Growth Phase-Dependent Transcription of the Streptomyces ramocissimus tuf1 Gene Occurs from Two Promoters

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The str operon of Streptomyces ramocissimus contains the genes for ribosomal proteins S12 (rpsL) and S7 (rpsG) and for the polypeptide chain elongation factors G (EF-G) (fus) and Tu (EF-Tu) (tuf). This kirromycin producer contains three tuf or tuf-like genes; tuf1 encodes the regular EF-Tu and is located immediately downstream of fus. In vivo and in vitro transcription analysis revealed a transcription start site directly upstream of S. ramocissimus tuf1, in addition to the operon promoter rpsLp. Transcription from these promoters appeared to be growth phase dependent, diminishing drastically upon entry into stationary phase and at the onset of production of the EF-Tu-targeted antibiotic kirromycin. In surface-grown cultures, a second round of tuf1 transcription, coinciding with aerial mycelium formation and kirromycin production, was observed. The tuf1-specific promoter (tuf1p) was located in the intercistronic region between fus and tuf1 by high-resolution S1 mapping, in vitro transcription, and in vivo promoter probing. During logarithmic growth, the tuf1p and rpsLp transcripts are present at comparable levels. In contrast to Escherichia coli, which has two almost identical tuf genes, the gram-positive S. ramocissimus contains only tuf1 for its regular EF-Tu. High levels of EF-Tu may therefore be achieved by the compensatory activity of tuf1p.

The polypeptide chain elongation factor Tu (EF-Tu), responsible for mediating the binding of aminoacyl-tRNA to the translating ribosome, is one of the most abundant proteins in the prokaryotic cell; in Escherichia coli, EF-Tu can constitute up to 10% of the total cellular protein under rapid growth conditions (35). Two unlinked, nearly identical copies of the tuf gene, which encodes EF-Tu, are present in E. coli (2, 19, 43) as well as in other gram-negative bacteria (13, 29). This tuf gene duplication has been suggested to be required to maintain the high levels of EF-Tu during rapid growth (2). In contrast, most gram-positive bacteria contain only a single copy of tuf (13, 29).

The E. coli tufA and tufB genes contribute about equally to the total EF-Tu concentration and are regulated coordinately under most growth conditions (35). The tufA gene is the promoter-distal gene in the str or S12 operon (27), which also includes the genes for ribosomal proteins S12 (rpsL) and S7 (rpsG) and for EF-G (fus). This operon organization is typical of the major tuf gene in eubacteria (reference 11 and references therein) and some archaea (20). The str operon is expressed from a promoter upstream of rpsL, via a polycistronic mRNA (19). Furthermore, weak secondary promoters were reported within the coding region of the fus gene (3, 44–46), which are exclusively used for transcription of tufA. The tufB gene is cotranscribed with four upstream tRNA genes (21) and is processed into separate tRNAs and a tufB mRNA (33).

The gram-positive streptomycetes are soil bacteria that undergo a complex process of morphological differentiation. The vegetative mycelium produces aerial hyphae which septate and differentiate into spores at their termini. The onset of morphological differentiation usually coincides with the production of a number of secondary metabolites, including a wide variety of antibiotics (9). In liquid cultures, antibiotic production is generally confined to the stationary phase.

Streptomyces ramocissimus produces the antibiotic kirromycin, which immobilizes the EF-Tu–aminoacyl-tRNA complex on the ribosome and thus inhibits protein synthesis. S. ramocissimus EF-Tu is sensitive to kirromycin (34), which indicates that resistance of this organism against its own antibiotic is not due to alteration of the target protein EF-Tu. Recently it was shown that S. ramocissimus contains three strikingly divergent tuf genes (40). The tuf1 gene is located in the str operon and encodes the kirromycin-sensitive elongation factor EF-Tu1. While tuf1 is expressed at a very high level, no expression of tuf2 or tuf3 has been detected so far during normal growth, and apparently these genes do not contribute significantly to the total EF-Tu pool in S. ramocissimus (40).

The aim of this work was to study in detail the transcription of the S. ramocissimus tuf1 gene and to assess the relationship between tuf1 transcript levels, growth phase, and kirromycin production. Our results indicate that, in addition to the major promoter for the str operon, another transcription start site, located in the fus-tuf1 intergenic region, makes an important contribution to the high EF-Tu1 level in the cell. Transcript levels of the promoters varied greatly during growth, perhaps reflecting the growth phase-dependent synthesis of different σ factors (7). The consequences of the additional promoter tuf1p in relation to the presence of only one active major tuf1 gene in S. ramocissimus is discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulations. E. coli JM101 (28) and ET12567 (23) were used as hosts for pUC18 derivatives (42). S. ramocissimus B7 and S. coelicolor M145 were obtained from Gist-brocades NV, Delft, The Netherlands, and the John Innes Centre, Norwich, United Kingdom, respectively. plSRT1xyE-1, plSRT1xyE-2, and plSRT1xyE-3 were made by cloning fragments of the tuf1-containing plasmids pUSR1 or pUSR1-1 (40) into puC18 into the xylE-based promoter probe vector pJH4053 (10). Inserts were BglII/SmaI (-1180 to -180) in plSRT1xyE-1, NruI/BclI (-180 to +280) in plSRT1xyE-2, and Smal/BclI (-70 to +280) in plSRT1xyE-3 (Fig. 1). All DNA manipulations were performed according to standard protocols (28). Transformation of S. coelicolor M145 was performed as described elsewhere (17).
numbers under inserts correspond to nucleotide positions relative to the translation start site of tuf1. the only small BamHI and NruI sites are shown. the scale applies to construct pUSR1 and derivatives.

**Culture conditions.** E. coli JM101 and ET12567 were cultured in LB medium (28). S. coelicolor M145 was grown in YEME and on R2YE (17). High-titer spore suspensions of S. ramocissimus B7 were obtained from SFM plates (8). Reproducibly dispersed growth (doubling time of 2.5 h) was obtained when spores were pregeminated in 2% YT medium (8) for 8 h, subsequently inoculated in NMMP containing 1% (wt/vol) glucose (17), and grown at 28°C with vigorous shaking (300 rpm). Estimates of growth were made by measuring optical density at 450 nm (OD450). Karromycin production was determined by extraction of the filtrate with ethyl acetate and analysis by thin-layer chromatography. Surface-grown cells of S. ramocissimus B7 were cultured on AMMAT medium, containing, per liter, 375 mg of NaNO3, 375 mg of K2HPO4, 375 mg of K2HPO4, 7.5 mg of FeSO4, 127.5 mg of MgCl2, 30 mg of MnSO4, 7.5 mg of ZnSO4, 337.5 mg of NaNO3, 375 mg of K2HPO4, 1.725 mg of MnSO4, and 7.5 mg of ZnSO4. Surface-grown cells were plated on cellophane disks to facilitate harvesting of the mycelium. Morphology of the surface-grown cultures was determined by phase-contrast microscopy, while karromycin secretion into the agar was detected by using E. coli JM101 as the indicator strain.

**Promoter-probe experiments.** The xylE gene from Pseudomonas putida was used as a reporter gene (47) for in vivo detection of promoter activity. Fragments containing various parts of the tuf1 upstream region were cloned upstream of the promoterless xylE gene of plJ4083 (10). Transformants containing plUSR1xylE-1, plUSR1xylE-2, and plUSR1xylE-3 were grown on R2YE agar plates in the presence of 25 μg of tiamulin (a gift from Squibb, Princeton, N.J.) per ml. Plates were sprayed with 0.5 M catechol after 2, 3, 4, and 5 days of growth, and the amount of catechol converted into yellow 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase was assessed visually.

**RNA isolation.** RNA was isolated as described by Hopwood et al. (17) from cultures of S. ramocissimus B7 grown in NMMP liquid medium containing 1% glucose and from surface-grown S. ramocissimus B7 cultured on AMMAT medium. RNA concentrations were determined spectrophotometrically, and the quality of the preparations was checked by gel electrophoresis.

**Northern blotting.** RNA samples were glyoxylated and run in 1% agarose in 15 mM NaH2PO4-Na2HPO4 buffer (pH 6.5). After electrophoresis, the RNA was transferred onto Hybond N+ nylon membranes (Amersham), using 25 mM NaH2PO4-Na2HPO4 buffer (pH 6.5) as the blotting buffer. The filters were hybridized for 16 to 20 h at 65°C in 10 ml of hybridization solution consisting of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.6% sodium dodecyl sulfate, 1% blocking reagent (Boehringer), and DNA probes 32P labelled by random priming (12). DNA probes (10 to 50 ng) were the 1.0-kb MluI/NcoI fragment from the S. ramocissimus tuf1 gene (designated tuf1) and the 0.75-kb PstI/BamHI fragment containing part of the S. ramocissimus rpsL gene, the entire rpsG gene (designated rpsG/L). Filters were washed twice in 2× SSC-0.1% sodium dodecyl sulfate at 65°C for 30 min.

**RESULTS**

**Transcription of tuf1 occurs from two promoters.** In E. coli, tuf4 is the most distal gene in the transcription unit comprising the genes rpsL, rpsG, fus, and tuf (19). Since EF-TuA is synthesized in three- to fivefold larger amounts than the other proteins of the str operon, additional promoter activity within the fus gene has been suggested and indeed observed (3, 44–46). To investigate transcription of the S. ramocissimus tuf1 gene, we carried out Northern blotting experiments using RNA isolated from exponentially growing cultures harvested 17 h after inoculation of liquid medium (NMMP-1% glucose) with pregeminated spores (see below for details). Using the S. ramocissimus 1.0-kb MluI/NcoI fragment of pUSRstr (32a) as a probe (Fig. 1, tuf1 probe), we identified two major in vivo tuf1 transcripts, hybridizing to similar extents (Fig. 2). The large transcript of approximately 5 kb was also visualized when the 0.75-kb PstI/BamHI fragment of pUSRstr was used as a probe (Fig. 1, rpsL/G probe), indicating that this transcript includes at least rpsG and fus in addition to tuf1. The strongly hybridizing tuf1 transcript of approximately 1.8 kb suggested the presence of a prominent tuf1-specific promoter. It is unlikely that this tuf1 transcript is a processing product of the large transcript, since only one band was found with the rpsL/G probe.

To determine the presence and approximate location of any tuf1-specific promoter(s), S. coelicolor M145 was transformed with various derivatives of the multicopy vector pJ4083, each containing a different S. ramocissimus tuf1 upstream fragment cloned in front of the promoterless xylE gene. The origin of
each of these fragments is shown in Fig. 1. Transformants containing pISRTxylE-2, which has the region from −180 to +280 (relative to the tuf1 translation start site) of tuf1 in front of xyIE, yielded colonies with bright-yellow aerial mycelium when sprayed with catechol after at least 3 days growth on R2YE agar plates. M145 transformants containing pISRTxylE-1 or pISRTxylE-3 displayed no or hardly any yellow color, respectively, upon spraying with catechol, reducing the tuf1-specific promoter region to the NruI/SmaI fragment (−180 to −70).

To map precisely possible transcription start sites directly upstream of tuf1, S1 nuclease protection experiments were carried out with RNA isolated from exponentially growing S. ramocissimus, harvested 17 h after inoculation of liquid medium (NMMP–1% glucose) with pregerminated spores (see below for details). The 490-bp PvuII/MluI fragment from pUSRTI-1 (Fig. 1, S1 probe), uniquely 32P labelled at the 5′ end of the MluI site, was used as a probe; the 200-bp nonhomologous pUC18-derived extension allowed discrimination between full-length RNA-protected fragments (transcription read-through from fus) and reannealed probe. A protected fragment corresponding in size to the NruI/MluI segment of the S1 probe (Fig. 3, FLP) indicated transcription of tuf1 from a promoter upstream of, or within, fus (most likely rpsL). An additional transcription start site was identified at nt −127 (Fig. 3, tuf1p). Figure 3 also shows a possible minor transcription start site (band X), located at nt −97. We cannot exclude the possibility that this S1-protected fragment is a processing product of the tuf1p-derived mRNA. Transcripts derived from tuf1p and the read-through promoter rpsLp were present in almost equal amounts, whereas the additional transcript (band X in Fig. 3) was barely detectable. The location of the tuf1p transcription start site is depicted in Fig. 4A.

To confirm that the RNA-protected fragment assigned to tuf1p, which was identified by the in vivo analyses described above, represents a transcription start site rather than a processing event, the 290-bp BamHI/MluI fragment of pUSRT1-1 was used as a template for in vitro transcription assays using different fractions of partially purified RNA polymerase holoenzymes isolated from translation-phase cultures of S. coelicolor M145. A runoff transcript of approximately 240 nt corresponding to the expected size for tuf1p, as was predicted from the S1 nuclease mapping data (data not shown).

Evidence that this additional tuf1 transcription start site is not unique for S. ramocissimus was obtained by comparison of homologous DNA sequences from S. coelicolor, S. mobaraense, S. netropsis, and S. collinus (Fig. 4B). The first 80 nt of the fus-tuf1 intergenic region were only 39% conserved between S. ramocissimus and the other streptomycetes. However, the putative recognition sequences for transcription were 100% conserved. Sequences immediately upstream of the translation start site (−64 to −1) were highly conserved among the five species (83%) (data not shown). The significance of the conservation of this part of the untranslated leader mRNA is not known.

**Growth-phase-dependent transcription of tuf1 in liquid culture.** To study the growth phase dependence of tuf1 transcription and to assess the relationship between growth rate, tuf1 transcription, and kirromycin production, we developed conditions which gave reproducible, dispersed growth of S. ramocissimus mycelia. In NMMP–1% glucose, exponential growth was obtained with a doubling time of about 2.5 h and the culture entered stationary phase (OD650 of 2.2) usually about 22 h after inoculation (Fig. 5A). At the same time, the pH of the medium dropped from about 6.6 to 5.0, and small amounts of kirromycin were detected about 4 h later. The doubling times and onset of antibiotic production were highly reproducible. In S medium, an almost identical growth curve was observed, but kirromycin production was significantly increased (data not shown).

To establish the level and timing of tuf1 transcription from tuf1p and rpsLp, S1 nuclease protection experiments were carried out with RNA isolated from S. ramocissimus NMMP cultures at different time points, between 13 h (OD650 of 0.6, corresponding to early exponential phase) and 34 h (OD650 of 2.2, late stationary phase) after inoculation of NMMP–1%.
glucose with pregerminated spores. The 490-bp PvuII/MluI fragment from pUSRT1-1 (described above) was used as a probe. As shown in Fig. 5B, the \( tuf1p \) and \( rpsLp \) transcripts were readily detected in RNA isolated from fast-growing cultures and after 16 h contributed almost equally to the total \( tuf1 \) mRNA concentration. Both transcripts reached maximal levels during early exponential growth and decreased markedly when the growth rate declined. Twenty-three hours after inoculation (corresponding to early stationary phase), transcription from \( rpsLp \) was barely detectable, and stationary-phase transcription of \( tuf1 \), during kirromycin production, occurred mainly from \( tuf1p \), although at a low level. Degradation products of the \( tuf1 \) transcripts were observed throughout growth (data not shown), presumably reflecting their relatively short half-lives.

Transcription of \( tuf1 \) in surface-grown cultures is growth phase dependent. To study \( tuf1 \) transcription during the life cycle of \( S. \) ramocissimus B7 on solid media, S1 nuclease mapping experiments were performed with RNA isolated from various growth phases, using again the 490-bp PvuII/MluI fragment from pUSRT1-1 as a probe (Fig. 6). High levels of \( tuf1 \) transcripts were observed during the formation of vegetative hyphae, with transcription initiating from the same start sites as found for the liquid culture (Fig. 5B). Transcript levels of \( tuf1p \) mRNA were even higher than those of the \( str \) operon promoter \( rpsLp \). A dramatic drop in \( tuf1 \) transcription occurred in the transition phase from vegetative growth to aerial mycelium development, coinciding with the production of kirromycin. At the onset of morphological differentiation, \( tuf1 \) transcript levels increased again, mainly due to the activity of \( tuf1p \). Transcripts corresponding to \( rpsLp \) also reappeared during the later stages of development.

**DISCUSSION**

In this report, we show that \( tuf1 \) of \( S. \) ramocissimus is transcribed from a promoter within the \( fus-tuf1 \) intergenic region, in addition to the \( str \) operon promoter \( rpsLp \). S1 nuclease protection experiments revealed a transcript with a 5′ end within the \( fus-tuf1 \) intergenic region at nt − 127 relative to the \( tuf1 \) translation start site. The XylE + phenotype of pSRT1xylE-2, revealing promoter activity within 180 bp of the \( tuf1 \) translation start site, and in vitro runoff transcription analysis with \( S. \) coelicolor M145 RNA polymerase indicated that the potential promoter start site found by S1 mapping does indeed serve to initiate transcription in vivo and is not the result of posttranscriptional mRNA processing. An additional
band which might reflect a weak second promoter in the fus-
tuf1 intergenic region was observed in the S1 nuclease mapping
experiments. Compared to the other two tuf1 transcripts, this
mRNA is sparsely present, and the transcript might be a deg-
radation or processing product of the tuf1p transcript.

The tuf1p transcript, which persists at a relatively high level
in both exponential and stationary phases, has a start site
preceded by a −10 region (TAGGCT) that is similar to the
consensus sequence (TAGRRT, where R = G or A) deduced
by Strohlf (31) for streptomycetes. These results
from kirromycin-sensitive EF-Tu1, occurred during kirromycin produc-
tion in both liquid and surface-grown cultures. These results
might simply reflect a second round of spore germination on
the solid surface, thereby creating the GTAAC potential promoter
core sequence of the tuf1p region. Should this be true, the
inverted repeat (Fig. 4A) extending from nt 126 to 144
binds a transcriptional activator that would obviate the need
for a conventional −35 sequence. In addition to the 5′-nt
inverted repeat, a direct repeat of 17 nt is located at nt 178 to
−161 (3′ end of the fus gene) and +4 to +21 (5′ end of the tuf1
gene) (Fig. 4A). Further analysis of the tuf1p region revealed
the sequence GTAAC 19 bp upstream of the consensus
sequence. This region shows a direct match with the consensus
for class II (GNAACN19/20T) potential promoter core se-
quenences (5) that might be of the σE class (formally σ32) (22).

The presence of two different promoter elements suggests
that tuf1p transcripts might be the result of both σA and σE-
containing RNA polymerase holoenzymes. Wright and Bibb
(41) noticed a strong preference for UGA over UAG, and
indicated that the translational start sites of tuf1p transcripts
are required to attain high EF-Tu concentrations during rapid
growth. Since gram-positive organisms contain only a single
operon promoter, and thus the two transcripts contribute more or
less equally to the total tuf1 mRNA concentration. An extra pro-
moter for tuf1 may allow S. ramocissimus to differentially
regulate EF-Tu synthesis from tuf1 without affecting the synthesis
of EF-G, S7, and S12, permitting relative increase in tuf1 gene
expression compared to the expression of upstream genes of
the str operon.

The observation that the duplication of tuf is widespread
among gram-negative genera suggests that for these bacteria,
tuf gene duplication fulfills an important role. No functional
differences between the nearly identical gene products of
E. coli tufA and tufB could be detected (25, 39), and both genes in
Salmonella typhimurium were shown to be individually dispens-
able for growth (18). However, cells with one disrupted tuf
gene have a decreased growth rate, suggesting that two genes
are required to attain high EF-Tu concentrations during rapid
growth. Since gram-positive organisms contain only a single
copy of the regular tuf gene, we propose that the additional
strong tuf1p promoter in the str operon of streptomycetes func-
tions to provide adequate levels of EF-Tu at high growth rates.

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