Regulation of Gene Expression by Macrolide-Induced Ribosomal Frameshifting

Pulkit Gupta,1 Krishna Kannan,1 Alexander S. Mankin,1,* and Nora Vázquez-Laslop1,*
1Center for Pharmaceutical Biotechnology, University of Illinois at Chicago, 900 South Ashland Avenue, Chicago, IL 60607, USA
*Correspondence: shura@uic.edu (A.S.M.), nvazquez@uic.edu (N.V.-L.)
http://dx.doi.org/10.1016/j.molcel.2013.10.013

SUMMARY

The expression of many genes is controlled by upstream ORFs (uORFs). Typically, the progression of the ribosome through a regulatory uORF, which depends on the physiological state of the cell, influences the expression of the downstream gene. In the classic mechanism of induction of macrolide resistance genes, antibiotics promote translation arrest within the uORF, and the static ribosome induces a conformational change in mRNA, resulting in the activation of translation of the resistance cistron. We show that ketolide antibiotics, which do not induce ribosome stalling at the uORF of the ermC resistance gene, trigger its expression via a unique mechanism. Ketolides promote framesshifting at the uORF, allowing the translating ribosome to invade the intergenic spacer. The dynamic unfolding of the mRNA structure leads to the activation of resistance. Conceptually similar mechanisms may control other cellular genes. The identified property of ketolides to reduce the fidelity of reading frame maintenance may have medical implications.

INTRODUCTION

Regulatory upstream open reading frames (uORFs, also known as leader ORFs) control the expression of many genes in bacteria, archaea, and eukaryotes. The activation of the downstream gene(s) depends on the progression of the ribosome through the uORF. Because uORF translation is generally regulated by exogenous factors, such as the presence and concentration of specific metabolites, this mode of gene control is highly sensitive to the physiological state of the cell and is used to adapt to the changing environment.

Most commonly, uORF-dependent gene regulation in bacteria involves programmed translation arrest, wherein ribosome stalling within the leader ORF controls the expression of downstream genes (Yanofsky, 1990; Atkins et al., 1990; Gurvich et al., 2003). However, in spite of ingenious attempts to identify this type of gene control (Gurvich et al., 2011), such a regulatory mechanism has not been discovered, and its existence remains purely hypothetical.

One of the most well-studied models of uORF-dependent gene regulation is the inducible expression of the gene ermC, which renders bacteria resistant to erythromycin (ERY) and other macrolide antibiotics (Weisblum, 1995a). Macrolides are among the most clinically important antibacterial protein synthesis inhibitors (Alvarez-Elcoro and Enzler, 1999). The newest generation of macrolides, the ketolides (e.g., telithromycin [TEL]) (Figure 1A), are characterized by higher potency, improved activity against some resistant strains, and, in comparison to ERY, enhanced bactericidal properties (Brysryker, 2000).

Macrolide antibiotics bind in the ribosomal nascent peptide exit tunnel (NPET) in the vicinity of the peptidyl transferase center and inhibit the synthesis of most proteins at early rounds of translation by promoting peptidyl-tRNA drop off (Menninger et al., 1994; Otaka and Kaji, 1975; Tenson et al., 2003). However, some polypeptides are able to bypass the antibiotic in the NPET with ketolides allowing considerably more proteins to escape inhibition (Kannan et al., 2012; Starosta et al., 2010). The major mechanism of resistance to macrolides is based on the dimethylation of an adenine residue (A2058 in the 23S rRNA) in the drug binding site of the ribosome (Weisblum, 1995b). This modification is catalyzed by methyltransferases encoded in the erm genes, which are most commonly inducible (Subramanian et al., 2011). The prototype of these inducible genes is ermC, whose expression is controlled by a well-characterized translation attenuation mechanism (Figure 1B) (Horinouchi and Weisblum, 1980; Shivakumar et al., 1980; Vazquez-Laslop et al., 2008). The resistance gene is preceded by the 19-codon leader ORF ermCL. In the absence of antibiotic, the leader ORF is constitutively translated, but ermC is translationally repressed because its ribosome binding site is sequestered by mRNA secondary structure. The presence of subinhibitory concentrations of a macrolide (e.g., ERY) causes the ribosome to stall at the ninth codon of ermCL. Translation arrest leads to the isomerization of the mRNA secondary structure, releasing the ribosome binding site of ermC, thereby activating its translation (Figure 1B) (Horinouchi and Weisblum, 1980; Shivakumar et al., 1980). Other inducible erms are regulated by conceptually similar mechanisms (reviewed in Subramanian et al., 2011). The strategic site of programmed translation arrest at ermCL is controlled by the amino acid sequence of the ErmCL peptide and by the structure of the drug (Mayford and Weisblum, 1989; Vazquez-Laslop et al., 2008).
modifications of the C3 cladinose of ERY dramatically reduce the efficiency of translation arrest (Vázquez-Laslop et al., 2011). In agreement with this observation, ketolides, which lack the C3 cladinose, fail to promote ribosome stalling at the ermCL ORF (Vázquez-Laslop et al., 2011; Vazquez-Laslop et al., 2008).

Although the initial studies generated the general perception that ketolides do not activate emr (Rosato et al., 1998; Van Bambeke et al., 2008), some reports have indicated that these drugs can induce the expression of erm genes in clinical pathogens, albeit to a lesser extent in comparison to macrolides (Bailey et al., 2008; Clarebout and Leclercq, 2002; Giovanetti et al., 2000; Schmitz et al., 2002a; Zhong et al., 1999). The puzzling observation that antibiotics, which apparently do not promote canonical programmed translation arrest at the uORF, can nevertheless activate the expression of erm raised the possibility that a principally different mechanism underlies the inducing activity of ketolides.

In this work, we uncovered that the mechanism of ketolide-dependent induction of ermC utilizes a previously unknown ability of these antibiotics to induce ribosomal frameshift errors. The ketolide-mediated frameshifting within the ermC leader ORF allows for bypass of the ermCL stop codon, inducing the isomerization of mRNA secondary structure due to the progression of the ribosome through the IGS of the bicistronic operon. Our findings unveil a principle of gene regulation wherein a frameshift event occurring at the leader ORF regulates the expression of a downstream gene.

RESULTS

TEL-Dependent Induction of ermC Does Not Involve Ribosome Stalling at the ermCL Regulatory uORF

To analyze the mechanisms of ERY- and TEL-dependent induction of ermC, we used the previously engineered reporter pERMZx, where the ermC sequence, starting from the third codon, is replaced with the lacZ sequence encoding the β-galactosidase x peptide (Bailey et al., 2008) (Figure 1C). In E. coli cells capable of α complementation (e.g., strain TB1), inducibility of the reporter is assessed by the disk diffusion assay with the use of paper disks impregnated with antibiotics. As shown previously, the reporter is readily inducible by ERY or TEL (Bailey et al., 2008) (Figure 1C).

Although ERY-mediated activation of ermC is controlled by ribosome stalling at a specific codon of the leader ORF, the mechanism of induction by ketolides remained unknown. Therefore, we tested whether TEL induction requires the translation of ermCL. When the initiation codon of ermCL was mutated to UAA (Figure 1C, construct pERMZx), induction by either ERY or TEL was completely abolished. This suggests that the TEL-dependent activation of ermC relies on events that occur during the translation of the leader ORF.

Programmed ribosome stalling at the ninth codon of ermCL, which is the centerpiece of the classic mechanism of macrolide-dependent ermC induction, requires the integrity of the four-amino-acid motif Ile6-Phe7-Val8-Ile9 in the ErmCL nascent peptide (Mayford and Weisblum, 1989; Vazquez-Laslop et al., 2008). In order to test whether this sequence is also important for TEL-mediated ermC activation, compensatory frameshift mutations that changed the identity of the ermCL codons 5 to 9 were introduced (construct pERMZx.FS[5–9] in Figure 1C). As anticipated, these mutations abrogated the induction of the reporter by ERY. In contrast, induction by TEL remained at the level observed with the wild-type (WT) construct (Figure 1C), reaffirming that stalling at the ninth codon of the leader ORF is not required for ketolide activation of ermC.

Ketolide Induction of ermC Requires a Shift-Prone Sequence at the End of the ermCL Regulatory ORF

The induction of ermC by ERY entails specific contacts between the ErmCL nascent peptide and the antibiotic in the ribosomal tunnel (Vázquez-Laslop et al., 2011; Weisblum, 1995b). Conceivably, TEL activation of ermC could also rely upon interaction of the drug perhaps with a different segment of the nascent peptide. In order to elucidate which segment of ErmCL could be important for induction, a progressive series of compensatory frameshift mutations in the leader ORF were engineered. The two most revealing mutants are shown in Figure 1C. The frameshift that started at codon five and reached as far as the 16th codon of ermCL (pERMZx.FS[5–16]) did not affect TEL induction of the reporter. However, deleting a nucleotide from the last sense codon of ermCL to extend the frameshift to the very end of the ORF (pERMZx.FS[5–19]) completely abolished ketolide induction. This finding indicated that either the nature of the C-terminal amino acid residues of the ErmCL peptide or, perhaps, the sequence of the last few codons of the ermCL mRNA were critical for TEL-induced expression.

To get further insights into the mechanism of TEL-dependent induction, we introduced mutations at the last three codons of the ermCL ORF. Codon 17 did not appear to be associated with any critical peptide or RNA sequence element, given that TEL induction remained unaffected when this codon was mutated to Lys (AAG), Gly (GGC), or the synonymous Asn

Figure 1. Ketolide-Dependent Induction of the ermC Gene Does Not Require Ribosome Stalling at the ermCL Ile9 Codon

(A) Chemical structures of ermC-inducing antibiotics: erythromycin (ERY), a macrolide, and telithromycin (TEL), a ketolide. (B) Top: the organization of the ermC operon. The regulatory 19-codon leader ermCL ORF (purple) is separated by a 60 nt-long intergenic region from the ermC gene (orange). Bottom: the classic scheme of ermC induction by erythromycin, where ribosome stalling within the ermCL ORF promotes switching the mRNA structure from the noninduced (Off) to induced (On) state. The encoded amino acids are indicated over the corresponding ermCL and ermC codons. The Shine-Dalgarno sequences and the start codons for ermCL and ermC genes are highlighted in bold. The amino acid residues of ErmCL critical for ERY-dependent stalling are in bold, and the codon occupying the P site of the stalled ribosome is indicated by an arrow.

(C) Mutation analysis of ERY- and TEL-dependent ermC induction. Top: the structure of the pERMZx reporter in which codons 3-244 of the ermC gene are replaced with 57 codons of the lacZ gene encoding the LacZ x peptide. Nucleotide alterations are indicated by red dots, insertions and deletions are shown by arrows, and the altered amino acid sequences are highlighted in red. Right: disc diffusion test plates with TEL or ERY antibiotic disks. Green and red check marks highlight the induction of the reporter by the antibiotic or a lack thereof, respectively. See also Figure S6.
Effects of the mutations of the last two Lys (AAA) codons (codons 18 and 19) were by far more intriguing. The codon 19 mutations initially seemed to support the role of the nascent peptide because activation remained intact when the third position of the codon was changed to G (converting Lys [AAA] to the synonymous [AAG]) but was disrupted upon the alteration of the first nucleotide of the AAA codon to G (which converted it to Glu [GAA]) (Figure 2, right). However, mutations in codon 18 clearly pointed to a different scenario. TEL induction was abolished when codon 18 was changed to the synonymous Lys (AAG), hinting that, rather than the encoded peptide, it is the mRNA sequence that plays the central role in induction. Consistently, mutating Lys18 (AAA) codon to two different (but synonymous) Ile codons had a differential effect; the AUA codon preserved the induction, whereas the AUU codon abolished it (Figure 2, middle). We ruled out the effect of the mutations on mRNA stability because all these reporters remained equally inducible by ERY (Figure S1 available online).

Careful analysis of the broad collection of mutants brought us to an unanticipated conclusion that reconciled the seemingly controversial results; the most recognizable common feature among the mutant reporters that retained inducibility was not the conservation of a particular peptide sequence but rather the preservation of a run of adenine residues (XXA18 AAA/G19) in the last two sense codons of ermCL. Such a structure strikingly resembles a shift-prone sequence (Fayet and Prère, 2010), which can hypothetically allow the tRNA Lys (anticodon UUU) that decodes codon 19 (either WT AAA or mutant AAG) to re-pair to the alternative Lys codon in the (−1) frame possibly via P site tRNA slippage. Such frameshifting would lead to readthrough of the ermCL stop codon and the possible activation of ermC.

Translation of the Entire ermCL by Ribosomes with Bound TEL Is Required for Induction of ermC

The finding that the events pertaining to TEL activation of ermC occur close to the end of the ermCL ORF was puzzling, given that antibiotics of the macrolide family are known to inhibit the translation of most proteins at the early rounds of elongation (Menninger and Otto, 1982; Otaka and Kaji, 1975; Tenson et al., 2003). We wondered whether ErmCL belonged to the class of proteins that could be synthesized in spite of the presence of the ketolide (Kannan et al., 2012). If this were the case, ribosomes with bound TEL could reach the last codons of the regulatory ORF. This possibility was tested in a cell-free protein synthesis system. Translation of the ermCL ORF produced a protein whose migration in a SDS-PAGE gel corresponded to an apparent molecular weight of ca. 3 kDa—reasonably close to the 2.2 kDa full-size ErmCL polypeptide (Figure 3A). Remarkably, although the production of the protein was abolished by ERY (because it causes translation arrest at the ninth codon of ermCL), the full-length ErmCL continued to be synthesized in the presence of even saturating concentrations of TEL (50 μM), demonstrating that the antibiotic does not prevent the ribosome from reaching the end of the ermCL ORF (Figure 3A). In addition to the WT ermCL, the mutant constructs explored in the previous experiments (Figure 1C) could also be translated in the presence of TEL but not ERY (Figure S2).

Figure 2. The Shift-Prone Sequence at the End of the ermCL ORF Is Critical for TEL-Dependent Induction

Inducibility by TEL of the pERMZ reporter mutants with alterations in the 17th, 18th, or 19th codons of the ermCL ORF. Top: the sequence of the WT ermCL and the encoded peptide. Bottom: the mutations in codons 17–19 of ermCL with nucleotide changes highlighted in red and changes in the encoded amino acids indicated by red dots. The preserved shift prone sequence XXA AAA/G is underlined by a solid line, and the disrupted shift-prone sequence is underlined by a dotted line. See also Figure S1.
Furthermore, the inability of the ribosome to reach the end of the luc-ermCL hybrid ORF in the presence of TEL completely abolished the reporter expression (Figure 3C). These data reinforce the notion that the translation of the entire ermCL is a prerequisite for TEL induction.

The ribosomes translating ermCL in the presence of TEL could still retain the antibiotic by the time they reach the last codons of the ORF, or, alternatively, the drug could be displaced by the growing nascent peptide early in the translation cycle (Kannan et al., 2012; Tripathi et al., 1998). To distinguish between these scenarios, we took advantage of the ability of ketolides to cause ribosome stalling at the Asp10 codon of the ermBL ORF (data not shown). Ribosome stalling at the ermBL ORF can be detected by toe printing (Hartz et al., 1988) (Figure 3D, lanes 1 and 2). To test whether TEL remains bound to the ribosome reaching the end of the ermCL ORF, we fused the ermBL ORF (starting from the second codon) in frame to the last sense codon of ermCL, and translation in the absence or presence of TEL was analyzed. A strong toe-print signal at the Asp codon of ermBL was observed when the ermCL ermBL hybrid was translated in the presence of TEL (Figure 3D, lane 4). This means that TEL was retained by the ribosome that translated the entire ermCL (and continued until reaching the arrest codon at ermBL). These results show that when the ribosome reaches the shift-prone sequence at the end of the ermCL ORF it still carries the TEL molecule bound in the tunnel.
Figure 4. Translation of the ermCL-ermC Intergenic Region Leads to ermC Induction

(A) The three reading frames in the ermCL-ermC intergenic segment of the ermC operon. The ermCL sequence is purple, ermC is orange, and the intergenic segment is black. The amino acid sequences that would be synthesized by the ribosome after direct (0 frame) readthrough of the stop codon or after (-1) or (+1) frameshifting within the last two codons of the ermCL ORF are shown above the mRNA-coding sequences. The stop codons in the respective reading frames are underlined and indicated by asterisks.

(B) Translation of the intergenic region in the (-1) reading frame activates the ermC expression. Top: the sequence of the ermCL-ermC intergenic region and the encoded peptide in the WT pERMZα. Middle: the pERMZα.FS reporter with a (-1) frameshift mutation in the ermCL coding sequence. Sequences are color coded as in (A), lacZ is in blue, and the inserted nucleotide is indicated by an arrow. pERMZα* and pERMZα.FS* are the counterparts of the corresponding reporters (legend continued on next page).
Frameshifting at \textit{ermCL} and Resulting Translation through the IGS Can Activate \textit{ermC} Expression

The \((-1\) \) frameshift that may take place within the two last sense codons of the \textit{ermCL} regulatory ORF would allow the ribosome to bypass the \textit{ermCL} stop codon. Remarkably, if this happens, then the ribosome would continue translation through the entire IGS until it encounters the first in-frame stop codon within the \textit{ermC} coding sequence (Figure 4A). Neither the 0 frame read-through of the \textit{ermCL} stop codon nor a \((+1)\) frameshift would make translation of any large segment of the IGS possible because of the presence of stop codons within these frames (Figure 4A). The unexpected picture that emerged from these observations suggested that TEL induction of \textit{ermC} might operate via an antibiotic-induced \((-1\) \) frameshift near the end of \textit{ermCL} that allows the ribosome to traverse the \textit{ermCL}-\textit{ermC} IGS.

We carried out two experiments to test whether IGS translation could activate \textit{ermC} expression. When an uncompensated frameshift mutation was introduced after the fourth \textit{ermCL} codon (pERMZ\textsubscript{z}x.FS; Figure 4B) forcing the ribosomes to translate the rest of \textit{ermCL} and the IGS in the \((-1)\) frame, the reporter was activated even in the absence of the inducing antibiotic (Figure 4B). No reporter expression was observed when the start codon of the mutant \textit{ermCL} gene was altered (pERMZ\textsubscript{z}x.FS\textsuperscript{*}), confirming that activation was contingent on the translation of mutant \textit{ermCL} and the spacer segment. Second, a UAA stop codon in the \((-1\) \) frame was introduced in the IGS of the pERMZ\textsubscript{SS} reporter (pERMZ\textsubscript{SS}; Figure 4C). This mutation, which had no effect on ERY-dependent induction, completely abolished activation by TEL (Figure 4C). The observation that a nonsense mutation in the normally untranslated mRNA segment negatively influences \textit{ermC} activation strongly argues that translation through the intergenic region in \((-1\) \) frame is required for the ketolide-dependent induction.

Translation through the \textit{ermCL}-\textit{ermC} IGS Stimulates Translation Initiation at the \textit{ermC} Start Codon

The ketolide-induced \((-1\) \) frameshifting within the \textit{ermCL} ORF and the resulting stop codon bypass and translation of the intergenic region would bring the ribosome to the \textit{ermC} cistron in the \((-1\) \) frame relative to the \textit{ermC} coding sequence (Figure 4A). The translation is expected to be terminated at the first stop codon in this frame located at a distance of 49 nt from the \textit{ermC} authentic start codon. We envisioned two scenarios of how the TEL-bound ribosomes traversing the IGS could activate the expression of the \textit{ermC} gene. Such ribosomes could possibly shift to the 0 frame within the \textit{ermC} coding region, thereby generating a hybrid protein that carries the ErmCL sequence at its N terminus (Narayanan and Dubnau, 1987). Alternatively, unwinding of the translation attenuator structure by the ribosome navigating through the IGS could provide a window of opportunity for another, presumably drug-free, ribosome to initiate translation at the \textit{ermC} start codon and synthesize a “regular” ErmC protein. To distinguish between these possibilities, we determined the N-terminal sequence of the TEL-induced protein. To isolate the polypeptide product, we appended a 3X-FLAG tag to the C terminus of the \textit{ermC}-based reporter, and additionally, the \textit{ermC}-coding segment of the reporter was extended to include the 22 5’ terminal codons of the gene in order to better mimic the native operon (Figure S3A). The TEL-induced translation product was affinity purified from \textit{E. coli}, and its N-terminal sequence was determined by Edman degradation (Edman, 1950). The first three amino acids (Met-Asn-Glu…) matched those of the native \textit{ermC} exactly, indicating that ketolide-induced translation of \textit{ermC} was initiated at its canonical start site. Furthermore, because translation of the \textit{ermC} gene is sensitive to TEL (Figure S3D), the ribosome that retained TEL during the ErmCL synthesis should be unable to continue translation of the ErmC polypeptide. Therefore, the available data argue that the disruption of the translational attenuator by the ribosome traversing the \textit{ermCL}-\textit{ermC} IGS stimulates the initiation of \textit{ermC} translation from its authentic start codon by another ribosome.

TEL Stimulates Frameshifting within the \textit{ermCL} Shift-Prone Sequence

The data presented above offer consistent but indirect evidence that ketolides can prod the ribosome in order to switch to a different reading frame within the shift-prone sequence at the end of the \textit{ermCL} ORF. This idea contradicts the conventionally accepted mode of action of the macrolide drugs, which are known to cause peptidyl-tRNA drop off or ribosomal arrest most commonly at the early rounds of translation. Thus, the possibility that these drugs induce frameshifting well into the ORF appeared rather unorthodox. Therefore, we felt compelled to unequivocally determine whether TEL is able to induce frameshifting at the slippery sequence at the end of the \textit{ermCL} gene. For this purpose, the reporter construct \textit{ermCL-BL}\textsuperscript{\((-1\) \)} was designed so that in vitro drug-induced frameshift (Figure 5A) could be directly monitored. Here, the sequence of the \textit{ermCL} ORF, including its stop codon, is followed in a \((-1\) \) frame by 16 codons (codons 2–17) of the \textit{ermBL} ORF. This \((-1\) \) frameshift error within the \textit{ermCL} coding region allows the ribosome to translate \textit{ermBL} in the correct frame and, if antibiotic is bound, stall at the Asp\textsubscript{10} codon of \textit{ermBL}. Additionally, in order to account for the possibility of drug-independent frameshifting, the Thr\textsubscript{12} (ACA) codon of \textit{ermBL} was mutated to Trp (UGG). If
the drug-free ribosome shifts to a (−1) frame within the ermCL ORF, then presence of the tryptophanyl-tRNA synthetase inhibitor indolmycin would cause translation stalling at the Lys11 codon of ermBL. Bypassing the ermCL stop codon in the 0 frame without frameshift would lead to the translation of the ermCL-BL(−1) reporter in the absence of TEL rendered no indolmycin-dependent ribosome stalling at the Lys11 codon (Figure 5B, lane 5), showing that the frequency
of drug-independent frameshift within the ermCL ORF is insignificant. In contrast, upon the addition of TEL, the characteristic toe-print band at the Asp10 codon of ermBL appeared (Figure 5B, lane 6), indicating that a fraction of ribosomes that retained the antibiotic molecule in the NPET switched to the (−1) reading frame while translating the ermCL ORF.

In order to verify this conclusion, the FLAG-coding sequence was introduced into the ermCL-ermC spacer sequence in the (−1) frame relative to the ermCL ORF. The resulting construct was translated in vitro in the absence or presence of TEL, and the appearance of the FLAG-bearing translation product was detected by western blotting (Figure 5D). Only a negligible amount of the product was detected in the absence of TEL, whereas the presence of the antibiotic greatly stimulated the accumulation of the FLAG-containing polypeptide whose size corresponded precisely to the anticipated frameshifted hybrid protein (Figure 5D, lanes 1, 2, and 3). Because the ermCL start codon mutation completely eliminated the accumulation of the FLAG-positive material (Figure 5D, lanes 4 and 5), we concluded that TEL-induced (−1) frameshifting takes place during the translation of the ermCL gene and leads to stop codon bypass and the continuation of translation of the spacer sequence in the (−1) frame.

In order to determine whether TEL-dependent (−1) frameshift occurs within the XAA AAA slippery sequence at the end of ermCL, mutations that affected TEL-dependent ermC induction in vivo (Figure 2) were tested in the in vitro frameshift assay with the ermCL-BL(−1) reporter (Figure 5C). Toe-printing analysis showed that TEL-promoted frameshifting at ermCL occurred when the four-adenine slippery sequence within the last two sense codons was preserved (Figure 5C, lanes 4, 6, and 12), but not when it was disrupted in the mutants AUU AAA, AAG AAA, and AAA GAA (Figure 5C, lanes 8, 10, and 14). This result is compatible with the single-tRNA slippage scenario (Baranov et al., 2004). Notably, the pERMZz.FS[5–19] construct, which retained a run of five adenines, was not inducible by TEL (Figure 1C). Testing this mutant in a toe-printing assay showed that the TEL-bound ribosome was arrested at the penultimate codon of the mutant ermCL, thereby making frameshifting impossible (data not shown). Because of a perfect correlation between occurrence of the frameshift and ermC induction, we concluded that TEL activates ermC expression by promoting (−1) frameshifting within the slippery sequence in the leader ORF.

Macrolides Induce Frameshift Errors Irrespective of the Nascent Peptide

The ability of macrolides to promote ribosome frameshifting was previously unknown. Therefore, we designed experiments to gain some insights into this activity of the NPET-binding antibiotics. Specifically, we wanted to understand whether frameshifting is mediated by the combined action of the antibiotic and the nascent peptide or by an intrinsic property of the antibiotic itself. Remarkably, when the ermCL portion of the frameshift reporter ermCL-BL(−1) (Figure 5A) was truncated in order to leave only three sense codons (the initiator codon followed by the two 3′ terminal codons containing the shift-prone sequence, MKK-BL(−1), Figure 6A), TEL was able to efficiently induce frameshifting (Figure 6A). The MKK tripeptide in the ribosome positioned at the shift-prone sequence of the MKK-BL(−1) reporter can barely reach the antibiotic. Hence, frameshifting activity of ketolides does not require extensive interaction of the drug with the nascent peptide. Other ketolides, including solithromycin, cethromycin, and HMR3004, as well as cladinose-containing macro- lides ERY and RU89874, were also able to induce frameshifting in the MKK-BL(−1) construct (Figure 6A), albeit with different efficiency. Notably, the pERMZz.FS[5–9] and pERMZz.FS[5–16] constructs, were not inducible by ERY because ERY-bound ribosome does not reach the 3′ end of the mutant ermCL ORFs, as indicated by the inhibition of translation in presence of ERY (Figure S2). Interestingly, ERY and TEL retained their capacity to promote (−1) frameshifting when the Lys (AAA) codons in the reporter were replaced with Phe (UUU) codons (Figure 6B). These antibiotics also induced (+1) frameshift within the (+1) shift-prone sequence UUU UAA in the MF-BL(+1) construct (Figure 6C). From these results, we concluded that (1) frameshifting activity is an intrinsic property of antibiotics of the macrolide family and (2) the propensity for reducing translation fidelity depends on the chemical structure of the drug.

DISCUSSION

We have shown that ketolide-dependent induction of the resistance gene ermC operates via regulated translational bypass of the leader ORF stop codon. The antibiotic promotes frameshifting at a defined site in the ermCL ORF and allows the continuation of translation through the IGS. This leads to the unwinding of the spacer secondary structure and activation of the resistance gene. Induction in vivo occurs at subinhibitory concentrations of TEL when a sufficient fraction of ribosomes translating ermCL carry TEL and, hence, are prone to frameshifting, whereas enough drug-free ribosomes remain in the cell in order to synthesize the ErmC methyltransferase. Our findings have two important implications: first, they reveal an example of gene regulation on the basis of a controlled recoding event that takes place during the translation of the leader ORF, and, second, in the particular case of ermC induction, this mechanism is powered by the previously unknown frameshifting activity of macrolide antibiotics, which could be explored for medical purposes.

The Frameshifting Activity of Ketolide Antibiotics Makes the Induction of ermC Possible

The induction of ermC expression by ketolides is possible because of the identified ability of these antibiotics, and macrolides in general, to induce ribosome frameshifting. At the moment, we have a very limited understanding of the mechanism of macrolide-induced frame maintenance errors. Because the slippery site in the ermC ORF immediately precedes the stop codon, we initially considered the possibility that the TEL-promoted ribosome slippage could be due to the inefficient termination of translation, resulting in the increased dwelling time at the last sense codon of ermC (Meskauskas et al., 2003). However, this scenario seems unlikely, given that the insertion of an extra sense codon after the ermCL slippery sequence did not prevent ketolide-induced frameshifting (Figure S4B).

Macrolides are known to promote peptidyl-tRNA drop off (Menninger and Otto, 1982; Tenson et al., 2003). For dissociation to occur, peptidyl-tRNA affinity to the ribosome needs to be
reduced, perhaps including a weakening of the tRNA-mRNA interaction. Therefore, the ability of macrolides to promote frameshifting could be a facet of the long-known peptidyl-tRNA drop off activity of these antibiotics. How macrolides achieve this remains unknown. Notably, drug-induced frameshifting was prominent even when the nascent peptides esterifying peptidyl-tRNA were as short as two to three amino acids, barely reaching the antibiotic in the NPET, suggesting that the steric hindrance of the peptide progression through the tunnel plays a minor role in the mechanism of frameshifting. Therefore, we favor the model in which the mere presence of the antibiotic in the NPET is sufficient to change the ribosomal properties involved in reading frame maintenance. Codon-anticodon interactions and, thus, reading frame maintenance, are controlled by the small ribosomal subunit, whereas macrolides bind in the NPET in the large subunit some 90 Å away. Hence, the influence of the antibiotics on reading frame maintenance must be allosteric. Previous structural studies of ribosome-antibiotic complexes, which have been carried out with vacant ribosomes, provided little clues about the possible effects of macrolides on ribosome-tRNA and tRNA-mRNA interactions. Expanding the structural analysis to more physiologically relevant complexes that include tRNA ligands may provide insights into the critical yet cryptic aspects of action of macrolides and other ribosome-targeting antibiotics.

Figure 6. Ketolides and Cladinose-Containing Macrolides Induce Frameshifting in a Nascent Peptide-Independent Manner

(A) Macrolide and ketoide antibiotics promote frameshifting even in the absence of the EmrCL nascent peptide in the NPET. Top: the structure of the reporter construct MKK-BL(−1) in which a three-codon minigene is fused in (+1) frame with the ermBL reporter. (−1) frameshifting within the second or third codons is required for macrolide-dependent translation arrest at the D10 codon of the ermBL segment. Bottom: toe-printing analysis of the frameshifting induced by cladinose-containing macrolides (ERY and RU69874) or ketolides (the rest of the antibiotics). All drugs were used at saturating concentrations (50 μM).

(B) Macrolides induce (−1) frameshifting irrespective of the structure of the nascent peptide. ERY- or TEL-dependent arrest at D10 of ermBL of the MFF-BL(−1) reporter (arrow) requires (−1) frameshifting within the three-codon leader segment encoding the MFF peptide.

(C) Macrolides can induce not only (−1) but also (+1) frameshifting. (+1) frameshifting within the 5′ terminal AUGUUUA sequence is required to produce a toe-print band (arrow) within the ermBL segment of the MF-BL(+1) reporter.

See also Figure S4.

The Miscoding Activity of Macrolide Antibiotics Can Be Medically Relevant

Thompson et al. (2004) had previously shown that macrolides are able to stimulate nonsense suppression when illegitimate stop codons are placed at the beginning of a reporter gene. At the time, this report seemed puzzling and hardly physiologically relevant because macrolide antibiotics were thought to inhibit synthesis of every protein at the early rounds of translation. However, the recent findings showing that a number of polypeptides escape erythromycin inhibition and that even more proteins (up to 25% of the entire E. coli proteome) continue to be synthesized in the presence of ketolides (Kannan et al., 2012), making miscoding, as well as frameshifting, activity highly relevant to the mode of action of these drugs. Due to the macrolide-instigated errors, translation of the macrolide-resistant proteins could potentially lead to the production of aberrant polypeptides. In support of this view, we observed that TEL induces frameshifting within a long polypeptide given that it could stimulate the synthesis of functionally active β-lactamase from a gene that contained a frameshift mutation introduced 109 codons from the start of the bla ORF (Figures S5A–S5C). Therefore, ketoide-induced production of aberrant cellular proteins could be an important component of the antibacterial action of these antibiotics. In agreement with this conclusion, we found that cells lacking translation release factor RF3, which has been implicated in maintaining high-fidelity protein synthesis (Zaher and Green, 2011), are hypersensitive to TEL (Figure S5D).
The “traditional” miscoding-promoting antibiotics (e.g., aminoglycosides) are highly bactericidal. It is possible that some bactericidal activity known to be associated with ketolide antibiotics (Hamilton-Miller and Shah, 1998; Kannan et al., 2012) stems, at least in part, from their misincorporation and frameshifting capacities. Notably, chemically diverse ketolides differ in their ability to activate $ermC$ expression (Bailey et al., 2008). Therefore, the ability to induce frameshifting could most likely be manipulated by altering the antibiotic structure. This opens a venue for improving the antibacterial potency of ketolide drugs or exploiting them for the treatment of disorders caused by missense or nonsense mutations (Zilberberg et al., 2010).

**Induction of $ermC$ by Ketolides Unmasks a Mode of Gene Regulation**

The mechanism of $ermC$ activation by ketolides is remarkably different from the classic induction by ERY and other cladinose-containing macrolides. ERY-dependent activation is controlled by the ErmCL nascent peptide. In cooperation with the antibiotic, the leader peptide induces ribosome stalling within the $ermCL$ ORF. The arrested ribosomes facilitate isomerization of the $ermCL$-$ermC$ IGS structure, leading to $ermC$ expression (Figure 7). In contrast, ketolide induction utilizes the structural features of both the leader peptide and the leader ORF mRNA. The N-terminal structure of the peptide accounts for its ability to bypass the antibiotic in the NPET and, thus, ensures that the drug-bound ribosome reaches the end of the $ermCL$ ORF. It is here, within the shift-prone sequence, that the antibiotic promotes slippage of the translating ribosome to the (−1) reading frame, leading to bypass of the $ermCL$ stop codon and the invasion of the $ermCL$-$ermC$ IGS. Although it is the presence of a static ribosome at a specific $ermCL$ codon that leads to the mRNA conformational switch in ERY-dependent induction, TEL-dependent activation of $ermC$ operates on the basis of cotranslational dynamic unfolding of the mRNA secondary structure (Figure 7).

Other macrolide resistance genes (e.g., $ermA$, $ermT$, and $erm33$) (Ramu et al., 2009; Subramanian et al., 2011) are controlled by the regulatory ORFs carrying an $ermCL$-like slippery sequence. The expression of such genes could be activated by ketolide-induced frameshifting. At first glance, this mode of gene regulation may appear “artificial,” given that the resistance genes acquired by clinical pathogens are not “native” to these organisms and that the medically useful ketolides are semisynthetic compounds. However, the inducible resistance genes and ketolide antibiotics, as well as other types of cladinose-lacking macrolides, are, in fact, rather abundant in nature (Vazquez, 1979); therefore, the recoding-based mechanism of induction could help organisms carrying $ermC$-like genes survive in antibiotic-containing habitats. Indeed, the natural ketolide pikromycin readily activates the expression of the $ermCL$-based pERM2 reporter via drug-induced frameshifting (Figure S6). Although ketolide induction of $ermC$ is often insufficient to render pathogens resistant to the clinically relevant high concentrations of these drugs, in the natural environment, increasing resistance 2- to 4-fold may make a difference between life and death.

Programmed frameshifting within protein-coding genes is used by various organisms and even more frequently by their invading viruses to regulate the expression of specific genes.
Molecular Cell

Frameshifting Activates Gene Expression

Induction by ketolides is powered by the previously unknown frameshifting activity of these antibiotics which can be exploited for medical purposes.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids
E. coli strains and plasmids used in this study are listed in Tables S1 and S2, respectively.

Inducibility Assay
Disc diffusion assays for testing the inducibility of the pERMZ2 reporters were carried out as described previously (Bailey et al., 2006) with the exception that the E. coli strain TB1 (Table S1) (Johnston et al., 1986) was the host. Expression of the reporter plasmids was carried out on Luria-Bertani (LB)-agar supplemented with X-gal, Isopropyl (IPTG), and ampicillin. Antibiotic discs contained 300 μg of ERY or 200 μg of TEL.

Cell-free Translation
Translation of the genes of interest was performed as indicated in either the E. coli S30 cell-free transcription-translation system for linear DNA templates (Promega) or PURExpress (New England Biolabs) following the protocols described previously (Kannan et al., 2012). When required, translation reactions were carried out in the presence of 50 μM ERY or TEL.

Toe-Printing Assay
Toe-printing analysis of drug-dependent ribosome stalling was carried out as described previously (Vazquez-Laslop et al., 2008). The DNA templates (0.1 pmol) were used in a total volume of 5 μl of PURExpress (NE Biolabs) reactions. Samples were incubated for 15 min at 37°C and [35S]-NVP1 primer was added (Table S3) designed to anneal ~100 nt downstream from the anticipated ribosome stalling site. The primer was extended by reverse transcriptase, and the reaction products were analyzed in sequencing gels as described previously (Vazquez-Laslop et al., 2008).

Analysis of ermC Induction by Western Blotting and N-Terminal Sequencing of the Induction Product
E. coli JM109 cells transformed with pERMZ22FLAG were grown in 5 ml LB with 100 μg/ml ampicillin and 0.2 mM IPTG. At an OD600 of 0.2, cultures were split into two flasks, one of which had TEL added to a final concentration of 15 μg/ml. Then, cells were incubated at 37°C for 4 hr, pelleted, and resuspended in 200 μl of B–Per reagent (Thermo Scientific) supplemented with 100 μl of Omnileave endonuclease (Epicerent). Lysis was completed by incubating at 37°C for 30 min. Samples with 20 μg of protein (ca. 20 μl of the lysate) were loaded onto 4%–12% NuPAGE Bis-Tris gel (Life Technologies) and resolved by electrophoresis. Proteins were transferred to PVDF membrane and probed with antibodies against FLAG (Santa Cruz Biotechnology). TEL-induced product was affinity purified by immunoprecipitation with the anti-FLAG M2 Affinity Gel (Sigma–Aldrich) according to the manufacturer’s protocol. The purified product was fractionated in a 20 cm-long, 16.5% polyacrylamide Tris-Tricine gel (Schägger and von Jagow, 1987), transferred to PVDF membrane, and stained with 0.1% Coomassie Blue R-250. The band of interest was excised and subjected to Edman sequencing carried out at the Columbia University Protein Core Facility.

SUPPLEMENTAL INFORMATION
Supplemental Information contains Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.10.013.

ACKNOWLEDGMENTS
We thank Dorota Klepacki, Yijia Luo, Keri Garlick, and Elena M. Guerra for help with some experiments. We are grateful to Dr. Knud Nierhaus (Max Planck Institute for Immunology and Neurosciences, Germany) for helpful discussions and the generous gift of anti-FLAG antibodies.

Bailey, M., Chettiath, T., and Mankin, A.S. (2008). Induction of erm(C) expression by macrolides to induce production of ribosomal methylase. 


