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The tuf3 gene of Streptomyces coelicolor A3(2) encodes an inessential elongation factor Tu that is apparently subject to positive stringent control

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In Streptomyces coelicolor A3(2), two genes, tuf1 and tuf3, encode the apparent polypeptide chain elongation factors EF-Tu1 and EF-Tu3, respectively. While tuf1 appears to code for the major EF-Tu, the function of tuf3 is unknown. To assess the role of EF-Tu3, tuf3 was subjected to mutational and transcriptional analyses. Replacement of the 5′-half of tuf3 by an antibiotic resistance cassette had no detectable effect on phenotype, indicating that tuf3 is not essential for growth or differentiation. The transcription start site of tuf3 was located approximately 195 nt upstream of the translation start site. The sequence of the tuf3 promoter (P_tuf3) resembles the consensus for the major class of eubacterial promoters, and P_tuf3 was recognized preferentially by an RNA polymerase fraction enriched in α_Escherichia coli, the principal α factor of S. coelicolor. Nuclease S1 mapping failed to reveal tuf3 transcripts during growth of S. coelicolor in liquid culture, consistent with the apparent absence of EF-Tu3 in total protein extracts of the same strain. However, tuf3 transcription was observed in cultures of S. coelicolor M145 shortly after nutritional shiftdown (which resulted in the disappearance of tuf1 transcripts) and after addition of serine hydroxamate, both of which induce the stringent response. Transcription of tuf3 was also observed in transition-phase and stationary-phase cultures of S. coelicolor J1681, a strain deleted for bldA (which specifies a tRNA_Lec for the rare leucine codon UUA). In all of these examples, transcription of tuf3 followed the production of ppGpp, consistent with the hypothesis that tuf3 is subject to positive stringent control.

Keywords: elongation factor Tu, tuf3, Streptomyces coelicolor A3(2), stringent control, ppGpp

INTRODUCTION

The polypeptide chain elongation factor Tu (EF-Tu), responsible for delivering amino-acyl tRNAs to the translating ribosome, is one of the most abundant proteins in micro-organisms: it can constitute up to 10% of total cell protein in rapidly growing Escherichia coli cells (van der Meide et al., 1983). Two tuf genes, tufA and tufB, encode EF-Tu in E. coli, and are very similar in nucleotide sequence. The Gram-positive actinomycete Streptomyces ramossimus produces the antibiotic kirromycin, which binds specifically to EF-Tu, and possesses three tuf genes, designated tuf1, tuf2 and tuf3. EF-Tu1 and EF-Tu2 show 85% amino acid sequence identity, and both of them share about 65% amino acid sequence identity with EF-Tu3 (Vijgenboom et al., 1994). While tuf1 encodes the major elongation factor EF-Tu1, the roles of tuf2 and tuf3 are unknown: polyclonal antibodies raised against EF-Tu2 and EF-Tu3 that had been produced in E. coli failed to detect either protein in extracts of S. ramossimus cultures grown under a variety of conditions (Vijgenboom et al., 1994). While tuf2 appears to be absent...
from most Streptomyces species, Streptomyces coelicolor A3(2) and many other streptomycetes contain close homologues of both tfuA and tfu3 (van Wezel, 1994). Like tfuA of E. coli, tfu1 of S. coelicolor is located in the str operon (van Wezel et al., 1994a, 1995), and is well-separated from tfu3 on the physical map of the S. coelicolor chromosome (van Wezel et al., 1995). While EF-Tu1 is abundant in S. coelicolor, EF-Tu3 could not be detected (van Wezel et al., 1994a).

The stringent response, which was first characterized as a rapid reduction in stable RNA synthesis upon amino acid starvation, is a pleiotropic response to the accumulation of uncharged tRNAs, and there is considerable evidence to suggest that the highly phosphorylated nucleotide ppGpp plays a key role in mediating stringent control (Cashel, 1991). ppGpp is synthesized in response to amino acid starvation, and is characterized by ppGpp synthesis in the presence of the -1900/ -260 tuf3 upstream region and on the other by the 2-kb fragment containing the second half of tfu3 and 3 kb of downstream sequence. Thus a double recombination event will replace the -260/ +600 segment of tfu3 by addA. Additional selectable markers on pJ2559 are thr (conferring thiotrepton (Thio) resistance in Streptomyces), cloned as a 1 kb Bgl II fragment from pJ4083, and gkA encoding glucose kinase, which was available as a 1.3 kb Pst I fragment on the plasmid pJ2423 (Angell et al., 1994). J1501/gkA-A is resistant to 2-deoxyglucose (2-dog), but the presence of the pJ2559-derived gkA renders this strain 2-dog sensitive. Successful inactivation of tfu3 by the required double crossover results in loss of both thr and gkA, yielding a Thio<sup>8</sup> 2-dog<sup>8</sup> phenotype.

Culture conditions. Surface-grown cultures were cultivated on R2YE or on minimal medium (MM) agar plates, using glucose or mannitol as the carbon source (Hopwood et al., 1985). Liquid cultures were grown in a minimal medium supplemented with Casamino acids (SM) (Takano & Bibb, 1992), or in yeast extract/malt extract medium (YEME) with 0.5% glucose (Hopwood et al., 1985), and were inoculated at a density of 5 x 10<sup>6</sup> c.f.u. ml<sup>-1</sup> and grown at 30°C with vigorous shaking (300 r.p.m. min<sup>-1</sup>). Reproducibly dispersed growth was obtained, with a doubling time of 2.2 h in SM and 1.9 h in YEME. In SM, rapid transition into stationary phase occurred approximately 14 h after inoculation (Strauch et al., 1991). Nutritional shiftdown and treatment with 25 mM SHX were performed as described by Strauch et al. (1991).

Promoter-probe experiments. The xylE gene from Pseudomonas putida (Zukowski et al., 1983) present in pJ4083 (Clayton & Bibb, 1990) was used as a reporter gene for in vivo promoter activity. Transformants containing pISCT3-U1 were grown on R2YE and MM (Hopwood et al., 1985) in the presence of 10 μg Thio ml<sup>-1</sup> (a gift from Bristol-Meyers Squibb). Plates were sprayed with 0.5 M catechol after 1, 2, 3 and 4 d growth and the amount of catechol converted into yellow 2-hydroxyxymonconic semialdehyde by catechol 2,3-dioxygenase was assessed visually.

In vitro transcription analysis. RNA polymerase was isolated from a culture of S. coelicolor M145 in the transition between exponential growth and stationary phase, as described previously by Buttner & Brown (1985), and partially separated into different holoenzyme forms by Superose-6 FPLC. In vivo run-off
transcription experiments were performed as described by Buttner & Brown (1985). Products were analysed on denaturing 6% (w/v) polyacrylamide gels using 32P-end-labelled HpaII fragments of pBR322 as size markers. The 600 bp PvuII-StyI (−500/+100) fragment and the 360 bp KpnI-StyI fragment were isolated from pISCT3-1 and pISCT3-2, respectively (Fig. 2), and used as templates.

**RNA isolation.** RNA was isolated from M145, J1501 or J1681 according to Hopwood et al. (1985). To remove residual DNA, the RNA was salt-precipitated in 3 M NaOAc (pH 6.0). The RNA samples were then treated with DNaseI (1 h at 37 °C with 0·1 U DNaseI per 50 ml initial culture sample), extracted with a 1:1 mixture of phenol/chloroform (saturated with 100 mM Tris, pH 7·0) and precipitated in 0·4 M NaOAc (pH 6·0) with 2-propanol. The RNA was resuspended in water and the concentration was determined spectrophotometrically.

**Nuclease S1 mapping.** Hybridization of 10 µg RNA with the appropriate DNA probe was performed according to Murray (1986) in NaTCA buffer (Summerton et al., 1983). All subsequent steps were carried out as described previously by Strauch et al. (1991), using an excess of probe. The 600 bp PvuII–StyI fragment from pISCT3-1 (Fig. 2), 32P-labelled at the 5′ end of the StyI site, was used for mapping tuf3 transcripts; the 25 bp non-homologous extension upstream of the PvuII site allowed discrimination between DNA–RNA hybrids and reannealed probe. The 530 bp Smal–XhoI (−70/+460) fragment from pASCT1-1 (van Wezel et al., 1994a; Fig. 2), 32P-labelled at the 5′ end of the XhoI site, was used for mapping tufl transcripts. The 558 bp FspI–HincII fragment, corresponding to nt positions −708 to −151 with respect to the 5′ end of the 16S rRNA coding sequence and 32P-labelled at the 5′ end of the HincII site, was used for mapping rrnA transcripts (van Wezel et al., 1994b).

**ppGpp measurements.** ppGpp levels were determined as described by Strauch et al. (1991).

**RESULTS**

**Inactivation of tuf3 by gene replacement**

To study the role of tuf3 in *S. coelicolor* we inactivated the gene by replacing the segment of tuf3 that extends from −260 to +600 (with respect to the translation start site of
Fig. 3. In vitro transcription analysis of S. coelicolor tuf3. P, Run-off transcript originating at P_133; RNAP fraction, RNA polymerase fractions eluting from a Superose-6 FPLC column. The right-hand side of the figure shows a DNA size marker (denatured HpaII-digested pBR322) in nt. GATC, tuf3 nt sequence ladder generated using a 19 nt primer whose 5' end corresponds precisely to the 5' end of the labelled probe used for 51 nuclease mapping (see Methods).

Expression of the tuf3 promoter occurs in surface-grown cultures when present on a high-copy-number plasmid

M145 transformants containing the multi-copy plasmid pSCT3-U1, which has the −1900/−5 region (relative to the translation start site) of tuf3 in front of xylE (Fig. 2), gave colonies that rapidly became yellow when sprayed with catechol after 2 d growth on solid MM containing mannitol as carbon source. After 4 d, spraying with catechol produced bright-yellow aerial hyphae. This suggests that catechol dioxygenase production (i.e. xylE transcription) takes place in both vegetative and aerial mycelium, although the pigmentation of the aerial hyphae might have been the result of diffusion of the yellow compound from the vegetative mycelium (diffusion of the product of XylE activity, the yellow compound 2-hydroxymuconic semialdehyde, into the agar was also clearly apparent). Interestingly, pSCT3-U1 transformants produced neither aerial mycelium nor the pigmented antibiotics actinorhodin and undecylprodigiosin on MM with glucose or on R2YE plates, even

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**Fig. 5.** Comparison of *tuf1* and *rrnA* transcription in *S. coelicolor* M145. (a) Growth curve (○) and ppGpp production (□) of a SMM-grown culture. (b) Transcription of *tuf1* and *rrnA*. *tuf1*, full-length protection of the *tuf1* probe; *rrnA* P1 and P2, transcripts initiated at the *rrnA* P1 and P2 promoters, respectively. M, DNA size marker as in Fig. 3; C, control lane (30 μg tRNA). Times indicate hours after inoculation with germinated spores.

after prolonged incubation at 30 °C, a phenotype comparable to that of an *S. coelicolor* *bldA* mutant (Lawlor et al., 1987). M145 transformants containing pISCT3-1 or pISCT3-2 displayed normal growth and differentiation, regardless of the medium used.

**In vitro transcription of *tuf3***

RNA polymerase from a transition phase culture of *S. coelicolor* M145, partially fractionated into different holo-enzyme forms by Superose-6 FPLC, was used in *in vitro* run-off transcription assays to identify promoters in the *tuf3* upstream region. The *PvuII*–*StyI* (−500/+100) and the *KpnI*–*StyI* (−260/+100) fragments were used as templates. Since both fragments gave identical results, only those obtained with the *KpnI*–*StyI* template are shown (Fig. 3). In each case, two major transcripts were observed. One corresponded to the size of the template and was probably the result of end-to-end transcription. The second band (designated P in Fig. 3) corresponded to a transcript of approximately 295 nt and suggests a transcription start site around nt position −195 (with respect to the translation start site), which was confirmed by nuclease S1 mapping (see below). The putative transcription start site is preceded by the sequences TCGACG and GATGAT, separated by 17 bp (Fig. 4), which resemble the consensus −35 and −10 sequences, respectively, for the major class of eubacterial promoters.

In *S. coelicolor*, such sequences appear to be recognized by *rpoB* (Brown et al., 1992), whose activity was most abundant in RNA polymerase fraction 28 (data not shown), the one that gave the strongest signal for *Ptuf3* (Fig. 3).

**Transcription of *tuf1* and *tuf3* during liquid culture**

To establish the level and timing of *tuf3* transcription *in vivo*, RNA from M145 and J1501 cultures grown in SMM was analysed by nuclease S1 mapping using the 620 bp *PvuII*–*StyI* fragment as probe. Growth of, and ppGpp production by, the M145 culture used for RNA isolation are shown in Fig. 5(a). No protection of the *tuf3* probe was observed. We also analysed the transcription of *tuf1* in the *S. coelicolor* M145 culture, using the same RNA preparations. *tuf1* lies in the S12 ribosomal protein operon (van Wezel et al., 1994a, 1995) and, by analogy to *E. coli* (Lindahl & Zengel, 1986, and references therein), is likely to be transcribed from a promoter upstream of *rpsL* (encoding S12). For mapping of *tuf1* transcripts we used the 530 bp *SmaI*–*XhoI* (−70/+460) fragment from pASCT1-1 (van Wezel et al., 1994a), uniquely end-labelled at the *XhoI* site, as probe (Fig. 2), and therefore expected full-length protection of the probe. Since different probes were used, strict quantitative comparisons are not possible, but it is interesting to note that...
the levels of the *tuf1* transcripts, which reflect rates of synthesis and degradation, were considerably higher than those derived from the *rrnA* P1 and P2 promoters (Fig. 5b), even though P2 is the strongest promoter of the *rrnA* operon (van Wezel *et al.*, 1994b). The control, 30 μg tRNA, failed to protect the probe, indicating that the full-length protection was not due to probe reannealing. Transcripts corresponding to *tuf1* and *rrnA* were barely detectable approximately 16 h after inoculation, corresponding to early stationary phase. Thus, transcription of *tuf1* shows the same growth-phase dependence as *rrnA*.

**tuf3** is transcribed after nutritional shiftdown and after addition of SHX

Since transcription of *E. coli* *tufA* and *tufB* is negatively stringently controlled (Reeh *et al.*, 1976), we analysed how *tuf1* and *tuf3* transcription responded to amino acid starvation provoked by either nutritional shiftdown or by addition of SHX. Nutritional shiftdown of an *S. coelicolor* M145 culture at an OD<sub>450</sub> of 0.6 led to a marked increase in the level of ppGpp, with a maximum of about 200 pmol (mg dry weight)<sup>−1</sup> 15 min after shiftdown (Fig. 7a), falling to 45 pmol (mg dry weight)<sup>−1</sup> 2 h after shiftdown. The culture continued growing (albeit at a slow rate) and reached stationary phase 6–7 h after shiftdown. *tuf1* transcripts were barely detectable 30 min after shiftdown (Fig. 7b), suggesting that expression of the major EF-Tu gene of *S. coelicolor* is also subject to negative stringent control. Reappearance of *tuf1* transcripts 4–8 h after amino acid depletion presumably reflects adaptation to the new growth rate and the turnover of excess EF-Tu1 present after shiftdown.

In contrast, *tuf3* transcripts appeared within 30 min of shiftdown, and reached a maximum after 1 h (Fig. 7c). The amount of *tuf3* transcript under these conditions was about ten times higher than that in transition-phase and stationary-phase cultures of J1681 (comparable amounts of RNA were used in each experiment, and autoradiogram exposure times were typically ten times longer for identification of the *tuf3* transcripts in J1681 than after nutritional shiftdown of M145). *tuf3* transcripts were barely detectable 3 h after shiftdown of M145.

The stringent response can also be elicited by treatment with SHX, although lower levels of ppGpp are induced than after nutritional shiftdown (75 and 200 pmol (mg dry weight)<sup>−1</sup>, respectively; Fig. 8a). While addition of SHX leads to a marked reduction in the level of rRNA transcripts (Strauch *et al.*, 1991), addition of SHX to a final concentration of 50 mM led to the appearance, within 30 min, of low-abundance *tuf3* transcripts (Fig. 8b).

**DISCUSSION**

*tuf3* deletion mutants were not noticeably affected in growth or differentiation, demonstrating that EF-Tu3 does not play an essential role in the *Streptomyces* life-cycle. Western blotting using antibodies raised against the highly homologous *S. ramossimus* EF-Tu3 failed to identify the *tuf3* gene product in total protein extracts isolated from M145 after nutritional shiftdown (van Wezel, 1994). This suggests that EF-Tu3, if present at all in *S. coelicolor*, occurs only in trace amounts, even when *tuf3* transcripts can be detected.

The XylE<sup>+</sup> phenotype of pISCT3-U1 revealed a promoter within 1900 bp of the *tuf3* translation start site. Earlier work (van Wezel, 1994) had shown that M145 containing pISCT3-2, which has only 260 bp of sequence upstream of *tuf3*, produces EF-Tu3 in the absence of any additional

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**Fig. 6.** Transcription of *tuf3* in J1501 and J1681 (a *bldA* deletion mutant of J1501). *P*. *tuf3* transcript. Lanes: M, DNA size marker as in Fig. 3; J1681, RNA isolated from J1681 (J1501Δ*bldA*) during (1) exponential phase, (2) transition phase and (3) stationary phase; J1501, RNA isolated from J1501 during (1) transition phase and (2) stationary phase.
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**Fig. 7.** Transcription of *tuf1* and *tuf3* after nutritional shiftdown of *S. coelicolor* M145. (a) Growth curve (Δ, ○) and ppGpp production (□) of a SMM-grown culture, with (△) and without (○) nutritional shiftdown (SD) at an OD_{450} of 0.6. (b) Transcription of *tuf1* after nutritional shiftdown. (c) Transcription of *tuf3* after shiftdown. P, *tuf3* transcripts initiated at the *tuf3* promoter around nt position —195; M, DNA size marker as in Fig. 3; C, control lane (30 μg tRNA).

Therefore it is possible that the strong *xylE* expression observed with pISCT3-U1 may be entirely due to a promoter located within 260 bp of the *tuf3* translation start site.

*In vitro* run-off transcription assays with *S. coelicolor* RNA polymerase revealed a transcription initiation site at approximately nt position —195 relative to the *tuf3* translation start site. The sequence of the putative *tuf3* promoter (TCGACG — 17 bp — GATGAT; Fig. 4) shows an 8 out of 12 nt match with the consensus sequences for the major class of eubacterial promoters (TTGACA — 17 bp — TATAAT; Hawley & McClure, 1983). The RNA polymerase fraction that yielded the highest level of *tuf3* transcription in *vitro* (Fig. 3) was enriched in RNA polymerase containing σ^{ahrdB}, consistent with recognition by σ^{ahrdB} in *vivo*. The *tuf3* promoter is preceded by a stretch of seven As and one T (Fig. 4), a very unusual sequence in the G+C-rich streptomycetes. Similar A + T-rich sequences found approximately 45 bp upstream of the transcription start site of various *E. coli* promoters were shown to interact with the α subunit of RNA polymerase, resulting in a stimulation of transcription (Ross et al., 1993). The A + T-rich stretch near the *tuf3* promoter lies at a similar position, and it is possible that this sequence is involved in α-dependent transcriptional activation.

*tuf3* transcripts appeared immediately after invoking the stringent response, either after nutritional shiftdown or after addition of SHX, suggesting that transcription of *tuf3* may be subject to positive stringent control, which may also be true for the histidine biosynthetic operon of *Salmonella typhimurium* (Rudd et al., 1985; Cashel & Rudd, 1987). Another gene that may be subject to positive stringent control in *S. coelicolor* is actII-ORF4 (Fernández-Moreno et al., 1991), the putative activator gene for the actinorhodin biosynthetic pathway (Takano & Bibb, 1994). However, in contrast to *tuf3*, actII-ORF4 transcripts can be detected in stationary-phase cultures of M145. Whether ppGpp plays a causal role in triggering the expression of these genes remains an open question. Interestingly, expression of *rpoS*, which encodes the stationary-phase-specific α factor σ^{5S} of *E. coli* (reviewed by...
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Fig. 8. (a) Growth curve (○, △) and ppGpp production (□) by S. coelicolor M145 in SMM, in the absence (○) or presence (△) of 25 mM SHX. ‘SHX’ indicates time point of addition of SHX (OD650 0.6). ppGpp levels were only measured in the SHX-treated culture. (b) Transcription of tuf3 after addition of 25 mM SHX. M, DNA size marker as in Fig. 3.

Hengge-Aronis, 1993), appears to be positively regulated by increased levels of ppGpp (Gentry et al., 1993; Takayanagi et al., 1994; Lange & Hengge-Aronis, 1994). Is there a σ-like σ factor present in S. coelicolor that is responsible for ppGpp-dependent transcription of tuf3 and attII-ORF4? Although the experiments performed in this study suggest that P_tuf3 is a σ^brain-type promoter, E. coli σ^ and σ^0 (the major σ factor of E. coli) show overlapping promoter specificities (Tanaka et al., 1993).

A low level of tuf3 transcription was observed in late-exponential and stationary-phase cultures of the bldA deletion mutant J1681, but not in its bldA^ progenitor J1501. If ppGpp does play a role in activating tuf3 transcription, perhaps the sensitivity of the tuf3 promoter to ppGpp is enhanced in the bldA mutant; alternatively, perhaps bldA mutants have elevated levels of ppGpp, reducing the incremental increase required to activate tuf3 transcription.

While tuf1 transcripts were abundant during exponential growth, roughly coinciding with transcription from the rRNA P1 and P2 promoters, transcription of tuf3 was not observed in unstressed cultures. However, the tuf3 upstream region gave a high level of xylE expression when cloned in a multi-copy promoter-probe plasmid, and high levels of EF-Tu3 had been observed previously in transformants containing either pISCT3-1 or pISCT3-2 (van Wezel, 1994). These multi-copy effects might reflect repression of tuf3 by a protein that is readily titrated out when tuf3 is present at high-copy-number. Alternatively, differences in the extent of DNA supercoiling of the chromosomally and plasmid-located tuf3 promoters may (partly) explain the discrepancy in their activity: the degree of DNA supercoiling is known to influence the effect of ppGpp on transcription initiation in vitro, suggesting that ppGpp regulation may be affected by DNA supercoiling in vivo (Ohlsen & Gralla, 1992).

The bald phenotype observed on R2YE plates after introduction of the -1900/-5 segment of the tuf3 upstream region on a multi-copy vector (pISCT3-U1) into M145 might reflect the titration of a transcription factor required for differentiation by a regulatory element contained in the cloned fragment. Other DNA fragments have been cloned that inhibit or prevent differentiation when present in S. coelicolor in multiple copies, most of which probably sequester proteins involved in development of aerial hyphae or spores (Champness & Chater, 1994). Examples are the putative σ^white-dependent promoters P_THA and P_THA^70 which probably titrate out this σ factor, leading to absence of sporulation (Tan & Chater, 1993). pISCT3-U1 overlaps pISCT3-1 by 500 bp at the tuf3-proximal end, and pISCT3-1 displayed normal growth and differentiation on R2YE; thus the putative regulatory element cannot be located near the tuf3 promoter, and hence it seems unlikely that it is involved in regulation of tuf3 transcription.

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