells organize their transcriptional response can be examined by analyzing the biodegradation of $m$-xylene by *Pseudomonas putida* mt-2, a function encoded by the catabolic TOL-plasmid pWW0 which it carries (7). Individual cells failing to demonstrate the required catabolic ability at any given time or location are surely displaced by fitter members of a community (8, 9). When *P. putida* mt-2 is challenged with $m$-xylene in the medium, much of the available transcriptional machinery is reassigned to allow the bacterium to endure this general stress (10, 79). In theory this could reduce the availability of the RNAP and other transcription factors necessary for expression of the xyl genes borne by plasmid pWW0, thereby making the corresponding catabolic promoters more sensitive to cell-to-cell variations. However, this is not the case because TOL genes seem to be equally expressed in all cells under these conditions (11). These features of the TOL plasmid prompted us to examine in more detail the aspects of the catabolic promoters that might be related to noise suppression. A remarkable feature of this system is the blend of extraordinary transcriptional capacity with an extremely low basal expression of $Pu$, the main $m$-xylene-responsive promoter of pWW0 (Fig. 1). Although the output of $\beta$-galactosidase from a chromosomal *P. lacZ* fusion reaches 10,000–15,000 Miller units in *P. putida* cells exposed for a short time to $m$-xylene, promoter activity in noninduced cells is below the detection limits of this reporter (12). $Pu$ belongs to the class of promoters that depends on the alternative $\sigma$ factor, $\sigma^{32}$, and is activated at a distance by the toluene-responsive activator XylR (13, 14). This involves the binding of the regulator to upstream-activating sequences (UAS) and the looping out of the complex into close proximity to the $\sigma^{32}$-containing form of RNA polymerase bound to the $-12/-24$ region of the promoter. This event is assisted by the binding of the integration host factor (IHF) to the region between the UAS and the $\sigma^{32}$ RNAP attachment site (Fig. 1). This facilitates contact between distant proteins and aids in the recruitment of $\sigma^{32}$-RNAp to $-12/-24$ (15, 16). Moreover, IHF enhances the specificity of $Pu$ for its legitimate activator XylR (17).

During the course of experiments on the expression of plasmid-encoded proteins in maxicells, it was noticed that the $Pu$ promoter is endowed with the capacity to stop transcription that originated upstream (18). At that time, however, the phenomenon was not understood, other than being a nuisance when trying to determine the gene content of different fragments of pWW0, and it was given no biological significance. In this work, we

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2 The abbreviations used are: RNAP, RNA polymerase; UAS, upstream-activating sequences; IPTG, isopropyl $\beta$-D-galactopyranoside; ssDNA, single-stranded DNA; IHF, integration host factor; RT, reverse transcriptase; 3MBA, 3-methylbenzyl alcohol; nt, nucleotide.
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address in detail the ability of the Pu promoter to inhibit readthrough transcription from upstream promoters. The results show that this activity can be traced to an ~100-bp DNA sequence that spans the binding site for the activator protein XylR. Moreover, the mechanism of inhibition is unlike a typical transcription termination event. It is also argued that such an effect is instrumental in protecting the regulatory sub-network of the TOL system from the transcriptional noise of the host.

MATERIALS AND METHODS

Strains, Plasmids, and General Methods—P. putida strain KT2442, a rifampicin-resistant derivative of the reference P. putida strain KT2440 (19), was used for the reporter DNA segments indicated in each case. Escherichia coli CC118 (Δara-leu araD lacX74 galE galK phoA thi1 rpsE rpoB argE-Am recA1; see Ref. 20) was employed as a genetically reliable host for in vivo transcription assays. Two derivatives of this strain were used for different purposes: E. coli CC118 Pu-lacZ, with its chromosomal insertion of mini-Tn5 Sm Pu-lacZ, was used to monitor the activity of the Pu promoter in single copy gene dosage by measuring the output of β-galactosidase (21), and E. coli CC118 Apir (22) was used as a recipient for all mini-transposon delivery vectors with a Pu protein-dependent R6K origin of replication. The mobilizing strain E. coli S17-1 Apir (22) was used for conjugal transfer. This strain expresses the replication Pu protein as well as the tra genes of the broad host range plasmid RP4 (which encodes functions for conjugal transfer of plasmids endowed with an oriT sequence; see Ref. 22). The plasmids used in this work were as follows: pUG11 (a kind gift of C. Kane), which consists of the N gene of the A phage under the control of the left promoter (Pr), cloned in vector pUC18; pCI857, a Km′ plasmid derived from pMC931 (i.e. it has a p15A origin of replication) in which the thermosensitive variant of the A repressor is expressed through its own native promoter (23); and pFHR, a derivative of the Cmr monocopy vector pVDL8 (24), which has a 1.9-kb segment of the TOL plasmid spanning the xylR gene downstream of its native promoter Pr. Predictions of secondary mRNA structures were generated with the mfold program (25).

Assembly of Hybrid Mini-transposons—Recombinant DNA techniques were performed as described previously (26). Fig. 1 shows the DNA segments from Pu employed for the various constructs described below, all derived from pCG2Pu (27), an Ap′ ori ColE1 ori M13 phagemid derived from vector pCG2 containing an EcoRI-BamHI fragment of pEZ9 (21) spanning positions −211 to +107 of the promoter. The U-containing, single-stranded pCG2Pu DNA was employed as the substrate for the introduction of new EcoRI sites at locations U-containing, single-stranded pCG2

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(pCNB5::Ptrc → UAS-D → lacZ). At various stages of the process, automated DNA sequencing in an Applied Biosystems device verified the inclusion of the cloned inserts and DNA fragments.

Mobilization and Transposition—To generate P. putida and E. coli strains carrying the reporter DNA segments specified in each case, each of the pCNB5 derivatives mentioned above was transformed into the mobilizing bacterial strain E. coli S17-1 Apir and then passed by conjugation into the target cells using a filter-mating technique (21). After 8 h of incubation at 30 °C on LB plates, the cells were washed with 10 mM MgSO4 and plated on either M9 citrate medium with 50 μg/ml kanamycin for Pseudomonas or LB with 50 μg/ml rifampicin and kanamycin for E. coli. Exconjugants were then screened for the lacZ+ phenotype accompanied by the loss of the pipercillin/ampicillin marker to confirm the correct insertion of the reporter construct in the mini-transposon vector (21). The result was the insertion of the mini-Tn5 vectors with the built in functional segments indicated in Fig. 2 (top) into the chromosome of either P. putida KT2442 or E. coli CC118. The designations of the resulting strains were as follows: P. putida 19C (control, KT2442::mini-Tn5 Km (lacP′ Ptrc → lacZ)), P. putida 19 (KT2442::mini-Tn5 Km (lacP′ Ptrc → UAS/DP → lacZ)), P. putida 36 (KT2442::mini-Tn5 Km (lacP′ Ptrc → UAS-D → lacZ)), P. putida 37 (KT2442::mini-Tn5 Km (lacP′ Ptrc → UAS-P → lacZ)). The corresponding insertions in E. coli CC118 originated strains CC118-19C, CC118-19, CC118-36, and CC118-37. The organization of such insertions (Figs. 2 and 3) shields the reporter cassettes from readthrough transcription from upstream and downstream host promoters, thus minimizing positional effects on the mobile element.

Construction of Plasmids for Anti-termination Assays—The test plasmids for examining N-mediated anti-termination were assembled in vector pVTR-A (30). This is a single-copy Cmr plasmid in which a lacP′ plus the hybrid trp/ lac promoter, Ptrc, is followed by a multiple cloning site. pVTR-A was digested with BamHI and SalI and a BglII-SalI DNA fragment from pIZ280 (31) containing the fusion galK::lacZ inserted as a reporter. This gave rise to the reference plasmid pK1 (lacP′ Ptrc → galK::lacZ). A 180-bp segment of Pu spanning the UAS and the ISph site (Fig. 1) was then amplified by PCR using primers AUAS-L (5′-GCCGGTACCCGCGATAGCCCT-3′) and AUAS-R (5′-GCTTTAT-ACCAGATC CCGGTTTCA-3′) (restriction sites in bold). This added flanking Km′ and Xmal ends to the PCR fragment, which was then inserted at the corresponding sites of pK1, generating pL1. Alternatively, a synthetic TR2 A phage terminator (32–34) was produced by hybridizing oligonucleotides TER-A (5′-TATTTGATGCCCT-3′) as a 69-bp EcoRI fragment (55 bp correspond-
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under study to an absorbance of 0.05–0.5 at 600 nm (A600). At this point, 0.1–1.0 mM IPTG was added, and when specified, the growth temperature was raised to 42 °C. Where indicated, 1 mM of the upper TOL pathway inducer 3-methylbenzyl alcohol (3MBA) was added to the cultures. Four hours after induction, P. putida and E. coli cells were collected, permeabilized with chloroform and SDS, and subjected to β-galactosidase assays (79) to determine the output of the lacZ fusions under the conditions mentioned in each case. The linearity of the assay within the range of cell densities and the development of the reaction with o-nitrophenyl β-D-galactopyranoside were verified in all cases. The β-galactosidase activity values given throughout this paper are the mean of at least three independent experiments conducted in duplicate (deviations ±15%).

In Vitro Transcription Assays—The DNA fragments for transcription in vitro were generated by amplification of relevant portions of pFH19C (no UAS), pFH19 (UAS/DP), and pFH36 (UAS-D) with oligonucleotides TER2 (5’-CACTCCCCGTCTGGAATAAG-3’) and TER3 (5’-CAGATGCGTCCGGTGA-3’). These primers hybridized 51 bp upstream of the transcription initiation site of thePtrc promoter and immediately downstream of the BamHI restriction site, respectively, which precedes the reporter trp::lacZ gene (see above). The resulting fragments were purified and used as linear DNA templates at a concentration of 20 ng/ml in transcription reactions performed in 25 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl2 following a standard technique (37). Briefly, cold ATP, CTP, and GTP were added, and the reactions were incubated for 15 min at 37 °C. Heparin (100 Ci of [32P]UTP (3000 Ci/mmol) were added. Four hours after induction, the DNA were mixed in a volume of 25 µl and 1 µl of RNasin (10 units/µl) and 1 µCi of [α-32P]UTP (3000 Ci/mmol) were added. Four units of E. coli RNA polymerase (a kind gift from F. Rojo) were then added, and the reactions were incubated for 15 min at 37 °C. Heparin (10 µg/ml) was added and the incubation continued for 2 min to complete ongoing transcription rounds. Finally, the reactions were stopped with 25 µl of TE buffer, 1 µl of 0.5 M EDTA and 1 µl of 10 µg/µl carrier tRNA. Samples were filtered through 1 ml of Sephadex G-50 equilibrated with TE buffer and precipitated with ethanol. The sediment was resuspended in loading buffer with 90% formamide and electrophoresed in a denaturing 8 M urea, 6% polyacrylamide gel, and the transcripts were visualized by autoradiography.

S1 Nuclease Assays—The method described by Sambrook et al. (26) was used for extracting total RNA from E. coli cells. The RNA was treated with DNase to eliminate any DNA contamination and further treated with phenol/chloroform treatment to eliminate residues of the enzyme. To produce the labeled single-stranded DNA (ssDNA) probe used in the experiments, plasmid pK1 (see above) was cleaved with XmaI, hybridized with the oligonucleotide LacS1 (5’-GGTGTTAGTG-CCGGTACG-3’) labeled at the 5’ end with [α-32P]ATP and subjected to 30 cycles of linear amplification with Taq polymerase, similar to the procedure described by Ding et al. (38). Because the 5’ end of the labeled primer binds 63 nucleotides downstream of the galK::lacZ fusion, the ssDNA is produced by the amplification spanned positions −245 (5’ end) to +63 (3’ end) of the galK::lacZ coding sequence. For the S1 nuclease protection assays, 50 µg of RNA from each sample were hybridized with an excess of the labeled ssDNA probe, digested with S1, and processed as described by Ausubel et al. (39). Samples were loaded onto a DNA sequencing gel with 7 M urea, run at high voltage, and dried. Autoradiographic images were acquired on x-ray film.

Semi-quantitative RT-PCR—Two micrograms of total RNA extracted from the E. coli cells under analysis were retrotranscribed using the first-strand cDNA synthesis kit (Amersham Biosciences). 10-Fold serial dilutions of the resulting product were used as templates for a standard PCR with primers PSREV (5’-ATGAGCTTGTGACAATTAATAC-3’) and GALK (5’-CGGTGGCGGACGGCAGAAGG-3’). These hybridize just downstream of the Ptrc promoter and the leading sequence of the reporter gene, respectively, thereby amplifying transcript segments from pK1 (147 bp), pK3 (262 bp), and pLIN (373 bp). The PCR was set at 30 cycles of 30 s at 90 °C, 30 s at 60 °C, and 30 s at 72 °C followed by a final 10-min extension at 72 °C.

RESULTS

The UAS of the Pu Promoter Inhibited Readthrough Transcription—Initial observations on the ability of the whole α44 Pu promoter of the TOL plasmid, or parts of it, to impede the progress of upstream transcripts were made in maxicells (18). In these assays, DNA sequences downstream of the native Smal site at −205 bp from the transcription initiation site of Pu (Fig. 1) altogether prevented the expression of genes from a P1 promoter of λ phage artificially placed upstream. Although little importance was given at that time, this prompted us to re-examine the phenomenon from a fresh perspective. Experiments were therefore performed to confirm this fact and to trace its origin to a minimum of elements. For this, we created new EcoRI sites at several places in the DNA sequence encompassing the Pu promoter, from its Smal site at (−205) all the way down to the HaeIII site at +93. This generated four main EcoRI segments designated UAS/IHF (167 bp, including the UAS for the cognate activator of Pu, the XylR protein, and the IHF site), UAS/DP (105 bp, containing both the distal UAS-D and the proximal UAS-P), UAS-P (52 bp, the proximal UAS only), and UAS-D (53 bp, the distal UAS-only). Each of these fragments was cloned in front of a promoterless lacZ gene and placed downstream of an IPTG/lacP-controlled Prtc promoter within a mini-Tn5 transposon vector. The resulting DNA segments gave rise to a reporter system (Fig. 2, top) consisting of the DNA under examination placed between a strong IPTG-inducible promoter and lacZ. As explained below, this provided a dependable genetic test for the inhibition of incoming transcription from Prtc by DNA sequences located between the promoter and the β-galactosidase gene.
Combining the expression inhibition observed in the test system, the specific UAS sequences borne by each of the strains tested were inserted in front of but fully induced is indicated in each case. Note that because the removal of the site had no consequences. However, the observable phenomenon was identical in either strain (Fig. 2). In contrast, the presence of XylR or XylR activated by 3MBA (Fig. 3, panels c and d) is that the UAS/P segment completely prevented inhibitory activity. Taken together, these data pinpoint the UAS of Pu (Fig. 1) as the sequences responsible for the blockade of transcripts from Puc.

Transcriptional Inhibition Brought About by Pu UAS Is Independent of XylR—Because the UAS are the binding sites for XylR (the cognate, aromatic-responsive regulator of the Pu promoter with which it forms a large multicomponent complex) (40, 41), the above results raise the possibility that inhibition occurs through interference of ongoing transcription via a preformed nucleoprotein assembly (42, 43). To examine this, the assay system described above was recreated in E. coli strain CC118 by inserting the reporter transposon bearing the UAS-DP sequence, or a control devoid of UAS, into its chromosome. The strains generated were transformed with the xylR+ plasmid pFHR and exposed to various induction conditions with or without IPTG in the presence or absence of 1 mM of the XylR effector 3-methylbenzyl alcohol (44). As a positive control of the binding of XylR to the UAS, the same plasmid was transformed into the isogenic strain E. coli CC118 Pu-lacZ, which bears a transcriptional fusion between the Pu promoter and the β-galactosidase gene. This is expressed only upon activation of Pu because of the binding of the multimeric form of XylR activated by 3MBA (45). β-Galactosidase accumulation in this strain therefore faithfully reflects XylR binding to the UAS under the conditions of the assay.

Fig. 3 shows the results of the experiments performed in E. coli. Comparison of the data for strains with and without the UAS (Fig. 3, panels a and b) confirms that the expression inhibition phenomenon seen in P. putida can be reproduced in E. coli. Yet the maximum transcriptional output of the control strain Ptc-lacZ without the UAS was ~5000 Miller units in monocopy gene dosage in E. coli, roughly half that observed in P. putida. However, the observable phenomenon was identical in either strain (Fig. 2). In contrast, the presence of XylR or XylR activated by 3MBA (Fig. 3, panels c and d) made no difference to the inhibition of readthrough transcription from Ptc. That XylR was able to bind the UAS under the conditions of the experiment was shown by the induction observed in parallel in E. coli CC118 Pu-lacZ transformed with pFHR. This was exacerbated when 3MBA was added to the cells. These results rule out the involvement of XylR in transcription inhibi-
tion and similarly to IHF also rule out that any TOL-specific protein could be the cause. This was unexpected, because a XylR multimer (46) binds the UAS to form a bulky complex (41) that almost certainly gets in the way of the transcribing RNA polymerase. Perhaps the occupation of the binding sites is too transient (47) to impede the worm-like progress of the transcriptional machinery (48). In any event, these results directed our attention to the intrinsic properties of the UAS rather than those of possible binding factors.

The UAS of Pu Allow Incoming Transcription to Proceed in Vitro—Fig. 4 shows that the transcribed UAS form stable secondary structures with quite pronounced stem-loops. The so-called rho-independent transcriptional terminators include sequences that originate stable secondary structures (49–52). Such sequences have an intrinsic ability to stop transcription by forcing the RNAP off the mRNA. Hence, this class of terminators needs no other factors and is functional both in vivo and in vitro (51). Although typical rho-independent terminators include a poly(U) tract following the stem-loop, there are cases where a given secondary structure of mRNA suffices for the same function (53). On this basis, we hypothesized that the ability of the UAS to produce secondary mRNA structures (Fig. 4) was the cause of the transcriptional inhibition effects described above. To examine this possibility, in vitro transcription experiments were performed (Fig. 5) with linear DNA templates amplified with PCR from plasmids pH19C, pH19, and pH36 (see “Materials and Methods”). In the main template (the PCR product of pH19), the sequences under examination were placed downstream of the Ptrc promoter as a 105-bp segment (Fig. 5), thereby reproducing in vitro the situation described in vivo in Fig. 2, panel c. As controls, equivalent transcription templates were employed, either devoid of any sequence (PCR product of pH19C; compare with Fig. 2, panel a) or carrying a 53-bp insert of the distal-most UAS (UAS-D; compare with Fig. 2, panel d).

The runoff transcription experiment shown in Fig. 5 was performed under conditions allowing multiple (rather than a single) rounds of initiation by RNAP from the Ptrc promoter. This was expected to amplify weak termination signals. However, the results clearly show that the only transcripts generated under these assay conditions were those of the full-length mRNAs spanning the entirety of the linear templates. The minor RNAP slowdown signal detected ~15–20 bp before the full-length predominant transcript of the fragment with the UAS (Fig. 5) is not enough to explain the complete halt of transcription downstream of Ptrc observed in vivo (Figs. 2 and 3) with the same fragments. These results conflict with the idea that the UAS have inherent transcriptional termination capability, indicating instead that cellular factors are required for inhibition to occur.

A Genetic Assay to Categorize Transcription Termination—The question that follows from the in vitro experiments described above is whether
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The observed inhibition of incoming transcription by the UAS is a genuine termination event \textit{i.e.} whether there is any forced separation of the RNAP from the DNA template or whether it is the consequence of a different type of mechanism. To address this, the anti-termination system of the \( \lambda \) phage (35, 36) was used to discriminate whether the UAS of \( Pu \) truly terminates incoming transcripts or interferes with readthrough in another way. These experiments were based on the rationale that if the RNAP is loaded with the N protein encoded by the \( \lambda \) phage, the transcribing enzyme should be able to overcome any termination signal \textit{whether factor-dependent or factor-independent} placed downstream of a cognate \textit{nut} sequence (35, 36, 54).

This is because the passing of the N-RNAP through such a \textit{nut} sequence gear up transcription thus making it insensitive to termination. Whether or not the \( N/nut \) combination actually prevents a candidate DNA sequence from becoming transcribed thus becomes an indication of true versus apparent transcriptional termination.

Given the above, a genetic test was developed based on the co-transformation of \textit{E. coli} CC118 with three compatible plasmids (Fig. 6). One of these was a single-copy \textit{lac}\( ^{P^+} \), Cm\(^+\) vector in which the \textit{Ptrc} promoter was followed by the \textit{nut} sequence from phage \( \lambda \), the candidate terminator \( T \), and a downstream \textit{galK::lacZ} reporter; (ii) plasmid pUG11 determining expression of the N protein under the control of \( \lambda \), (iii) plasmid pCL857 encoding the cI857 thermosensitive \( \lambda \) repressor, expressed under its own promoter. Co-transformation of the three plasmids in the same \textit{E. coli} strain allows temperature-dependent anti-termination of any possible terminator cloned downstream of the \textit{nut} sequence.

**FIGURE 6. Outline of the three-plasmid system used to qualify candidate terminator sequences.** The genetic setup included the following: (i) a single-copy \textit{lac}\( ^{P^+} \), Cm\(^+\) vector in which the \textit{Ptrc} promoter was followed by the \textit{nut} sequence from phage \( \lambda \), the candidate terminator \( T \), and a downstream \textit{galK::lacZ} reporter; (ii) plasmid pUG11 determining expression of the N protein under the control of \( \lambda \), (iii) plasmid pCL857 encoding the cI857 thermosensitive \( \lambda \) repressor, expressed under its own promoter. Co-transformation of the three plasmids in the same \textit{E. coli} strain allows temperature-dependent anti-termination of any possible terminator cloned downstream of the \textit{nut} sequence.

**FIGURE 7. Detection of transcripts of DNA sequences inhibiting readthrough expression.** Panel a, a schematic representation of the relevant parts of reporter plasmids. The organization of the functional elements of \textit{pK1} and their derivatives \textit{pK3} and \textit{pL1N}, inserted respectively with either \textit{nut} \( \lambda\)-\textit{TR2} or \textit{nut} UAS/IHF downstream of \textit{Ptrc}. The length of the \textit{32P}-labeled ssDNA probe is indicated, as well as the location of the primers PSREV and GALK employed for the RT-PCR procedure. Panel b, S1 nuclelease protection assay of \textit{galK::lacZ} transcripts. The RNAs extracted from \textit{E. coli} CC118 transformed with \textit{pK1}, \textit{pK3}, or \textit{pL1N} were hybridized with an excess of a labeled ssDNA probe spanning the leading portion of the \textit{galK::lacZ} sequence and processed as explained under "Materials and Methods." The expected S1 product is 246 nt. The doublets probably reflect a residual activity of the S1 nuclease at the extremes of the mRNA/DNA hybrid (78). Size markers correspond to pUC19 digested withMspI. Panel c, detection of transcripts spanning TR2 and UAS sequences with RT-PCR. Serial 10-fold dilutions of RNA from \textit{E. coli} transformants with each plasmid were subjected to the RT-PCR assay described under "Materials and Methods" with oligonucleotides PSREV and GALK. Controls for each sample without reverse transcriptase showed no signals.
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![Graph showing transcription readthrough](image)

**Figure 8.** *In vivo* anti-termination assays with the UAS of *Pu*. *E. coli* CC118 (pUG11, pC857) was transformed with either plasmid pK1 (no inserts between *Ptrc* and *galk*:lacZ), pK2 (containing *λ*:TR2), pK3 (containing nut-TR2), or pL1N (inserted with nut UAS/IHF). The diagram for each of these inserts is show at the top of the corresponding panels. Each strain with its set of three plasmids was grown at 30 °C in LB with Cm, Km, and Ap, diluted to an A600 of ~0.05, and 0.1 mM IPTG added at the same time as temperature was increased to 42 °C. The accumulation of β-galactosidase was measured after continuing incubation at either 30 or 42 °C for 4 h. The growth rates of *E. coli* at both temperatures were not significantly different. Note the relief of termination caused by *λ*:TR2 at the higher temperature (panel c) and the lack of effect of the UAS (panel d).

each of the two segments inserted between *Ptrc* and *galk*:lacZ borne by pK3 and pL1N, compared with the reference plasmid pK1.

Unfortunately, the S1 assays performed with a probe covering the entire region under scrutiny were of no help in mapping the point at which the leading transcript of pL1N was discontinued by the UAS (not shown). This occurred because the corresponding RNA region forms stable secondary structures (Fig. 4) that yield unreliable RNA-DNA hybrids, thereby making the identification of the 3′ end of the transcript difficult. We therefore resorted to the rougher but still informative procedure of quantifying the transcript encompassing the UAS. To this end, the *E. coli* CC118 cells transformed with pK1, pK3, and pL1N were subjected to semi-quantitative RT-PCR to determine the presence of the mRNA segment sequences between the 5′ end of the mRNA initiated in *Ptrc* and the 3′ end of the inserts under consideration. RNA was extracted from each of the corresponding strains, retro-transcribed, and then amplified with primers targeted to the sequence just downstream of the transcription initiation site of *Ptrc* and the beginning of the *galk*:lacZ sequence. The 10-fold serial dilutions of the extracted mRNA used as templates for the amplification reactions generated the results shown in Fig. 7, panel c. In the most concentrated reaction concentration (1:10), the three amplification products of the inserts of pK1, pK3, and pL1N were readily detected. The differences in RNA sequences amplified between the samples became more pronounced with the 1:100 and 1:1000 dilutions. In particular, the results obtained with the latter showed the levels of the targeted mRNA segments to be lower with the construct bearing the TR2 *λ* terminator than with the UAS. This result is consistent with the S1 protection data (Fig. 7, panel b) in that the TR2 terminator was somehow more efficient than the UAS of *Pu* at inhibiting readthrough transcription from *Ptrc* to *galk*:lacZ.

The UAS Evade the Anti-termination System of λ—Fig. 8 summarizes the critical experiment required to determine whether the termination of incoming transcription by the UAS of *Pu* can be classified as a bona fide event. *E. coli* CC118 cells bearing the c857™ plasmid pC857 and the *N* construct pUG11 were transformed with pK1 derivatives containing either the TR2 terminator of *λ* (pK2), the same preceded by the *nut* sequence recognized by the N-loaded RNAP (pK3), or the UAS of *Pu* following the same *nut* sequence (pL1N). These bacteria were then grown at 30 °C until an A600 of ~0.05, at which point 0.1 mM IPTG was added. The cultures were then divided into separate aliquots and maintained at either 30 or 42 °C for the next 4 h. As explained earlier, growth at the higher temperature was expected to trigger overproduction of the anti-termination factor N, which ought to overcome any genuine termination. The behavior of the controls in the experiment outlined in Fig. 8 was as expected. (i) The TR2 terminator by itself blocked readthrough transcription at both temperatures (Fig. 8, panel b). (ii) The presence of the *nut* sequence before the TR2 terminator inhibited transcription at 30 °C but not at 42 °C (Fig. 8, panel c), surely because of the anti-termination effect of the overproduced N factor. The conduct of the test strain with the UAS (Fig. 8, panel d) unequivocally showed that the *λ* anti-termination system had no effect on the inhibition caused by the UAS on the readthrough transcription originated at *Ptrc*. This suggests that the UAS do not produce authentic termination signals (i.e. they do not provoke the release of RNAP from the transcribing complex). Instead, they seem to cause the inhibition of downstream expression by an alternative, post-transcriptional mechanism that, on the basis of the *in vitro* experiments presented above, probably involves host factors (perhaps acting on secondary RNA structures).

**Discussion**

Transcriptional noise in regulatory circuits is the consequence of illegitimate promoter activation (4, 5, 55). Noise can increase when a bacterium acquires a transmissible plasmid or another mobile element encoding a suite of genes, the regulation of which must occur within the larger context of the host. The catabolic operons for *m*-xylene biodegradation in the *P. putida* mt-2 TOL plasmid pWW0 are an example of such an implication of a structured transcriptional sub-network within the pre-existing global regulatory setup of a receiving bacterium (6). As it appears now, the transcriptional control of *xyl* genes involves an intricate two-way interplay between plasmid-encoded and chromosomally encoded factors and promoters (7). However, this is probably the result of a later adaptation and the increasing involvement of the incoming gene network with that of the host. One of first steps of such adaptation is to avoid the situation in which the existing expression circuits over-rule the regulation of the acquired genes because of their excessive noise.

*Pu* is the principal promoter of the TOL plasmid. It is the earliest responder to the presence of *m*-xylene in the medium and initiates the regulatory cascade associated with the expression of the *xyl* operons (56). However, both the type of promoter and certain accidental characteristics of *Pu* make it particularly susceptible to transcriptional noise. First, like other *σ*24 promoters, *Pu* can be activated “from solution” by noncognate, unrelated, prokaryotic enhancer-binding proteins of the AAA−-type; this activation bypasses the need to bind the specific UAS (17, 57). Second, the key factors involved in *Pu* functioning (σ24 and the *m*-xylene responsive regulator XyIIR) are in very short supply in *P. putida*. It has been calculated that there are ~50–60 σ24-dependent promoters in the genome of this bacterium (58), whereas the number of σ24 molecules is as small as 70–80 per cell (59). Similarly, the number of XyIIR multimers available for activation of *Pu* and *Ps* (a second σ24 promoter of the TOL plasmid) may not be above 10 per bacterium (60). These tiny figures may make *Pu* prone to stochastic fluctuations and cell-to-cell variability (4). Finally, the *Pu* promoter happens to be placed just adjacent to one of the two IS1246 elements that flank the *xyl* operons within the frame of the TOL plasmid pWW0 (61). The sporadic insertion of such a mobile segment in a new replicon may bring *Pu* under the influence of an external promoter, thus increasing the chances of transcriptional noise. Despite these features, however, the reality is that the *Pu* promoter is tightly regulated *in vivo* and that its activation seems to occur homogeneously in all members of an induced
bacterial population (11). This suggests the Pu promoter has specific features that actively suppress transcriptional noise.

A potent mechanism for restricting adventitious activation of Pu by proteins other than XyIR was reported in 1995. This involved the sharp bending of the DNA region between the UAS and the ς68-RNAP, achieved through binding with IHF (17). The involvement of DNA bending has since been reported in several cases of noise suppression in bacterial promoters (62, 63). However, other than a casual observation made in maxicells (18), a second type of noise-suppression property of the Pu promoter, instrumental in inhibiting transcription from upstream promoters, went unnoticed.

The present work documents that the UAS of Pu possess true transcription filter capability and provides in vivo evidence regarding the type of mechanism that could account for it, although this operative termination is certainly not standard. This molecular device is superficially reminiscent of eukaryotic transcriptional insulators (64–66). Yet the mechanisms and the biological functions of noise suppression are fundamentally different in bacterial and animal cells, e.g., the coordination of topological chromatin domains in eukaryotic systems (64).

Fig. 2 shows the primary genetic evidence for the inhibition of readthrough transcription caused by Pu sequences in P. putida. The same phenomenon can be recreated in E. coli with the same genetic setup (Fig. 3). These simple in vivo assays indicate that an ~100-bp DNA segment of Pu, spanning positions −106 to −205 of the transcription initiation site, altogether inhibits transcription from a strong upstream promoter (Ptrc) read-through to a reporter lacZ gene. Although the precise boundaries of the sequence active in this inhibition were not determined, they include the UAS of the promoter, i.e., the sites for binding XyIR, the native activator of the system. Yet, XyIR binding had no influence per se on the observable phenomenon (Fig. 3, panel c). This is noteworthy because, like other prokaryotic repressors, XyIR is likely to form a bulky nucleoprotein with the UAS (41, 67). However, unlike other cases of transcriptional interference caused by proteins bound to DNA (42), XyIR binding neither exacerbates nor relieves this inhibition. Because the mRNA generated upon transcription of the ~100-bp segment of Pu containing the UAS was able to form stable secondary structures (Fig. 4), it is likely that their inhibitory strength is intrinsically related to the sequence and not to specific proteins encoded by the TOL plasmid.

Although stem-loop structures in mRNA are characteristic of many factor-independent termination signals in eubacteria (48, 50, 52, 53), transcripts starting at Ptrc in vitro ran freely through the same UAS that completely inhibited transcription in vivo (Fig. 5). This suggests that cell factors are necessary for this to occur, but it says nothing about any possible mechanism. One plausible scenario is that this inhibition is the result of a factor-dependent termination (rho or otherwise). However, the data shown in Fig. 8 argue strongly against such a possibility. The λ phage nus/N anti-termination system, which is known to operate in both factor-dependent and -independent termination signals (36, 68), could not defeat the inhibitory effect of the UAS in the in vivo system prepared to detect termination (Fig. 8). Although these results do not rule out that an unusual termination device is behind the phenomenon, the evidence seems to suggest an alternative explanation. One option is that the nascent noncoding mRNA that includes the UAS folds into a secondary structure that becomes the substrate of mRNA nucleases (69, 70).

Preliminary experiments to recreate the in vivo assay of Fig. 3 in rnc mutants of E. coli lacking the double-stranded endoribonuclease RNase III (71) were inconclusive (data not shown) because the mRNA of the reporter gene may also be sensitive to the loss of this enzyme. Similarly, temperature-sensitive rnc mutants conditionally lacking the key ribonuclease E (72) were too pleiotropic to give reliable results. However, the degradation of the transcribed UAS by host ribonucleases remains a good hypothesis and fits well with the RT-PCR data of Fig. 7, panel c. This issue requires clarification and will be the subject of further studies.

Termination signals and anti-termination devices are common elements of gene expression control (48). In particular, anti-termination is a documented mechanism that checks certain catabolic promoters in Pseudomonas and other bacteria (73). Furthermore, the growing extent to which riboswitches (74, 75) and small RNAs (76) are thought to control gene expression suggests that many noncoding DNA sequences have nontrivial regulatory capacity. Their functions (for instance, noise suppression), however, may not be understood when promoters are studied in isolation because their role is associated with the global performance of regulatory circuits (77). In this context, it can be argued that the inhibition of readthrough transcription by the UAS of Pu (and perhaps by other UAS of other ς68 promoters) is no casual property. Rather, it is likely that their functions as binding sites for XyIR and for the cessation of transcripts from upstream promoters have co-evolved and to have been co-selected to facilitate the implantation of transposon-borne xyl genes into new bacterial hosts.

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