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Codon adjustment to maximise heterologous gene expression in *Streptomyces lividans* can lead to decreased mRNA stability and protein yield

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Abstract The impact of the codon bias of the mouse tumour necrosis factor α (mTNF) gene cloned in *Streptomyces lividans* on the efficiency of expression and secretion was analysed. Minor codons occurring in the mTNF gene were therefore adapted to the codon bias of *Streptomyces* by site-directed mutagenesis. No improvement in mTNF yield could be detected. The stability of the transcript derived from the construct was shown to be more important for determining the final level of mTNF production. A strong correlation was observed between the yield of secreted biologically active mTNF and the amount of mTNF mRNA present in the cells.

Key words Heterologous gene expression · *Streptomyces lividans* · Codon bias · mRNA stability

Introduction

For the last decade, *Streptomyces* species and, in particular, *Streptomyces lividans*, have been extensively investigated as host strains for the expression and secretion of heterologous proteins, as a valuable alternative to the currently predominantly used *Escherichia coli* (recently reviewed by Anné and Van Mellaert 1993; Brawner 1994). In order to obtain efficient expression and secretion in *S. lividans*, expression and secretion signals from genes of abundantly secreted homologous proteins were employed. Among the homologous regulatory sequences most often used are those from α-amylase (Chang and Chang 1988; Van Mellaert et al. 1994), β-galactosidase (Lichenstein et al. 1988), tyrosinase (MelC1) (Chang and Chang 1988), tendamistat (Bender et al. 1990) and subtilisin inhibitor (Taguchi 1992). Some of these studies led to yields of eukaryotic protein up to 300 mg/l, and more, as in the case of soluble CD4 T-cell receptor derivatives produced under the control of the expression and secretion signals from STI-II, a serine protease inhibitor of *S. longisporus* (Fornwald et al. 1993). In most cases, however, the yields of foreign proteins turned out to be substantially lower than those obtained for proteins of actinomycete origin. In consequence, these amounts remained below commercially significant levels.

Similar difficulties were encountered using other host cells such as *Saccharomyces cerevisiae* (Chen 1984). The reasons for this generally lower yield in the case of eukaryotic proteins are not yet understood. Before such systems can become really competitive with expression systems such as *E. coli*, an improvement in knowledge concerning the secretion process and the factors influencing expression and secretion is required. For instance, codon choice in the eukaryotic gene could be one of the parameters responsible for relatively low expression levels. The codon bias of foreign genes is quite different from the codon preference of *Streptomyces*, which is characterised by a GC content of 70–74% (Wright and Bibb 1992). The absence of a correlation between codon bias of the eukaryotic gene and the relative tRNA amounts in the host cell could possibly be responsible for the lower expression levels. Furthermore, mRNA instability, inefficient processing of the preprotein (Chang and Chang 1988; Bender et al. 1990), difficulties with translocation through the membrane and proteolytic degradation are undoubtedly important parameters influencing the final protein yield. In this study we used mouse tumour necrosis factor α (mTNF) indirectly fused to the signal peptide of α-amylase of *S. venezuelae* as a model for investigating

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Table 1 Plasmids used in this study. (*Ap*^{R} ampicillin resistant, *Tc*^{R} tetracycline resistant, *Ap*^{S} ampicillin sensitive, *Tsr*^{R} thiostrepton resistant, *mTNF* mouse tumour necrosis factor α)

Name	Description	Reference
Escherichia coli plasmids		
pUC19	Multiple cloning site, Ap^R, LacZα	Yanisch-Perron et al. (1985)
pAlter-1	Multiple cloning site, Tc^R, Ap^S, LacZα	Promega
pIG2mTNF	Tc^R, mTNF cDNA	Innogenetics
Streptomyces plasmids		
pIJ486	Multiple cloning site, Tsr^R	Ward et al. (1986)
pLMS164mTNF	Tsr^R, mTNF cDNA	Van Mellaert et al. (1994)
pLMS164mTNF-M1	pLMS164mTNF mutated at codons 1–5 of mTNF cDNA	This work
pLMS164mTNF-M94	pLMS164mTNF mutated at codon 94 of mTNF cDNA	This work
pLMS164mTNF-M141	pLMS164mTNF mutated at codon 141 of mTNF cDNA	This work
pLMS164mTNF-GS519	pLMS164mTNF with a deletion of nine nucleotides	Van Mellaert et al. (1994)
pLMS164mTNF-GS519-M1	pLMS164mTNF-GS519 mutated at codons 1–5 of mTNF cDNA	This work
pLMS164mTNF-GS519-M94	pLMS164mTNF-GS519 mutated at codon 94 of mTNF cDNA	This work
pLMS164mTNF-GS519-M141	pLMS164mTNF-GS519 mutated at codon 141 of mTNF cDNA	This work

Table 2 Oligonucleotides used for in vitro mutagenesis and DNA sequencing reactions

DNA sequence (5′ → 3′)	Name
GACTACTTGATCTTACGTAGATTTAAACCTCCTG	93H36
CACTCGAATTTTGGCTGCTCGAGCGGACCATGATTTC	EG24674
GGACTCCGCAAAGTCCAGGTACTTGGGCAG	EG25101
GGGCTCTTGACCGCGGAGAGGAGGTTG	EG25267
CTACTTGATCTTACCATGATTTCCTCGACGTCCTTCTCG	94C889
GTAAAACGACGGCCAGT	M13 universal primer
AACAGCTATGACCATG	M13 reverse primer

the impact of several of the factors outlined above on expression and secretion in *S. lividans*.

Materials and methods

Strains and plasmids

S. lividans TK24 (Hopwood et al. 1985) functioned as the host strain for maintenance and isolation of the plasmid pIJ486 (Ward et al. 1986) and derived mTNF secretion vectors. For primary vector constructions and in vitro mutagenesis the following *E. coli* strains were used: JM109 {*endA1, recA1, gyrA96, thi, hsdR17*(r_k^-, m_k^+), *relA1, supE44*, Δ(*lac-proAB*), [F′, *traD36, proAB, lacI*^{q}ZΔM*15*]}, BMH71-18mutS {*thi, supE*, Δ(*lac-proAB*), [*mutS*::Tn*10*] [F′, *proAB, lacI*^{q}ZΔM*15*]}. Table 1 indicates the *E. coli* and *Streptomyces* plasmids used in this work.

Media and buffers

S. lividans was grown at 27° C, with continuous shaking at 300 rpm, in Nutrient Broth number 2 (Lab M, Bury, UK) buffered at pH 7 with 0.05 M MOPS (3-[N-morpholino]propane sulfonic acid) (NM medium). When necessary, thiostrepton (10 μg/ml) was added. Protoplast formation and subsequent transformation of *S. lividans* were carried out as described by Hopwood et al. (1985). *E. coli* strains were grown at 37° C (300 rpm) in Luria Bertani medium (Miller 1972) in the presence of ampicillin (50 μg/ml) or tetracycline (15 μg/ml). For solid media, 15 g agar/l was added.

DNA manipulations

All general DNA manipulations used in this work were done essentially as described by Sambrook et al. (1989) and Hopwood et al. (1985). Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer (Mannheim, Germany), MBI Fermentas (Vilnius, Lithuania) and Gibco BRL (Gaithersburg, Md., USA) and used as indicated by the suppliers.

Site directed mutagenesis and DNA sequencing

Mutations in the α-amylase-mTNF fusion gene were carried out using the Altered Sites in vitro Mutagenesis kit of Promega (Madison, Wis., USA). Oligonucleotides for mutagenesis came from Pharmacia (Uppsala, Sweden) and Eurogentec (Seraing, Belgium) (Table 2). Prior to sequencing, the DNA fragments containing the newly inserted mutations were subcloned in pUC19. The plasmid DNA was subsequently purified by CsCl-ethidium bromide gradient centrifugation. Double-stranded plasmid DNA was sequenced using the dideoxy chain-terminating method developed by Sanger et al. (1977). This was done either manually using α-[^{35}S]-labelled dATP (1000 Ci/mmol, ICN, Costa Mesa, Calif., USA) or by means of an automated DNA sequencer (A.L.F., Pharmacia, Uppsala, Sweden) with fluoro-labelled primers as recommended by the manufacturer. Primers used for sequencing were the M13 universal and reverse primers (Table 2).

RNA analysis

Streptomyces cells were grown for 24 or 36 h. After harvesting the cells by centrifugation (6000 × *g*, 10 min), total RNA was extracted as previously described (Hopwood et al. 1985). After isolation, controls on the quality of the RNA samples were carried out by gel analysis and ethidium bromide staining. For northern blot experiments, 70 µg of total RNA was pelleted from the RNA-isopropanol stock solution, resuspended in 50% formamide, 6% formaldehyde, MOPS buffer (0.02 M MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) and 10% loading dye (50% sucrose, 1% orange G, 5 mM EDTA) and denaturated by heating at 65° C for 5 min. The RNAs were separated by 1.3% agarose-formaldehyde gel electrophoresis in MOPS buffer. A 0.24 to 9.5-kb RNA ladder (Gibco BRL, Gaithersburg, Md., USA) was used as a molecular size marker. After electrophoresis for 2–3 h at 8 V/cm, the lanes with the RNA marker were stained with ethidium bromide and photographed under UV light. Transfer of the RNAs to nylon membranes (Hybond N, Amersham, Buckinghamshire, UK) was performed by overnight capillary blotting using a solution of 10 × SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0) as transfer buffer. After 2 h of baking at 80° C, hybridisation with α-[^{32}P]-labelled mTNF cDNA was done for 36 h at 42° C. Radioactive labelling of the mTNF cDNA available as a *Dra*I-*Xba*I fragment of 0.73 kb in pIG2mTNF (Innogenetics, Gent, Belgium) was established with a Random Primed Labeling kit from Boehringer with α-[^{32}P]dCTP (3000 Ci/mmol, ICN) followed by purification of the labelled oligonucleotide by gel filtration over a NAP-10 column (Pharmacia, Uppsala, Sweden). Hybridisation solution contained 50% formamide, 1% blocking reagent (Boehringer, Mannheim, Germany), 0.02% SDS, 0.01% N-lauroyl sarcosine, and 5 × SSC. After hybridisation and a final washing in 2 × SSC, 0.1% SDS at room temperature, the mRNA bands were visualised by autoradiography for 24 h or longer.

Quantification of the mTNF yield

After sampling, culture supernatants of *S. lividans* TK24 containing the different mTNF secretion vectors were immediately stored at − 80° C. Subsequently, the amount of biologically active mTNF was determined as described by Heremans et al. (1990) using recombinant mTNF produced in *E. coli* (Innogenetics, Gent, Belgium,) as standard. Immunoblot analysis was done according to Van Mellaert et al. (1994).

Results

In order to analyse the feasibility of using *S. lividans* as a host for expressing and secreting the heterologous protein, mTNFα, the expression and secretion signals of the α-amylase gene of *S. venezuelae* ATCC15068 (Virolle et al. 1988) were applied. A derivative of the streptomycete plasmid pIJ486 containing the mTNF cDNA, fused to the α-amylase signal sequence at a distance of 36 nucleotides by means of a linker for in-frame fusion, was constructed, as described previously, and designated pLMS164mTNF (Van Mellaert et al. 1994). An analogue of this construction lacking nine nucleotides, including a second ribosome binding site (RBS) and the ATG start codon preceding the mTNFα gene, was designated pLMS164mTNF-GS519 (Fig. 1). Immunoblot analysis of the culture supernatants and cell lysates of *S. lividans* [pLMS164mTNF] and *S. lividans* [pLMS164mTNF-GS519] revealed that in both cases all expressed mTNF was completely secreted (data not shown) provided that mTNF was linked to a proper signal peptide. Our choice for an indirect fusion of mTNF with the α-amylase signal sequence was based on results described for hTNF (Chang and Chang 1988), a protein that is 86% homologous to mTNF. A comparison of these two cases shows the importance of a conserved signal peptide and adjacent amino acids. In the case of hTNF, the heterologous protein was fused directly to the signal peptides of MelC1 or α-amylase and part of the expressed hTNF was not secreted but remained in the cells as unprocessed preproteins. This could be due to inaccurate cleavage of the signal peptide or to inefficient translocation through the cell membrane.

Bioassays of the culture fluid of *S. lividans* [pLMS164mTNF] indicated that mTNF was secreted at concentrations up to 0.5–2.5 × 10^6 U/ml with a minimum specific activity of 2.5 × 10^8 U/mg mTNF protein. *S. lividans* transformants containing the plasmid lacking nine nucleotides showed a decrease in mTNF production by a factor of 100–1000. As the expression signals of the α-amylase gene were not changed, the negative effect on mTNF yield could neither be explained by a modification at the level of transcription, nor at the level of secretion. This indicates that the coding sequence itself must contain important information determining the final yield of the foreign protein.

Does codon adaptation affect mTNF yield?

The mTNF gene has a GC content of about 56%, significantly lower than that of the host strain, which results in a different codon composition. To investigate the influence of codon adaptation on the expression levels of mTNF, some minor codons were adapted to the codon preference of *Streptomyces* without changing the amino acid composition (Fig. 1). These mutations were introduced both in pLMS164mTNF and pLMS164mTNF-GS519 derivatives and confirmed by DNA sequence analysis. Different transformants carrying the same plasmid construction were screened for mTNF production by immunoblot analysis using rabbit anti-mTNF antisera and by determination of the biological activity. Although the mTNF production of each transformant remained constant, provided the clones were stored at − 80° C in 20% glycerol, different colonies of the same transformation experiment gave rise to varying yields of mTNF. This was also observed by Ueda et al. (1993), who investigated the effect of minor codons on the expression of subtilisin inhibitor protein. As can be concluded from Fig. 2, changing TCT^{Ser} to TCC^{Ser} did not affect mTNF production; neither did the replacement of TTA^{Leu}, an uncommon codon for *Streptomyces*, by CTG^{Leu}. On the contrary, changing five consecutive minor codons

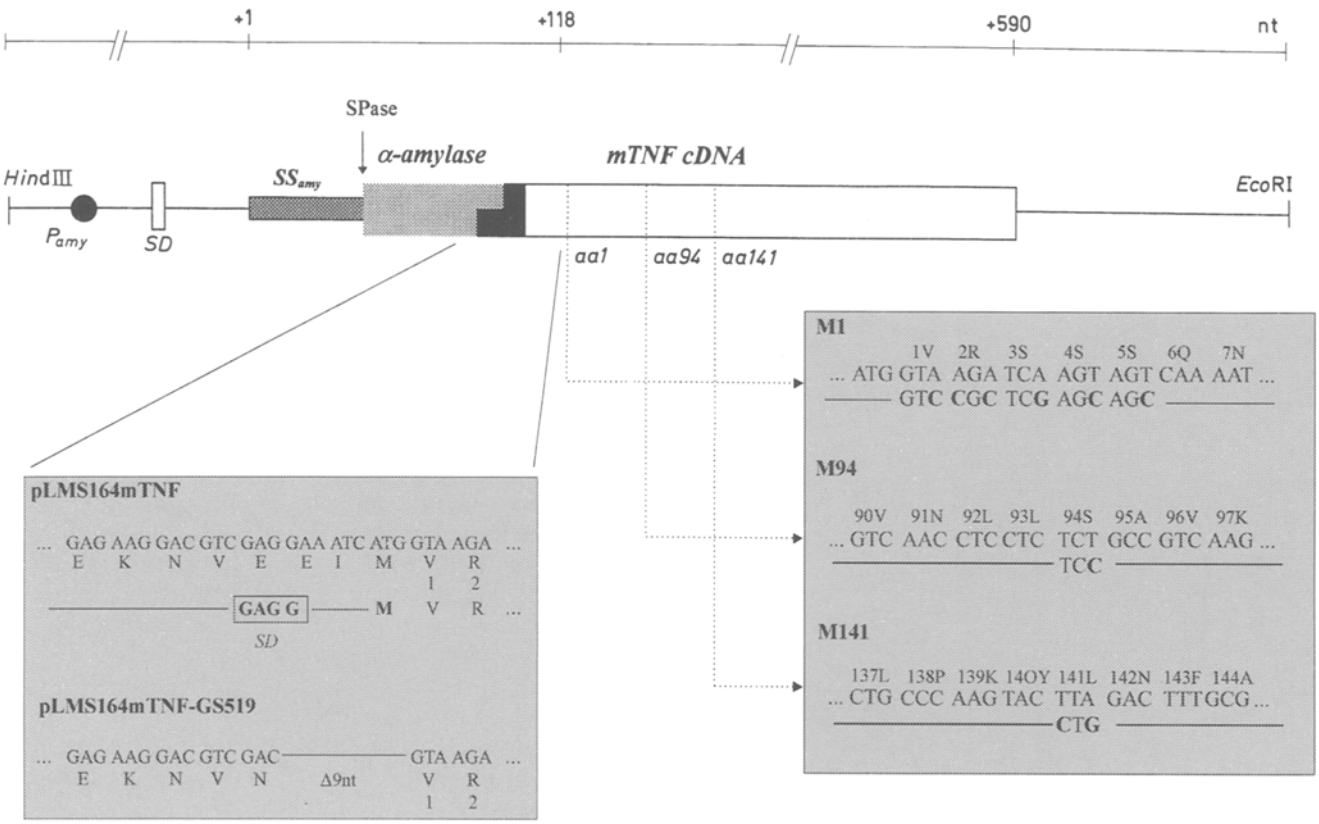

Fig. 1 Representation of the mouse tumour necrosis factor α (*mTNF*) secretion cassette from the plasmids pLMS164mTNF and pLMS164mTNF-GS519 and mutations introduced in the two original plasmids. (*aa* amino acid, *nt* nucleotide, P_{amy} promoter of the α-amylase gene, SS_{amy} signal sequence of the α-amylase gene, *SD* Shine-Dalgarno sequence, *SPase* signal peptidase cleavage site, *nt* + 1 first nucleotide of ATG start codon of α-amylase, *nt* + *118* first nucleotide of ATG start codon of mTNF, *nt* + *590* last nucleotide of TGA stop codon of mTNF)

downstream of the ATG codon preceding mTNF cDNA in favour of the codon bias of *Streptomyces* resulted in a drastic decrease in mTNF yield (factor of 10^4). As described above, the deletion of nine nucleotides from pLMS164mTNF also caused an unexpectedly significant reduction in levels of secreted mTNF. Moreover, reinsertion of the nine nucleotides led to a restoration of the original mTNF yield, which indicates that the decrease in production was not artefactual. As replacing a codon implies changing the mRNA molecule, it is possible that the altered secondary structure of the mRNA or a decreased mRNA stability could have influenced translation and, in consequence, the final mTNF yield.

Does mRNA stability improve mTNF yield?

To test this hypothesis, total RNA was extracted at different stages of growth from *Streptomyces* cultures harbouring the different mutant mTNF constructs. Isolated RNAs were analysed for the presence of traces of plasmid DNA and, subsequently, 70 μg total RNA was analysed by northern blotting. As can be seen in Fig. 3, the level of mTNF mRNA present in the cells was different for the different mutant mTNF constructions. In the case of low-producing strains, mRNA amounts were too low to be detected with our assay. A correlation exists between the amounts of biologically active mTNF and mRNA amounts. Besides full-length α-amylase-mTNF mRNA and a longer transcript, possibly originating from the lack of a transcription terminator, a series of lower molecular weight mRNAs was observed. The presence of these shorter mRNAs was confirmed by low resolution nuclease S1 mapping (Favaloro et al. 1980). Although it is not certain from where these shorter fragments arise, a specific degradation of the full-length α-amylase-mTNF mRNA is not excluded. A comparison of the amount of mTNF mRNA present in cultures grown for 24 h and 36 h showed that the relative amounts of mRNAs were maintained. The total mRNA amounts, however, seemed to be 10–50 times higher after 36 h than after 24 h of growth, which is in accordance with the increasing titre of biologically active mTNF (data not shown). To test the hypothesis of a decreased mRNA stability in the case of the low-producing strains, predictions concerning RNA secondary structures and corresponding stabilities were made by means of computer software (FOLD, SQUIGGLES, MFOLD and PLOTFOLD;

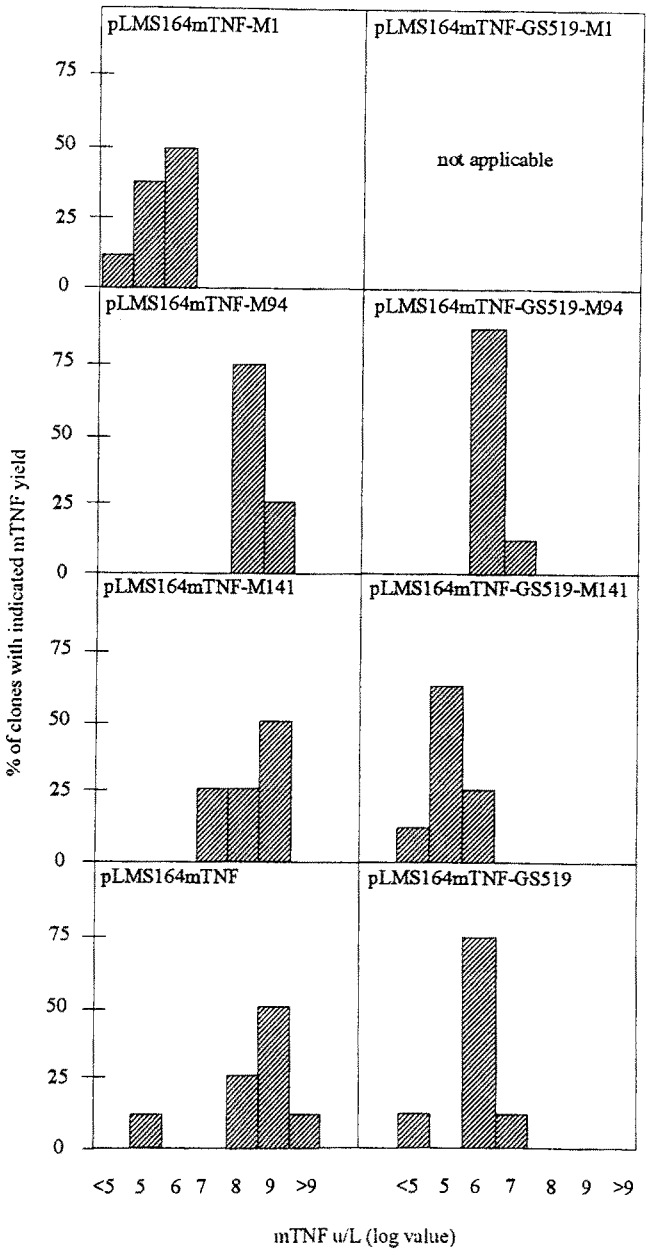

Fig. 2 Influence of the introduction of codons preferentially used by *Streptomyces* in mTNF cDNA on the yield of secreted mTNF by *S. lividans*. Yields for *S. lividans* containing pLMS164mTNF and pLMS164mTNF-GS519 and their derivatives are compared

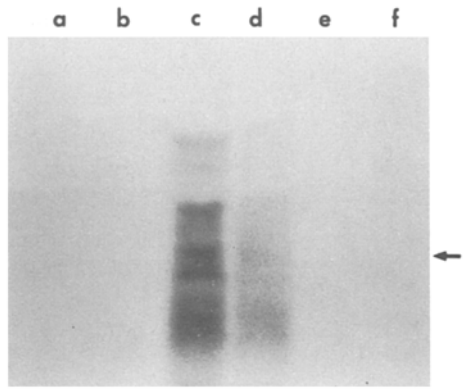

Fig. 3 Northern blot analysis of mRNA of *S. lividans* TK24 containing (*a*) pIJ486, (*b*) pLMS164mTNF-M1, (*c*) pLMS164mTNF-M141, (*d*) pLMS164mTNF, (*e*) pLMS164mTNF-GS519-M141 and (*f*) pLMS164mTNF-GS519. The band resulting from full length α-amylase-mTNF mRNA is indicated by an *arrow*

GCG package, Devereux et al. 1984), both for full-length mRNA and growing mRNA molecules. On the one hand, this analysis showed that there was not much difference in free energy of stabilisation between pLMS164mTNF (−208 kcal/mol), pLMS164mTNF-GS519 (−216 kcal/mol) and pLMS164mTNF-M1 (−210 kcal/mol). On the other hand, however, analysis of mRNA stability by means of a P-Num plot (Fig. 4) yielded interesting data. The FOLD algorithm calculates the structure with the minimum free energy, together with a population of other secondary structures with a slightly higher energy, according to the energy minimisation method of Zuker (1990). Statistical analysis of that population shows that a base can be associated with several other bases. The number of the different associations of one base is the P-Num value. For each position of the sequence along the horizontal axis, the height of the plot indicates how many different pairing partners are found in all predicted optimal and suboptimal foldings within an energy increment of 4.6 kcal/mol. A low P-Num value means a well-defined structure and a good prediction; a high P-Num value gives rise to many possible arrangements which are not very different in terms of free energy of stabilisation. Given that the initiation of translation, i.e. the binding of the ribosomes to the mRNA, is most important for efficient translation, we only considered the P-Num plot in the vicinity of the ribosome binding site and the ATG start codon of the α-amylase gene. Adding up the P-Num values in this neighbourhood resulted in the following values: 103 for pLMS164mTNF (high producer) and 140 and 194 for pLMS164mTNF-M1 and pLMS164mTNF-GS519, respectively (low producers).

Discussion

For *E. coli* and *S. cerevisiae* a strong positive correlation was found between the relative abundances of isoaccepting tRNAs in the cell and the codon choice pattern used in the genes (Ikemura 1981, 1982). In addition, a similar relationship exists between the level of gene expression and the degree of major codon bias of a gene (Gouy and Gauthier 1982). Extensive studies concerning the effect of minor codons on gene expression have already been carried out for different species,

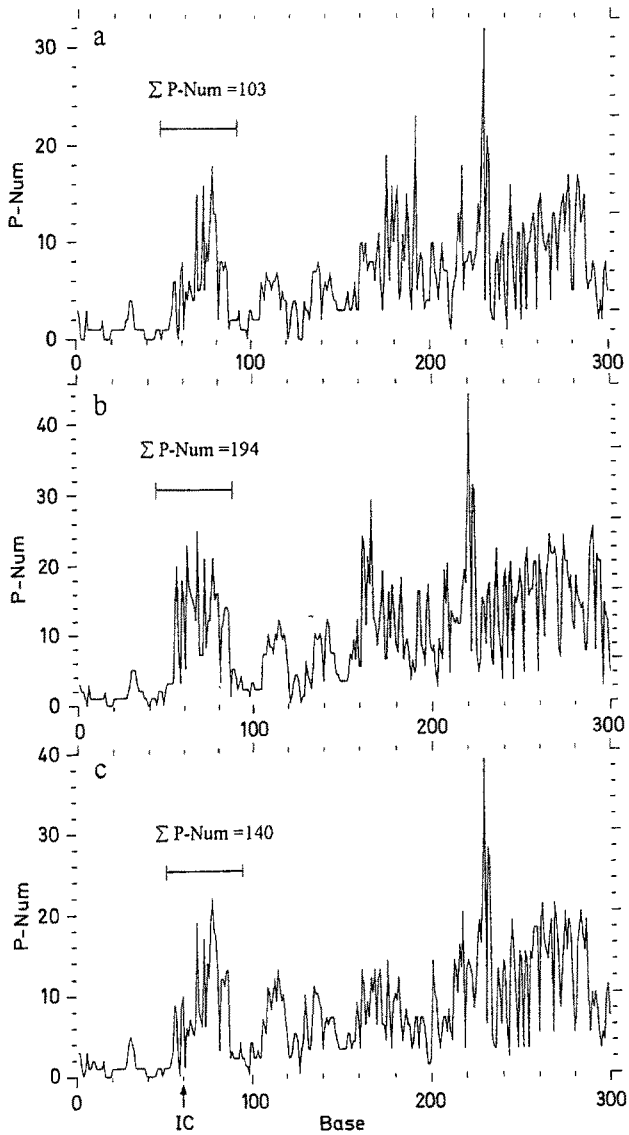

Fig. 4 Output of FOLD algorithm with indication of P-Num value for mRNA encoded by (*a*) pLMS164mTNF, (*b*) pLMS164mTNF-GS519 and (*c*) pLMS164mTNF-M1. The ATG initiation codon (*IC*) of the α-amylase gene is at nucleotide 60. ($\sum P\text{-}Num$ sum of the P-Num values in the vicinity of the initiation codon)

but led to controversial results (Robinson et al. 1984; Chen and Inouye 1990; Rosenberg et al. 1993). Clusters of minor codons seem to be capable of modulating gene expression, especially if they are introduced near the ATG initiation codon of the gene. Recently, Ueda et al. (1993) found that introducing TTA^{Leu}, a codon rarely used by *Streptomyces*, in the highly expressed subtilisin inhibitor (SSI) gene from *S. albogriseolus* resulted in a considerable reduction of SSI production by *S. lividans* until late in growth. This is in agreement with the transcriptional regulation of the *bldA* gene, which encodes the only tRNA for the rare TTA codon in *S. coelicolor* (Leskiw et al. 1993).

With these results in mind, we adapted several minor codons occurring in the mTNF gene towards the codon bias of *Streptomyces*. However, replacing TTA^{Leu} by CTG^{Leu} or TCT^{Ser} by TCC^{Ser} did not result in an improvement of mTNF yield. We observed a drastic reduction in mTNF production when changing five tandemly arranged minor codons at the beginning of the mTNF gene for more biased ones. Therefore, we concluded that it is not the use of minor codons, as such, which is responsible for lower expression levels of heterologous proteins. Variation in plasmid copy numbers of the different constructs as a possible explanation for the low mTNF yields was excluded experimentally. It seemed that the observed effects were due to the changes in the DNA sequence. After all, these changes involve changing the primary and secondary structures of the mRNA which, in turn, could affect mRNA stability. We found, indeed, that the decrease in mTNF yield was correlated with lower mRNA levels. It was shown by P-Num analysis that mRNA of high-producing strains was characterised by a low P-Num value while the predicted P-Num values for mRNA from low-producing strains were higher. An increase in P-Num is probably correlated with an increase in the population of RNA secondary structures. For RNAs with a higher P-Num value, the way to reach the conformation associated with the lower energy is probably more complicated and longer and, in consequence, the action of RNases could be increased. Parameters causing mRNA instability and ways to reduce it remain to be investigated. As shown for the expression of hIFNα2 by *S. lividans*, introduction of a transcriptional terminator downstream of the foreign gene has a stabilising effect on the mRNA transcripts (Pulido and Jiménez 1987).

A greater understanding of these and other parameters affecting (heterologous) gene expression and secretion, together with the development of protease-deficient host strains in order to minimise proteolytic degradation, are indispensable in making *Streptomyces* an even more attractive host for expression and secretion of eukaryotic proteins in commercially interesting quantities.

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