Alternate Paradigm for Intrinsic Transcription Termination in Eubacteria*§

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Intrinsic transcription terminators are functionally defined as sites that bring about termination in vitro with purified RNA polymerase alone. Based on studies in Escherichia coli, intrinsic termination requires a palindromic stretch followed by a trail of T (or U) residues in the coding strand. We have developed a highly efficient algorithm to identify hairpin potential sequences in bacterial genomes in order to build a general model for intrinsic transcription termination. The algorithm was applied to analyze the Mycobacterium tuberculosis genome. We find that hairpin potential sequences are concentrated in the immediate downstream of stop codons. However, most of these structures either lack the U trail entirely or have a mixed A/U trail reflecting an evolutionarily relaxed requirement for the U trail in the mycobacterial genome. Predicted atypical structures were shown to work efficiently as terminators both inside the mycobacterial cell and in vitro with purified RNA polymerase. The results are discussed in light of the kinetic competition models for transcription termination. The algorithm identifies >90% of experimentally tested terminators in bacteria and is an invaluable tool in identifying transcription units in whole genomes.

The interaction of the template DNA, RNA polymerase, and the nascent RNA chain has evolved so as to minimize the release of the transcript prematurely (1). At certain sequences, the release occurs at a rate comparable with that of elongation either spontaneously or in the presence of assisting factor(s). Based on exhaustive work in Escherichia coli, terminators are classified into two groups (2). Functionally, if a sequence can bring about transcript release in an in vitro system with purified RNA polymerase alone, it is defined as an intrinsic terminator. These are also referred to as simple or factor-independent terminators. Terminators that require the presence of additional factors are classified as complex or factor-dependent terminators. In E. coli, most complex terminators depend on the action of the Rho termination factor (3). These two classes of terminators are not sharply defined as the efficiency of many intrinsic terminators is enhanced by the presence of additional factors (4).

Intrinsic terminators are characterized by the presence of a G/C-rich (interrupted) palindromic region followed by a trail of A residues on the template strand (5–7). There is evidence from multiple sources that the palindromic region extrudes out as a hairpin in the nascent transcript (8–12). Furthermore, there is a strong, although not absolute, correlation between the predicted stability of the stem-loop structure and termination efficiency (12). The stem-loop structure is believed to cause pausing of the polymerase (13–15) and weaken the interaction of the polymerase with the nascent RNA and template DNA (16, 17). The release is facilitated by the presence of a U trail (10, 17) probably due to the unusually weak hybrid formed by the dArU base pairing (18). In addition to these two primary determinants, sequences further downstream have also been shown to affect the efficiency of termination probably by being an impediment to transcription elongation (19).

Although intrinsic terminators have been studied extensively in E. coli, little is known about their orthologs, if any, in other bacteria. Intrinsic terminators from E. coli have been shown to function in many bacteria. However, recent theoretical analysis indicates that only a minority of bacteria may employ this mechanism of transcription termination (20, 21). In an attempt to formulate a general model for intrinsic transcription termination in eubacteria, we have developed an algorithm to identify hairpin potential sequences in bacterial genomes and have applied it to the Mycobacterium tuberculosis genome. Such sequences appear to be concentrated in the immediate downstream region of stop codons, a feature one would expect of intrinsic terminators. Surprisingly, nearly 90% of these structures lack a U trail entirely or possess a mixed A/U trail. We show experimentally that these atypical structures work efficiently as terminators both in vivo and in vitro. Based on our results, the present algorithm represents the most efficient and accurate software for the identification of intrinsic terminators in eubacteria.

EXPERIMENTAL PROCEDURES

Algorithm—The GeSTer (Genome Scanner for Terminators) algorithm first segregates the coding, upstream, and downstream regions based on the feature table entries of the genome sequence. Next it searches for palindromic sequences downstream of each gene (20 to +270 nucleotides of the stop codon) without entering adjacent coding regions. The search is initiated at a G/C-rich (>50%) tetranucleotide, and a reverse complementary match is sought within the next 70 nucleotides. This defines the base of the stem, and the match is extended inward. Once a mismatch is encountered, all possible structures are computed allowing for different combinations of mismatches and gaps. The ΔG of formation of each of these structures was computed using the parameters from Turner et al. (22) and Jaeger et al. (23). Among all these structures, the one with the lowest ΔG was retained. Then the program moved to the next G/C-rich tetranucleotide and reiniated the search.

The final set of structures used a minimal ΔG filter based on the G/C content of the bacteria. The species-specific ΔG_cutoff was set at −0.230 × (G_c) + 3.44 based on two premises. Firstly, the basal ΔG of the

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downstream region (20) is strongly correlated with the G/C content of the genome. Secondly, the algorithm should identify preferentially structures in the downstream rather than in the upstream region. The optimized cutoff value for ∆G was derived by iteratively weighing down ∆G downstream, so as to maximize the likelihood of identifying only downstream structures in the analysis, and 5% glycerol. The reaction was started by adding NTPs (final concentrations of 100 µM ATP, CTP, and GTP, 0.4 µM UTP, and 1 µCi of [α-32P]UTP) and shifting to 37 °C. After 1 min, the reaction was supplemented with UTP (final concentration of 100 µM) and heparin (final concentration of 150 µg/ml). Reactions were stopped by the addition of equal volumes of formamide containing 0.25% bromphenol blue and 0.025% xylene cyanol and resolved on an 8% denaturing polyacrylamide gel. The reactions were visualized and quantitated by phosphorimaging (Fujiﬁlm). The termination efﬁciency (TE) was calculated as follows.

For single structures, TE = 100 × TP/(RO + TP) where TP is the amount of terminated product and RO is the amount of runoff transcript. For total termination efﬁciency of the tandem structures, TE = 100 × (TP + TP_1 + TP_2 + TP_3) where TP, TP_1, and TP_2 are the amounts of product terminated downstream of the ﬁrst and second structures, respectively.

For the first structure present in tandem, TE = 100 × TP/(RO + TP_1 + TP_3). For the second structure present in tandem, TE = 100 × TP_1/(RO + TP + TP_3).

RESULTS

Algorithm—To delineate the elements involved in intrinsic transcription termination conserved in all bacteria, we have developed the GeSter algorithm that identiﬁes and classiﬁes structures based on the trailing nucleotides and the position of adjacent structures. The algorithm identiﬁes hairpin structures using the following parameters, a stem length ranging from 4 to 20 nucleotides with a loop of 3 to 10 nucleotides with a maximum of 3 unpaired nucleotides in the form of gaps or mismatches. These parameters are based on the qualitative assessment of all known terminators from different bacteria (29–32). The list of terminators compiled from previous literature has been provided as Supplemental Material. –20 to 270 nucleotides around the stop codon for each gene were searched, without entering adjacent coding regions. In the case of overlapping structures, the one with the lower ∆G was retained. Finally, structures were ﬁltered using a minimal ∆G requirement based on the G/C content of the genome. With these parameters, the algorithm identiﬁed more than 90% of all experimentally shown terminators in different bacteria (29–32, Supplemental Material). Structures were classiﬁed as follows: (a) E. coli type L-shaped, those with >3 U residues present in the 10 nucleotides trailing the structure; (b) Mycobacterium type I-shaped, those with 3 or fewer U residues in the trail; (c) V-shaped, structures that are immediately followed (or preceded) by another structure; (d) Tandem/U-shaped, multiple structures that are present downstream of a single gene; and (e) Convergent/X-shaped, structures present between adjacent convergently oriented genes. It should be noted that all structures, other than the L-shaped ones, are symmetric and could potentially work in either orientation.

Whole Genome Analysis—When structures identiﬁed by the program were compiled, we found that there was a preponderance of hairpin potential sequences within 50 nucleotides downstream of the stop codon in bacterial genomes (Table I, Fig. 1), which include what one would expect of transcription terminators. In a few species either the L-shaped (B. subtilis and N. meningitidis in Table I) or I-shaped structures (M. tuberculosis and M. leprae in Table I) predominate. However, in many cases, these two classes constitute signiﬁcant fractions of the structures identiﬁed (E. coli and X. fastidiosa in Table I).

A detailed analysis of M. tuberculosis and E. coli genomes revealed many interesting features. Firstly, the algorithm detects putative terminators downstream of 20–40% of genes. This is probably due to the operonic arrangement of many...
Secondly, there is dramatic concentration of genes. In addition, some of the other genes may rely on the Rho protein for transcription termination. In agreement with this, a Rho homologue has been identified in the \emph{M. tuberculosis} genome as well. Secondly, there is dramatic concentration of structures about 21 nucleotides downstream of the stop codon in \emph{E. coli} with relatively few structures present in the rest of the downstream region. In \emph{M. tuberculosis}, on the other hand, the structures peak 37 nucleotides downstream of the stop codon with a significant fraction spread throughout the downstream region. Furthermore, as discussed above, \emph{E. coli} shows marginal preference in using L-shaped structures over I-shaped structures whereas \emph{M. tuberculosis} almost exclusively uses I-shaped structures. However, it is noteworthy that irrespective of their frequency of occurrence, the non-L-shaped structures are concentrated at the same position as the L-shaped structure (Fig. 1) indicating that they serve a similar purpose, i.e. of transcription termination. Thirdly, a significant portion of the genes employ multiple structures, either V- or U-type, for bringing about termination. Of these the tandem structures are more frequent. For instance, 10% of the putative terminators in \emph{M. tuberculosis} and 15% in \emph{E. coli} are of the U-type. Lastly, few convergently oriented genes use a single structure present in the shared downstream region between them.

\textit{Structures without a U Trail Are Efficient Terminators in \emph{M. smegmatis}}—Although L-shaped structures function in many species including \emph{E. coli}, the V-shaped structures have been identified previously at least in \emph{Streptomyces} (31). In addition, X-shaped structures function both in \emph{E. coli} (33) and \emph{Streptococcus} (32). Therefore, we decided to test the ability of structures that lack an obvious U trail to bring about transcription termination in mycobacteria. Toward this end, we constructed a mycobacteria-specific termination selection vector (pTER5; Fig. 2) by cloning the \emph{M. smegmatis} gyrA promoter (27) upstream of a CAT reporter gene. A fragment cloned between the promoter and the reporter gene would reduce transcription read-through if it were a terminator, thereby leading to chloramphenicol sensitivity and a quantitative decrease in specific CAT activity.

Representative I-shaped terminators were PCR-amplified from the \emph{M. tuberculosis} genome and cloned into the termination vector (see “Experimental Procedures”). The terminator downstream of \emph{tuf} gene harbors an AU-rich trail. When present upstream of the CAT gene, it reduces transcription read-through by \~ 80% (Fig. 3, A and D), indicating that a classical U trail as defined in \emph{E. coli} is not essential for transcription termination. Surprisingly, the terminator showed comparable efficiency in the reverse orientation that lacks an appreciable AU-rich trail. To substantiate this observation, we tested the terminator present downstream of \emph{Rv1324} for its ability to bring about transcription termination bidirectionally. This structure is flanked on both sides by G/C-rich stretches. In agreement with the above results, this terminator also functions with comparable efficiency in both orientations (Fig. 3, B and D). This clearly demonstrates that the U trail is not essential for the functioning of the terminator in mycobacteria. To analyze the termination efficiency of U-shaped structures, we used the putative terminator present downstream of the \emph{gyrA} gene in \emph{M. smegmatis} (Fig. 3C). The individual structures here are weaker than the structures tested so far; however, in tan-

\renewcommand{\arraystretch}{1.2}
\begin{table}[h]
\centering
\caption{Representative whole genome analysis of bacterial sequences}
\begin{tabular}{|l|c|c|c|c|c|c|c|c|c|}
\hline
Species & Genome & Genes & All & L & I & U & V & X & Peak & p value* \\
\hline
\emph{B. subtilis} & 4214814 & 4218 & 1608 & 1422 & 196 & 104 & 0 & 153 & +21 & 0.0023 \\
\emph{E. coli} & 4639221 & 4397 & 1734 & 918 & 816 & 256 & 13 & 139 & +21 & 0.0004 \\
\emph{M. leprae} & 3268203 & 1653 & 342 & 67 & 275 & 20 & 1 & 2 & +32 & 0.0011 \\
\emph{M. tuberculosis} & 4411529 & 3970 & 890 & 83 & 807 & 87 & 5 & 31 & +37 & 0.0002 \\
\emph{N. meningitidis} & 2272351 & 2096 & 885 & 621 & 264 & 93 & 2 & 67 & +33 & 0.0002 \\
\emph{X. fastidiosa} & 2679306 & 2821 & 481 & 198 & 283 & 31 & 3 & 23 & +25 & 0.0039 \\
\hline
\end{tabular}
\footnote{* t test performed as described under “Experimental Procedures.”}
\end{table}
dem they show a similar termination efficiency in both orientations (Fig. 3, C and D). Thus, both I- and U-shaped structures function with high efficacy in vivo.

Terminators Work Only in the Untranslated Region—In the experiments described so far, the terminators were cloned more than 50 nucleotides downstream of the promoter in the 5′-untranslated region. When such a structure was moved closer to the promoter (27 nucleotides downstream), there was no detectable effect on termination efficiency (compare pTER1 and pTER5 in Fig. 4). However, when cloned within the coding region (1.1 kb downstream of the promoter), the structure had no detectable effect on transcription read-through in either orientation (compare pTER7 and pTER5 in Fig. 4). On the other hand, in the same construct, when translation was moved out of frame, leading to a premature stop codon, the structure brings about termination with efficiency comparable with the 5′-untranslated region context (compare pTER7Δ and pTER7 in Fig. 4). Thus, terminators appear to be effective only in the non-coding region. The close coupling of transcription and translation in bacteria probably prevents the extrusion of these structures in the RNA in the coding region.

Terminators Work Efficiently in Vitro—To ensure that the structures were genuine intrinsic transcription terminators, we analyzed their ability to bring about termination in vitro using purified RNA polymerase from M. smegmatis. The templates containing the promoter and the various terminators in either orientation were generated as described under “Experimental Procedures.” Fig. 5 shows results of a representative in vitro transcription termination assay. The majority of the transcript appears to terminate a few nucleotides downstream of the structure in every case. The termination efficiency of these structures is comparable with those obtained in vivo (Table II). Furthermore, in agreement with the results obtained in the in vivo experiments, all three structures work bidirectionally (compare “Forward” and “Reverse” in Table II). Of particular interest is the tandem terminator in which transcription terminates downstream of each structure. Notably, the first structure encountered by the polymerase (t1 in the forward orientation and t2 in the reverse orientation) works at low efficiency (~45%, Table II). On the other hand, the same structure when encountered second (t2 in the forward orientation and t1 in the reverse orientation) shows appreciably higher termination ef-
Intrinsic Transcription Termination in Eubacteria

**TABLE II**

<table>
<thead>
<tr>
<th>Terminator</th>
<th>Termination efficiency ± S.D. *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
</tr>
<tr>
<td>AT trail</td>
<td>84.4 ± 3.5</td>
</tr>
<tr>
<td>No trail</td>
<td>82.9 ± 4.1</td>
</tr>
<tr>
<td>Tandem (total)</td>
<td>82.6 ± 4.3</td>
</tr>
<tr>
<td>Tandem (t₁)</td>
<td>43.2 ± 3.9</td>
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* Calculated as described under "Experimental Procedures."

**DISCUSSION**

Intrinsic terminators represent an extremely economical mechanism of transcription termination. Earlier attempts to identify intrinsic terminators have, in general, had limited success in bacterial species other than *E. coli* (21, 30, 34, 35). This is probably because they fail to take into account the possibility that secondary structure alone could work as a terminator. As a result, they identify only the L (and possibly the X) subsets of the terminators identified by the present algorithm. The only other theoretical analysis of the distribution of secondary structures in the non-coding region similarly failed to detect a concentration of structures downstream of the stop codon in the majority of genomes (20). This is probably because of the 60-base window (moved in steps of 10 bases) employed in the study. Such a rigid window and large step size would lead to blunting of peaks, which reduces the resolution of their analysis. Therefore, in many organisms, including *M. tuberculosis*, the modest concentration of structures is no longer statistically distinguishable from the background ΔG. In contrast, the present algorithm varies the window size dynamically to specifically identify individual stem-loop structures, thereby improving the sensitivity and accuracy of the prediction.

We used the *M. tuberculosis* genome as a test for our algorithm. Surprisingly, although there were many secondary structure potential sequences present downstream of genes, most of these were devoid of a trail of U (Table I). Notably, irrespective of whether the structures are followed by a U trail or not, they are concentrated approximately within 50 nucleotides downstream of the stop codon (Fig. 1). Thus, both classes of structures appear to have evolved for a conserved function in transcription termination. Interestingly, even in *E. coli*, a significant fraction of the structures lack a discernible U trail. In agreement with this, we have experimentally shown that the U trail does not play a primary role in transcription termination both inside the mycobacterial cell (Fig. 3) and *in vitro* with purified mycobacterial RNA polymerase (Fig. 4). On the other hand, the significance of the trail sequence in *E. coli* is not completely clear. In different systems, the U trail has been shown to be either essential (10, 17), unnecessary (36), or necessary only in the absence of appropriate elements downstream of the termination site (19).

The efficiency of termination is believed to be determined by the opposing influences of the rates of elongation and release (1, 37). Recently, a paused form of the polymerase that reacts slowly with the nucleotides has been proposed as an intermediate prior to the actual step of release (38, 39). Revised models based on single molecule experiments evoke kinetic competition between elongation rates and the largely irreversible formation of the paused complex rather than the actual step of release (39). Most bacterial coding sequences have evolved to

**Fig. 4.** Effect of distance from the promoter and translation on termination efficiency. The terminator from Fig. 2A was placed at different distances from the promoter, –27 bp (pTER1), 77 bp (pTER5), and 1.1 kb (pTER7) downstream of the transcription start site. Representative CAT assays and the means obtained from at least three independent experiments is shown. The promoter (arrow), terminator (filled box), and translated regions (hatched box) are indicated.

**Fig. 5.** *In vitro* termination assay. A, schematic representation for the assay. Runoff transcription assays were performed with constructs harboring the terminators shown in Fig. 2, in either orientation. The terminators used in the assay are as follows, *tuf* terminator (B), *Rv1324* terminator (C), and the *gyrA* terminator (D). The positions of full-length runoff and terminated (Term. Prod.) products are indicated. A sequencing ladder was used as molecular mass marker.

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favor the former rather than latter reaction. Terminators represent sequences, which specifically alter one or both of these reactions leading to transcription termination.

Support for the above models came from the analysis of mutationally altered polymerases that have a lower elongation rate and show a concomitant increase in termination (40). Similar results are obtained using a wild-type polymerase in the presence of limiting concentrations of nucleotides (7, 40). Furthermore, recent work shows that the primary role of the U trail may be to decrease the rate of elongation (38) and thereby allow the hairpin to extrude and dislodge the nascent chain from the catalytic site. In _M. tuberculosis_, where the rate of RNA chain elongation is about 10-fold slower than _E. coli_ (41), such a role for the U trail would be redundant. Therefore, an I-shaped structure, even without the stalling effect of the U trail, could work as efficiently as an L-shaped structure. Thus, in the framework of the kinetic competition model, a lower elongation rate would mean that the enhancement required in the rate of pausing/release to bring about termination would be correspondingly lower. An alternative explanation for the low representation of the L-shaped structures in _M. tuberculosis_ could be the high G/C content of the organism. However, _M. leprae_, an organism closely related to _M. tuberculosis_, shows a similar preference for I-shaped structures although it has a considerably lower G/C content. In addition, we find no simple relationship between the G/C content of an organism and its preference for one or the other type of structure. On the other hand, our hypothesis predicts a correlation between the prevalence of the I-shaped structure with a lower rate of RNA chain elongation. In agreement with this prediction, when the _E. coli_ RNA polymerase itself is made to move slowly in the presence of limiting amounts of nucleotides, it terminates efficiently even in the absence of a U-trail (7). Thus, our results substantiate the model of kinetic competition between the rates of elongation and termination (1, 39).

Another point of interest is that we found that the cells are protected against premature termination at structures within the coding region by the translating ribosomes (Fig. 4). This mechanism would not be operational in tRNA and rRNA genes. These two classes of highly transcribed genes are known to have extensive secondary structure in their RNA without the protective influence of translation. Therefore, a terminator structure within the coding region of such genes would be disastrous to the cell. Significantly, the algorithm does not identify putative terminators in the coding regions of these genes, implying that the identified structures are genuine terminators.

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REFERENCES