

A hairpin near the 5' end stabilises the DNA gyrase mRNA in *Mycobacterium smegmatis*

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ABSTRACT

RNA is amongst the most labile macromolecules present in the cells. The steady-state levels of mRNA are regulated both at the stages of synthesis and degradation. Recent work in *Escherichia coli* suggests that controlling the rate of degradation is as important as the process of synthesis. The stability of mRNA is probably more important in slow-growing organisms like mycobacteria. Here, we present our analysis of the *cis* elements that determine the stability of the DNA gyrase message in *Mycobacterium smegmatis*. The message appears to be stabilised by a structure close to its 5' end. The effect is especially pronounced in a nutrient-depleted state. These results largely parallel the model proposed in *E.coli* for mRNA degradation/stability with subtle differences. Furthermore, these results suggest that the slow-growing organisms might use stable mRNAs as a method to reduce the load of transcription on the cell.

INTRODUCTION

Gene expression is determined by a combination of factors that regulate the efficiency of transcription, mRNA stability and translation of the message. While transcription and translation have been studied in depth for a long time, interest in mRNA stability has developed only recently. Work in the last decade has revealed many players that regulate the stability of mRNA in *Escherichia coli* (1–4).

In *E.coli*, the survival of a single message is determined by a combination of *cis* as well as *trans* factors. The *trans* factors consist of a large number of ribonucleases that have been identified over the years. These include both exo- as well as endoribonucleases. At least some of these enzymes physically associate to form a 'degradosome' that is believed to facilitate cooperative degradation of the entire message (2,3,5,6). In general, in *E.coli*, the exonucleases degrade the message

processively in a 3' to 5' direction. On the other hand, the endonucleases primarily act to remove *cis* elements present on the mRNA that would otherwise stabilise it. The *cis* elements in the mRNA can be grouped into two classes, stabilisers and destabilisers. Destabilising elements consist of sequences and structures that help provide binding sites to nucleases and thereby initiate the degradative process. In contrast, the stabilisers hinder the degradation of the mRNA by blocking the access or action of different nucleases. For instance, secondary structures at or near the 3' end of the message, such as *rho*-independent transcription terminators, act by blocking the processive progression of exonucleases from the 3' end of the message (7–9). On the other hand, secondary structures at the 5' ends of the mRNA appear to stabilise the message probably by preventing RNase E from interacting with the 5' end of the message (2,10–12). In addition, a strong Shine–Dalgarno (SD) sequence near the 5' end of the message would recruit ribosomes and stabilise the message by blocking access of nucleases to degradative signals present in the naked mRNA (11,13). While the half-life of mRNA is strictly determined by a combination of all these factors, in most cases the rate-determining step appears to be the removal of these stabilising elements and structures by an endonuclease (most often RNase E), which results in an mRNA species that is rapidly degraded by exoribonucleases (2).

The half-life of the bulk of the mRNA in *E.coli* is 2.4 min at 37°C (2). This short half-life is believed to reflect the fast-growing nature of *E.coli* (14) and probably allows rapid adaptation to environmental changes (14,15). Thus, one would expect that slow-growing organisms would firstly have more stable messages (16) and furthermore, the regulation of degradation of these messages would be at least quantitatively, if not qualitatively, different. Towards this end, we have analysed the stability of the DNA gyrase mRNA in *Mycobacterium smegmatis* and identified a secondary structure near the 5' end that protects the message against degradation. The effect is significantly pronounced in nutrient-deprived conditions. These results appear partly to parallel models proposed for the determination of mRNA stability in *E.coli*.

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Table 1. Primers used in the study

Primer name	Sequence
F	5'-cgaagcagatctgtatgccggacgtc-3'
R	5'-ttcggtgatccgggttgagtcgccgca-3'
Mut(F)	5'-gacggatctcagggcgtgtctgcaccgc-3'
Mut(R)	5'-gcggtgcagacacgcctgagatccgtc-3'
Reg(F)	5'-ggcgtgtctgcacgccctcccagtgaac-3'
Reg(R)	5'-gttcactgggagggcgtgcagacacgcc-3'

MATERIALS AND METHODS

Strains and growth conditions

The mutator strain RM111 [*W3110 Tn10(Tet^r) mutD5 F⁻*] of *E. coli* was used to generate random mutants. *Mycobacterium smegmatis* mc²155 strain was used as the mycobacterial host. RM111 cells were transformed with pSUN-WT (Fig. 1B, described below). These cells were subcultured for 10 rounds (1% inoculum every 24 h) in LB medium containing kanamycin (Kan, 35 µg/ml). *Mycobacterium smegmatis* cells were transformed with DNA prepared from different subcultures and selected on LB plates containing either Kan alone or with chloramphenicol (Chl, 25 µg/ml).

Screening

Individual colonies were patched on LB-Kan and LB-Kan+Chl plates at approximately 100 patches per plate. A second round of screening was performed at a density of approximately 20–30 patches per plate. Colonies sensitive to Chl (Chl^s) were inoculated in YK medium. After the cultures reached late-log phase, the cells were inoculated into fresh YK (17) or starvation medium (SM). SM contains YK with reduced glycerol (0.05% instead of 2%).

Immunoblotting and chloramphenicol acetyl transferase (CAT) activity

Protein extracts were prepared as described previously (18). Briefly, a single colony was inoculated into 3 ml of medium and grown to mid-log phase. The cells were harvested, washed with an equal volume of 250 mM Tris-HCl pH 8 and resuspended in one-quarter vol of the same buffer. The cells were sonicated and centrifuged at 15 000 g for 15 min at 4°C. Protein extract (2 µg) was resolved on a 1% SDS, 15% polyacrylamide gel. The proteins were transferred onto a PVDF membrane and probed with anti-CAT antibodies (Sigma Aldrich). CAT assays were performed with an appropriate dilution of the supernatant as described previously (18). Briefly, the reaction mix contained 100 µM ¹⁴C-chloramphenicol (5 mCi/mmol) and 300 µg/ml acetyl coenzyme A in 250 mM Tris-HCl pH 8. The reaction was carried out at 37°C for 30 min and stopped with 10 vol of cold ethyl acetate. The organic layer was dried. Samples were resuspended in minimal volume of ethyl acetate and resolved on a fluorescent silica thin layer chromatography plate with chloroform:methanol (95:1) as the mobile phase. The spots corresponding to the substrate and the products were quantified using a phosphorimager (Fujifilm). All CAT activities are an average of at least three experiments and the error between independent experiments was <10%.

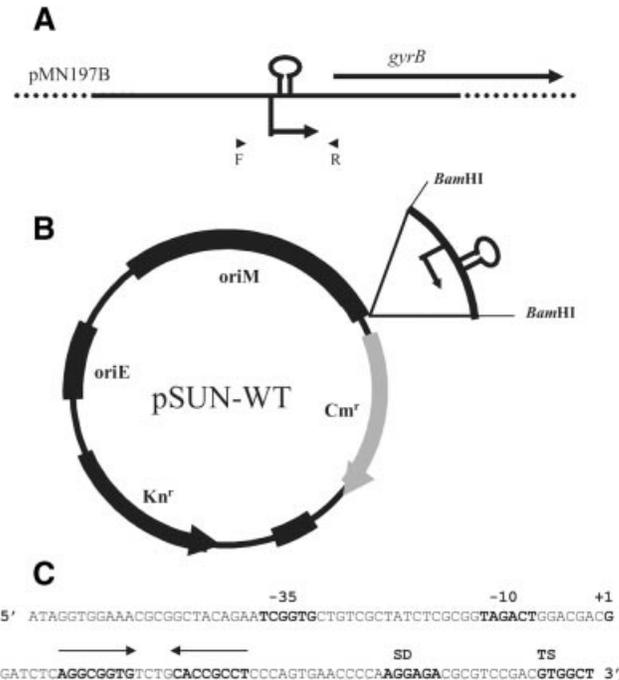


Figure 1. Templates used in the study. (A) The parent construct pMN197B contains part of *M. smegmatis gyrB* and 1.5 kb of the upstream region cloned in pUC19. The position of the transcription start site and the CHPS is indicated. The primers used for amplifying a minimal promoter region along with the 5' untranslated region are indicated (F,R). (B) The construct used for mutagenesis, pSUN-WT. It is derived from pSD7, it contains a promoterless CAT gene (Cm^r), a kanamycin resistance gene (Kn^r), origins of replication for mycobacteria (OriM) and *E. coli* (OriE), and three transcription terminators (black boxes) to prevent read-through. (C) The sequence around the transcriptional start site. The transcription start site, putative promoter elements, the dyad symmetric CHPS, the Shine–Dalgarno sequence (SD) and the protein coding region, translational start (TS), are highlighted.

DNA isolation from *M. smegmatis*

Aliquots of 1.5–3 ml of culture were harvested after treating with 0.2 M glycine for 2 h. After washing with 0.5 ml of STE (100 mM NaCl, 10 mM Tris-HCl pH 8 and 1 mM EDTA), the cells were resuspended in 200 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8 and 1 mM EDTA) and incubated at room temperature with 40 µl each of lysozyme (50 mg/ml) and lipase (5 mg/ml). After 1 h, 400 µl of solution II (200 mM NaOH, 1% SDS) was added and the samples were incubated on ice for 10 min. Subsequently, 300 µl of solution III was added and incubated for a further 10 min on ice. The precipitate was removed by centrifugation at 13 000 r.p.m. for 10 min (Eppendorf Microfuge). After phenol–chloroform extraction, the nucleic acids were precipitated with 2 vol of ethanol. The pellet was washed with 70% ethanol, dried and resuspended in 20 µl of water.

DNA manipulations and site-specific mutagenesis

All the primers used in the study are listed in Table 1. The parent construct pMN197B (Fig. 1A) contains a 2.5 kb *Bam*HI fragment that includes part of the *gyrB* and ~1.5 kb of the upstream region, cloned in pUC19. This was used as the template for both cloning and mutagenesis. The *E. coli*–*Mycobacterium* shuttle vector pSD7 (19) was used for

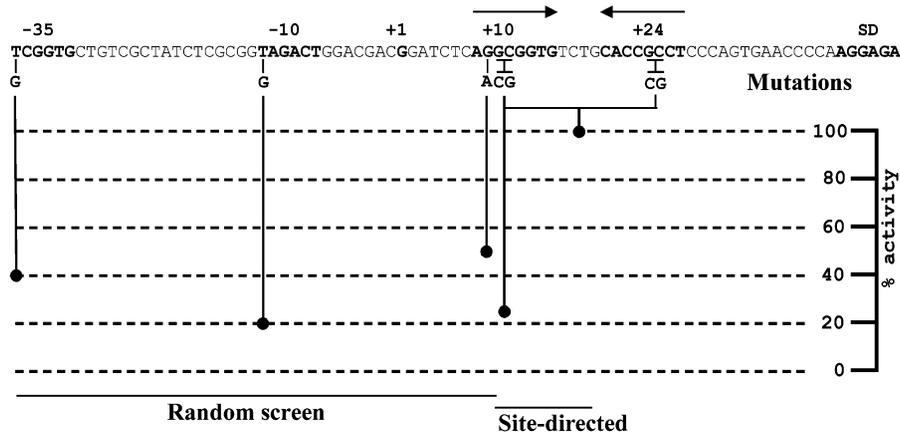


Figure 2. Mutants obtained by random as well as site-directed mutagenesis. The three single mutations obtained in the random screen and the mutations that were engineered to disrupt and regenerate the CHPS are shown along with their effect on CAT activity relative to the wild-type activity.

expression analysis. The pSUN-WT construct (Fig. 1B) was generated by amplifying the minimal *gyr* promoter of *M. smegmatis* along with the entire 5' untranslated region from pMN197B using the primers F and R and cloning into pSD7 at the *Bam*HI site.

Site-specific mutagenesis was performed by the double primer method based on the QuikChange protocol (Stratagene). The primers Mut(F) and Mut(R) were used to disrupt the palindromic sequence while Reg(F) and Reg(R) were used for regenerating an alternative palindrome. The mutants were identified by sequencing and the relevant region was cloned in pSD7 as described above. The ΔG of individual structures was calculated using the web interface of the *mfold* algorithm available at <http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html> (20). Figure 3 shows the idealised structures that would be formed if the altered base only were to be mismatched while the ΔG values are those calculated for the optimal structure determined by *mfold*. For instance, in the mutant obtained in the random screen, the solitary A-U base pair that initiates the stem is no longer stable according to *mfold*. However, this is shown to be base paired in the idealised structures for the sake of simplicity. Furthermore, the site-directed disruption of the structure is predicted to lead to the formation of two alternate forms of $\Delta G = -6.8$ and -7.7 kcal/mol. Therefore, the average of these two values has been reported.

RNA isolation, primer extension and half-life measurements

RNA was isolated from exponentially growing *M. smegmatis* cells using TRIzol Reagent (Gibco BRL) following the manufacturer's instructions. For half-life measurements, cultures were allowed to reach 1 OD₆₀₀ and treated with rifampicin (50 μ g/ml). At different time intervals, RNA was isolated and used for primer extension, which was performed using Superscript II reverse transcriptase (Gibco BRL) with a primer specific to the *gyrB* (5'-TCGAGAATGGTGATGGAATCGGC-3'). Prior to the reaction, the primer was end-labelled using [γ -³²P]ATP (>5000 Ci/mol, Amersham) and T4 polynucleotide kinase (Gibco BRL).

RESULTS AND DISCUSSION

Positive regulators of gene expression

Transcription of the *gyr* operon in *M. smegmatis* is driven by a solitary promoter present upstream of *gyrB* (18). Previous work has shown that the *gyr* promoter is amongst the strongest promoters identified in *M. smegmatis* (18). In addition, there appeared to be a positive regulator of gene expression located in the 5' untranslated region of the *gyr* message. To define the *cis*-acting positive regulators of expression, constructs were generated that harboured either the minimal promoter alone or the promoter with the 5' untranslated region cloned upstream of the CAT gene in the promoter selection vector pSD7 (19). Notably, constructs with the minimal promoter alone showed 3–3.5-fold less activity than those with the promoter with the 5' untranslated region (18). While these results were obtained in LB, the disparity was further increased to 6- and 40-fold when the cells are grown in YK and starvation medium, respectively.

Analysis of the sequence of the 5' untranslated region reveals an inverted repeat that could potentially extrude out to form a cruciform in DNA or a hairpin in RNA. This cruciform/hairpin potential sequence (CHPS) is present 7 nt downstream of the transcriptional start site (Fig. 1C). In addition, the 5' untranslated region used in these constructs included the ribosome binding site as well (Fig. 1C). As discussed above, both of these elements are known to stabilise mRNA in bacteria (12,13). To delineate the exact elements in the 5' untranslated region that stimulate gene expression, constructs harbouring the promoter with the 5' untranslated region were subjected to random mutagenesis in *E. coli* as described in the Materials and Methods. Mutated DNA was used to transform *M. smegmatis* cells and clones that showed reduced CAT expression were selected for analysis. Since reduced CAT expression results in sensitivity to chloramphenicol (Chl^s), colonies were first screened for chloramphenicol sensitivity on plates. Next, sensitive clones were tested for reduction in both CAT activity and CAT protein levels to minimise irrelevant mutations. Three single mutants obtained after analysis of over 2000 colonies showed reduced resistance to chloramphenicol that correlated with the levels of CAT protein (not shown) as well as specific CAT activity (Fig. 2). All three

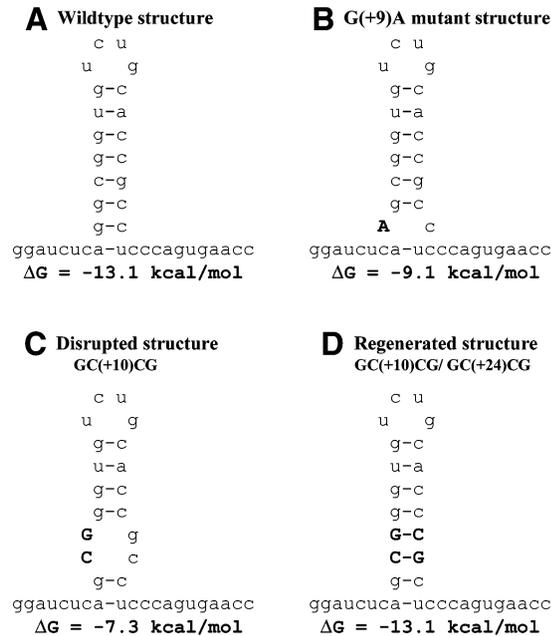


Figure 3. Disruption of the hairpin structure. Idealised structures of the wild-type (A), mutant from the random screen (B), site-directed disruption (C) and regenerated (D) forms of the CHPS are shown. ΔG was calculated using the web interface of the *mfold* algorithm as described in the Materials and Methods.

mutants carried mutations in the region surrounding the transcription start site. Two of them correspond to conserved residues in the putative -10 and -35 regions. These mutations would take these residues away from the mycobacterial promoter consensus (21,22) and therefore it is not surprising that they lead to a reduction in the expression of the CAT protein. Surprisingly, the third mutation that reduced CAT expression to about half the wild-type level is located downstream of the transcriptional start site. Notably, such a mutation would be predicted to weaken the stem-loop structure formed by the CHPS (Figs 2 and 3) suggesting that positive influence of the 5' untranslated region observed earlier (18) was probably related to the presence of this structure.

Structure as a positive regulator

To confirm the positive influence of the CHPS on gene expression, we mutated two consecutive bases to substantially weaken the putative structure (Fig. 3). In line with our prediction, this disruption reduced expression of CAT activity by >4-fold (Fig. 2 and Table 2). This effect is further enhanced when the cells are starved of glycerol with the difference increasing to 20-fold (Table 2) paralleling earlier results with the deletion of the 5' untranslated region (18). To delineate the function of the structure as opposed to the sequence in the region, the structure was restored by making compensatory mutations in the complementary stem (Fig. 3). Such a regeneration produces an equally stable structure in a different sequence context. Notably, this regeneration completely compensated for the defect and brought back the expression to wild-type levels in both YK as well as starvation medium (Fig. 2 and Table 2). Thus, it appears that the structure and not the sequence is the critical determinant for this positive influence on expression.

Table 2. Effect of CHPS on CAT activity

Medium	Construct	% Activity
YK	Wild-type	100.0 ± 9.2
	Disruption	23.3 ± 5.1
	Regeneration	102.1 ± 7.9
Starvation	Wild-type	104.1 ± 4.4
	Disruption	5.4 ± 2.3
	Regeneration	95.3 ± 8.1

CHPS stabilises the *gyr* transcript

The expression of a gene is determined by the combined effects of transcription, degradation and translation. Therefore, to ensure that the presence of the CHPS alters the steady-state level of the mRNA, RNA was isolated from cells harbouring different constructs and the *gyr-cat* hybrid was quantified by primer extension. Notably, the level of transcript (Fig. 4) paralleled the results obtained above with the CAT activity (Table 2). The disruption of the structure decreased the level of the mRNA 5- and 13-fold in YK and starvation medium, respectively. Furthermore, regeneration of the structure by compensatory mutations rescued this defect almost completely (Fig. 4). To ascertain whether the CHPS influences the rates of transcription or degradation, the half-life of the mRNA was measured in the context of wild-type, disrupted or regenerated forms of the structures. The hybrid message with the intact wild-type structure had a long half-life of ~49 min (Fig. 5). Disruption of the CHPS dramatically reduced this to ~5 min. This effect was further pronounced (2 min) in starvation medium. Finally, as with the steady-state level of the transcript and the CAT activity, regeneration of the structure restored the half-life to nearly wild-type levels (~42 min).

Lessons on the regulation of DNA gyrase

Earlier work has shown that DNA gyrase in *M. smegmatis* is subject to autoregulation in response to changes in the global topology of DNA (18). This phenomenon of relaxation-stimulated transcription is characteristic of gyrase and appears to be conserved throughout evolution (18,21,23,24). The nutrient-dependent stabilisation of the gyrase message reported here, and seen previously (18), represents a second, hitherto unexplored, level of regulation of the *gyr* genes in any organism (25). While, in general, stabilisation of a house-keeping message would be important for slow-growing organisms like mycobacteria, in the specific case of DNA gyrase, it probably has additional significance. The *gyrA* and *gyrB* genes in *M. smegmatis* are part of a single operon with transcription being driven from a single promoter present upstream of *gyrB* and for all known biological functions both proteins are required in equimolar amounts. Since genes present downstream in an operon are usually under-represented at the protein level, it would be useful for the organism to evolve methods to prevent this discrepancy. The mycobacterial *gyr* operon attempts to circumvent this problem by subtle changes in its primary sequence. For instance, the *gyrB* gene has a strong SD sequence (AGGAGA) upstream of a weak start codon (GTG) while *gyrA* has a poor SD sequence (AGGATT) with the more efficient ATG as the start codon. In such a context, the presence of a stabilising secondary

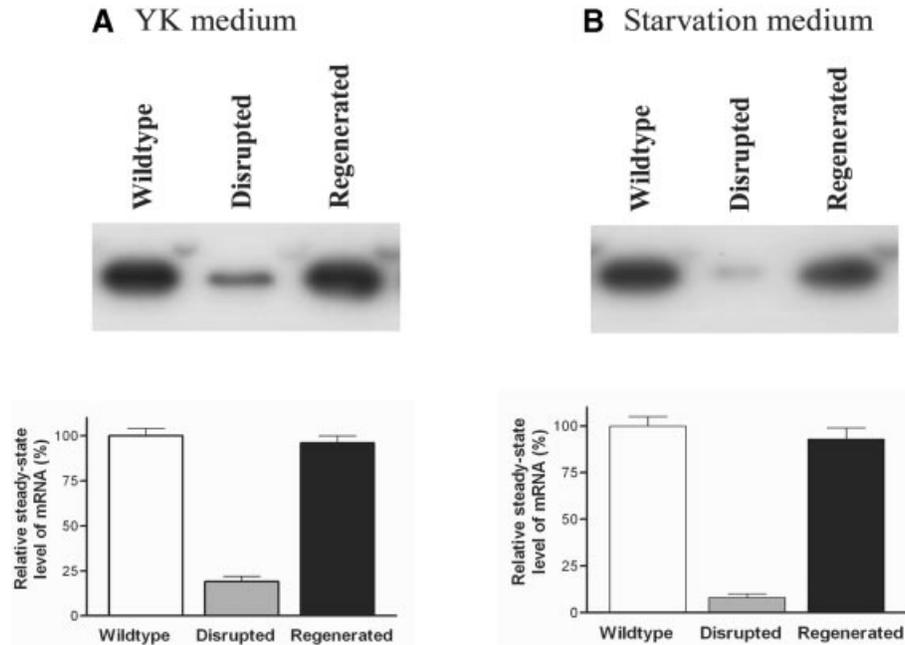


Figure 4. Effect of the disruption of the structure on the steady-state levels of the transcript. Primer extension analysis was performed on RNA isolated from exponentially growing cells harbouring different constructs. The wild-type, disrupted and regenerated structures are shown in Figure 3A, C and D, respectively. Representative results are shown with cultures grown in YK (A) or starvation medium (B). The average of three independent experiments is depicted graphically.

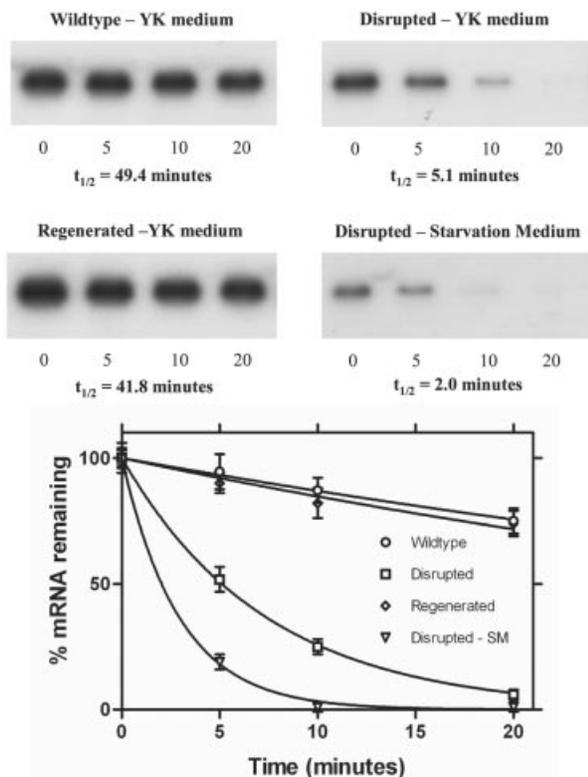


Figure 5. Effect of the disruption of the structure on the half-life of the transcript. RNA was isolated after exponentially growing cells harbouring different constructs were treated with rifampicin (50 $\mu\text{g}/\text{ml}$) for indicated time intervals. Primer extension was performed to quantify the level of transcript at each time point. Representative results with different cultures are shown. The wild-type, disrupted and regenerated structures are shown in Figure 3A, C and D, respectively. The average of three independent experiments is depicted graphically.

structure is probably an additional mechanism to ensure that the downstream message is maintained long enough to be translated efficiently. Interestingly, although the same CHPS can potentially extrude out in the DNA to form a cruciform in a supercoil-dependent manner, it has no discernible role in detecting changes in DNA topology to regulate the expression of gyrase (18). These changes are instead detected by regions far downstream of the promoter well within the *gyrB* gene (18). Thus, *M.smegmatis* appears to rely on two distinct sensors: a promoter-proximal sensor for nutrient levels and a promoter-distal sensor for DNA topology.

Evolutionary considerations

In conclusion, we have shown that the presence of hairpin structure near the 5' end of the message in *M.smegmatis* *gyr* transcript stabilises the downstream message considerably. Notably, the stabilising influence is more significant under conditions of nutrient deprivation. Enhanced stability of mRNA upon starvation has been reported in many organisms (14,26–28). This allows the cells to utilise the already synthesised messages to their fullest and conserve resources when they are scarce. This is arguably more important for organisms like mycobacteria that grow slowly even under nutrient-rich conditions. In these organisms, the lower rate of transcription elongation is probably compensated for by enhanced stability of the message, at least for housekeeping genes.

Secondary structures at or near the 5' end of the mRNA are known to stabilise downstream mRNA against degradation in *E.coli*. Extensive work has shown that, at least in *E.coli*, the structure is required to be present close to the 5' end of the mRNA and is believed to function by preventing access of RNase E to the 5' end of the mRNA (12). Furthermore, cleavage by RNase E appears to be the primary rate-determining step in the degradation of most messages in

E. coli (29). In the context of *M. smegmatis* there are three aspects of interest in the present study. First, at present, the status of RNase E is not clear from the shotgun sequence available from the *M. smegmatis* genome. However, other mycobacterial genomes such as *Mycobacterium tuberculosis* (30) and *Mycobacterium leprae* (31) appear to code for a homologue of this enzyme. Thus, RNase E probably represents an evolutionarily conserved mechanism of mRNA degradation. Second, to be protective in *E. coli*, the structure needs to be not more than 4 nt from the 5' end of the RNA (32). In contrast, the present study shows that a structure 7 nt from the 5' end of the message significantly protects the downstream mRNA against degradation. Third, genome-wide analysis of the distribution of secondary structures indicates that genes in slow-growing organisms like *M. tuberculosis* are more likely to have a strong secondary structure ahead of them than those in fast-growing organisms like *E. coli* (33). This probably protects a majority of the messages against the primary degradative activity in the cell, namely RNase E. Such a strategy would make economic sense for a slow-growing organism that does not necessarily need to respond to environmental changes rapidly (18,21,34). While it is tempting to propose such a model, extensive genetic experiments would be required to prove it.

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