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Optimization of gene expression in *Streptomyces lividans* by a transcription terminator

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ABSTRACT

The ability of an inverted repeat sequence (IRS) from the 3' end of the aph gene from Streptomyces fradiae to induce transcription termination in vivo has been examined. As a model system, a DNA fragment encoding the human interferon α 2 inserted in the Streptomyces plasmid pIJ702 was used. When the IRS was inserted downstream from this sequence and transcription assayed in Streptomyces lividans, highly efficient (~90%) transcription termination was observed occurring immediately after the 3'terminus of the dyad. In contrast, gene constructions lacking the IRS transcribed longer mRNAs. Moreover, the IRS gave rise to increased amounts of the hIFN α 2 suggesting that the putative stem-loop structure stabilised the transcript.

INTRODUCTION

Transcription termination in prokaryotic organisms is thought to involve specific DNA signal(s) that halt(s) polymerization and determine(s) release of the synthesized mRNA (1,2,3,4). Two types of transcription terminators have been described, one dependent of proteins factors, rho (5), NusA (6) and tau (7); and another independent of factors. Factor-independent terminators generally contain a region of dyad symetry (rich in G+C) which could give rise to a stem-loop structure in the mRNA. In general, a thymidine-rich sequence is located 3' to this potential hairpin structure. The region of dyad symmetry is important for both efficient transcription termination (8) and mRNA stability (9). In contrast, no clear specific DNA sequences have been found in factor dependent transcription terminators, although, in a few cases, the presence of stem-loop structures facilitates rho and Nus A mediated transcription termination (4).

The presence of transcription terminators has been profusely described for E. coli and some of its phages (1,2,3,4). However, very little is known of their presence in Gram-positive organism. Thus, in Bacillus pumilus a sequence determining transcription termination has been characterized and apparently it is involved in the induction of the cat-86 gene (10,11). A variety of genes from both Streptococcus sanquis and Staphylococcus aureus contain potential stem-loop structures which seem to be implicated in the processes of inducibility (12) and attenuation (13). Also, in the Ø29 phage from Bacillus subtilis, putative sequences determining transcription termination have been reported (14). Streptomyces are an important group of mycelial Gram-positive bacteria that produce, generally during the secondary metabolism, many useful antibiotics. These bacteria undergo a complex life cycle which includes a developmental process culminating in spore formation (15). These processes appears to be regulated by the timely expression of specific genes, which heightens the interest in characterizing the mechanisms involved in controlling their transcription. Moreover, the high G+C content (>73%) of the Streptomyces DNA (16), makes it likely that its transcriptionally regulatory regions are different from those found in other organisms. Indeed, most Streptomyces promoters examined so far do not function in E. coli, have structures that appear unrelated to those from this Gram-negative organism and can be grouped in distinct classes (17).

Concerning transcription termination in Streptomyces, putative hairpin structures have been found in several genes: aph (18) (see Fig 1), vio, tsr (19) and hyg (20). Although these structures could be involved in transcription termination, no experimental results are yet available to support this role.

In the present work, we have examined in Streptomyces lividans the transcription termination capacity of a DNA fragment containing the inverted repeat sequence (IRS) from the aph gene of Streptomyces fradiae on the expression of the human interferon α 2 (hIFNα2) gene, as governed by the Streptomyces aph or hyg gene promoters.

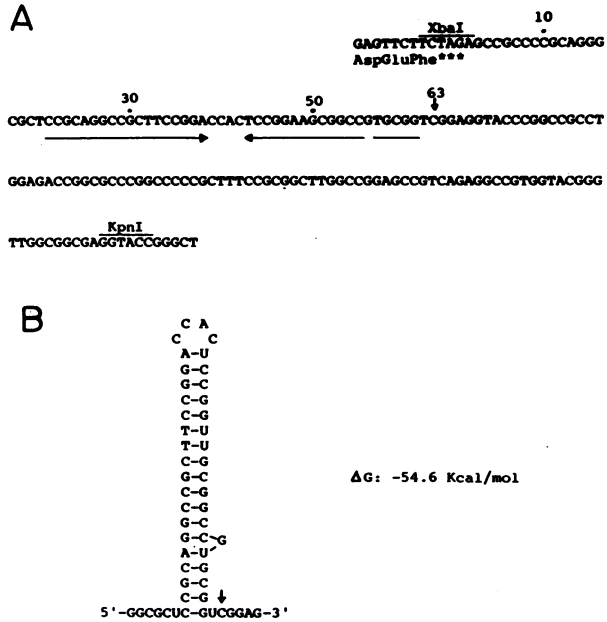

Figure 1. A) Nucleotide sequence of the 3' end of the aph gene. This sequence has been taken from Thompson & Gray (18). The inverted repeat region is indicated by the two horizontal arrows. The XbaI and KpnI restriction sites mark the cloned 160 bp fragment. The vertical arrow indicates the site of transcription termination. B) Putative secondary (stem-loop) structure of the transcripts across the terminator region. The theoretical free energy of formation (Δ G) of this structure was taken from Bibb et al. (19).

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

E. coli JM83 (21) and S. lividans 1326 (22) were obtained from J.E. Davies and D.A.Hopwood, respectively. E. coli plasmid pUC19 (23) and Streptomyces plasmid pIJ365 (24) were obtained from J.E. Davies and D.A. Hopwood, respectively. Streptomyces plasmids pNIS19 and pNIS91 (Fig 2) are derivatives of the Streptomyces plasmid pIJ702 (25). They contain a modified fragment of human DNA encoding the mature form of interferon ∝2 fused to the promoter of the aph gene from S. fradiae (26). The two plasmids differ in the relative orientation of the inserted hIFN∝2 gene. Plasmids pGIS12 and pGIS21 differ from pNIS19 and pNIS91 in that the former contain

the hIFN α2 gene fused to the promoter of the hyg gene from S. hygroscopicus (20) instead of the aph promoter (D. Pulido, M. Zalacain and A. Jiménez, in preparation).

E. coli was grown using either liquid or solid LB medium supplemenmted with X-gal (40µg/ml) and ampicillin (100µg/ml) as required (27). S. lividans was grown in liquid medium YEME (28) supplemented with 34% sucrose and 5mM $MgCl_2$. Solid medium R5 and overlay agar were as described elsewhere (29).

Both black and white phenotype of S. lividans clones was scored on R5 solid medium. The black colour shows the synthesis of melanine which is due to the expression of the tyrosinase (mel) gene carried by pIJ702. White colour indicates inactivation of the mel gene (25).

E. coli and S. lividans were transformed as described by Maniatis et al. (30) and Hopwood et al. (31), respectively.

Assay of interferon activity

Preparation of cell extracts from Streptomyces clones and assay of interferon activity were performed as described previously (26).

Northern analysis and S1 mapping

RNA from interferon-producing Streptomyces clones was prepared as described elsewhere (20). RNA-DNA hybridization experiments (Northern analysis) were carried out as described by Thomas (32), using formaldehyde as a denaturing agent in agarose gel electrophoresis. Approximately, 40µg RNA from each Streptomyces clone was loaded per lane.

The transcription termination site was determined by the S1 mapping procedure described by Favoloro et al. (33), except that DNA-RNA hybrids were formed at 60ºC, because of the high G+C content of Streptomyces DNA. After S1 digestion, the reaction products were loaded on a 6% acrylamide sequencing gel, in parallel with sequencing reactions (34) from a fragment of the 5'region of the hIFN α2 gene of known sequence, present in plasmid pIFXbal2 (26).

Quantitation of mRNAs in X-ray films

To determine the efficiency of transcription, autoradiograms prepared from Northern blots were analysed using a digital microdensitometer (Optronics model PDP 11/45). The

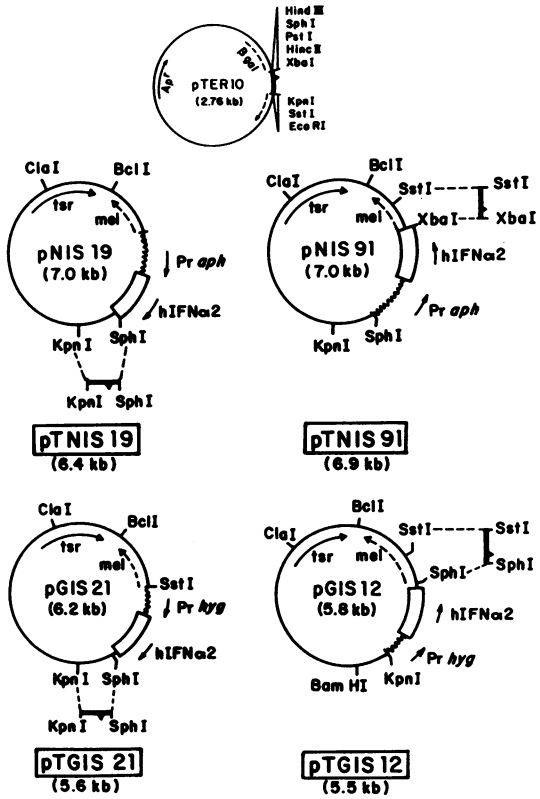

Figure 2. Construction map of several plasmids containing the IRS. Plasmid pTER10 is a derivative of E. coli plasmid pUC19 and contains the 160 bp XbaI-KpnI sequence from the aph gene (Fig 1) inserted in the polylinker region. This sequence was excised with the appropriate restriction enzymes to be inserted at the 3' end of aph or hyg gene promoter-hIFNα2 gene fusions in the Streptomyces plasmids pNIS19, pNIS91, pGIS21 and pGIS12. The resulting plasmids are boxed. Pr, indicates promoter. The arrows indicate the directions of transcription. The closed arrow heads indicate the approximate position of the IRS.

densitometric values obtained from each hybridization band, were represented in units of integration.

Source of materials

Restriction enzymes and T_4 DNA ligase were obtained from Boehringer Mannheim and Promega Biotech, respectively and were used as indicated by the suppliers. Other materials were obtained from Sigma and E. Merck (Darmstat). Radioactive nucleotides were obtained from Amersham.

TABLE 1. Chacteristics of the different *S. lividans* clones.

Clone	Plasmid	(Kb)	Promoter[a]	IRS[b]	Phenotype[c] (colour)	Tyrosinase[d] activity (induced)	IFN[e] activity (IU/L) x10^6
3131	pIJ702	(5.8)	-	-	Black	2.13	0.0
DP19	pNIS19	(7.0)	aph	-	White	< 0.01	1.0
DP91	pNIS91	(7.0)	aph	-	Black	1.41	1.0
DP21	pGIS21	(6.2)	hyg	-	White	< 0.01	0.5
DP12	pGIS12	(5.8)	hyg	-	Black	1.07	0.5
DT19	pTNIS19	(6.4)	aph	+	White	< 0.01	4.0
DT91	pTNIS91	(6.9)	aph	+	White	0.14	4.0
DT21	pTGIS21	(5.6)	hyg	+	White	< 0.01	1.5
DT12	pTGIS12	(5.5)	hyg	+	White	0.09	1.5

a) Promoter preceding the hIFN α2 gene.
b) (+) and (-) indicate presence or absence of the IRS downstream from the hIFN α2 gene.
c) The phenotype is given by the colours of the colonies in R5 agar medium. Black colour is due to the melanine synthesis promoted by tyrosinase expression from the *mel* gene. White colour indicates absence of tyrosinase activity due to lack of expression of the *mel* gene.
d) Determined in cell-free extracts as described by Katz *et al* (25), and expressed in units x mg protein^{-1}.
e) IFN activity was determined by the reduction of the cytopathic-effect of encephalomyocarditis virus on HeLa cells, and expressed in international units (IU) per liter of culture.

RESULTS

Fusion of the IRS from the aph gene to the 3'-end of the hIFN α2 gene

S. lividans clones carrying plasmids pNIS19, pNIS91, pGIS12 or pGIS21 (Fig 2) expressed interferon activity (26; Table 1). Curiously, clones containing plasmids pNIS91 and pGIS12 into which the hIFN α2 gene was inserted in the promoter region of the *mel* gene (35) appeared black (Table 1), indicating that expression of the *mel* gene took place (25). This finding was not surprising since hIFN α2 and *mel* are transcribed in the same direction in plasmids pNIS91 and pGIS12 (Fig 2). Although the insertion of the hIFN α2 gene in this site inactivates the *mel* gene (35), under these conditions transcription of the latter could start from either the *aph* or the *hyg* gene promoter as shown in Fig 4. If this is correct, insertion of a transcription termination signal between the hIFNα2 and *mel* genes would impede transcription of the latter, and, consequently, clones containing the termination signal would have a white (*mel*$^-$) phenotype. Therefore, the 160 bp

XbaI-KpnI sequence, containing the IRS from the aph gene (Fig 1) was inserted immediately next to the 3' terminus of the hIFN α2 gene in plasmids pNIS19, pNIS91, pGIS21 and pGIS12 (see Fig 2 for details). S. lividans clones carrying plasmids pTNIS19, pTNIS91, pTGIS21 and pTGIS12 were isolated (Fig 2). In all these plasmids the inserted 160 bp fragment did not affect the structural mel gene. Despite this observation colonies carrying plasmids pTNIS91 and pTGIS12 had a mel$^-$ phenotype (Table 1), consistent with the interpretation that the inserted IRS sequence determine transcription termination before transcription reaches the mel gene.

Assay of interferon activity

The interferon activity of the different Streptomyces clones is given in Table 1. Cells containing plasmids with an inserted IRS had higher antiviral activity than those carrying the parental plasmids. Thus, clones carrying plasmids pTGIS21 and pTGIS12 in which the hIFN α2 gene is governed by the hyg promoter have 3-fold more activity than clones containing plasmids pGIS21 and pGIS12. Similarly, clones containing plasmids pTNIS19 and pTNIS91 in which the hIFN α2 gene is directed by the aph promoter had 4-fold more activity than clones containing plasmids pNIS19 and pNIS91. (Table 1).

Analysis of the interferon transcripts

The model proposed, which postulates that the IRS sequence determines transcription termination, can be tested by measuring the size of the transcripts encoding hIFN α2 from the relevant plasmids. Therefore, total RNA from the different clones was obtained and analyzed in Northern blots. The results shown in Fig 3, indicate that plasmids lacking the IRS transcribe a single mRNA of either 1.7 or 1.1 kb, depending upon the orientation of the hIFN α2 gene (chanels B-E). However, a new transcript of 0.7 kb appears in the tracks with mRNAs from plasmids with an inserted IRS (chanels F-I), in addition to minor amounts of the 1.7 or 1.1 kb species. A diagram of the transcriptional events in the different plasmids is presented in Fig 4.

The size of the 0.7 kb transcript agrees well with the size (0.6 Kb) of the hIFN α2 gene present in the different

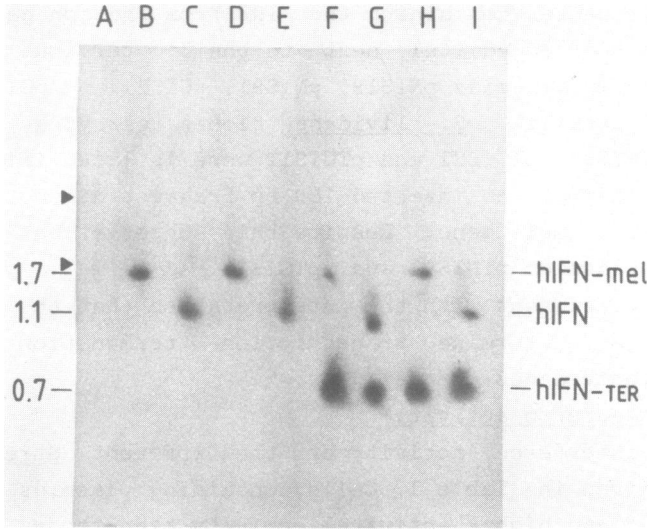

Figure 3. Northern blot analysis of the different transcripts. Northern blot analysis was performed with total RNA from S. lividans clones containing the indicated plasmids. A 420 bp AvaII-HincII DNA fragment, internal to the hIFNα2 gene (43) was used as probe. Numbers on the left indicate the sizes (in kb) of the hybridizing mRNAs. hIFN-mel, mRNA encoding mature human interferon and tyrosinase; hIFN, mRNA encoding human interferon and hIFN-TER, mRNA from the hIFN gene containing the IRS. (▶) Migration of the rRNA from S. lividans used as size markers. A) pIJ702; B) pNIS19; C) pNIS91; D) pGIS21; E) pGIS12; F) pTNIS19; G) pTNIS91; H) pTGIS21 and I) pTGIS12.

plasmids, indicating that the IRS determines transcription termination when inserted behind that gene. The 1.7 kb mRNA is only transcribed from plasmids where the hIFN α2 and the mel gene are transcribed in the same direction. The size of the transcript indicates, most probably, that it contains the coding sequences for both genes, terminating transcription at a site close to the mel structural gene. In agreement with this interpretation is the finding that plasmids pNIS91 and pGIS12 induce the accumulation of both tyrosinase and melanin, in addition to interferon α2, in their hosts (Table 1). Transcription termination to produce the 1.1 kb mRNA must occur 0.7 kb downstream from the end of the hIFNα2 gene, in a site pro-

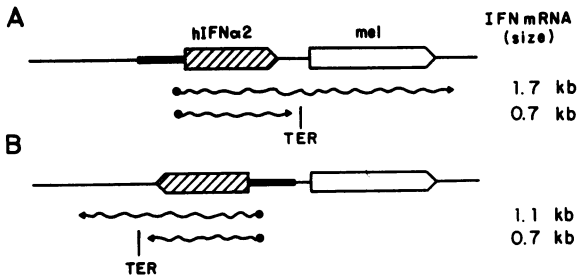

Figure 4. Diagram of the transcriptional products from the different plasmids construction. A) mRNA transcripts obtained using plasmids pNIS91 and pGIS12 DNA as templates. Plasmids pTNIS19 and pTGIS21, carrying an inserted IRS, terminate transcription just after the hIFNα2 gene rendering a 0.7 kb mRNA. B) mRNA transcripts obtained using plasmids pNIS91 and pGIS12 DNA as templates. Plasmids pTNIS19 and pTGIS21, carrying an inserted IRS sequence, terminate transcription just after the hIFNα2 gene, yielding a 0.7 kb mRNA. (▬) aph or hyg gene promoters. The arrow indicates the direction of transcription of the hIFNα2 and mel genes.

vided by the parental (pIJ702) plasmid (Fig 4). Curiously, this site is a highly active terminator, since it promotes, apparently, a 100% of termination events.

Densitometric analysis showed that the IRS induced approximately termination in 90% of the trancriptional events (Table 2); the process is, therefore, highly efficient. In addition, the amount of hIFNα2 mRNA transcribed from plasmids containing the IRS was significatively higher (3-4-fold) than that from plasmids lacking the IRS. This finding may explain, at least in part, the increase of interferon activity in the clones containing plasmids with an inserted IRS (Table 1). Considering that these clones did not show any increase in their plasmid content, which could affect gene dosage (results not shown), it is suggested that the presence of the IRS stabilizes the transcripts for hIFNα2, resulting in their increased accumulation in the cytoplasm. In this respect, it has been shown that aph gene terminates transcription immediately after the IRS element (see below).

TABLE 2. Densitometric analysis from the different hIFN mRNAs in S. lividans clones.

Clone	(Plasmid)	mRNAs			%
		1.7	1.1	0.7	0.7 mRNA
3131	(pIJ702)	-	-	-	-
DP19	(pNIS19)	11.6	-	-	-
DP91	(pNIS91)	-	10.1	-	-
DP21	(pGIS21)	8.7	-	-	-
DP12	(pGIS12)	-	8.1	-	-
DT19	(pTNIS19)	1.6	-	30.2	94.7
DT91	(pTNIS91)	-	7.5	27.1	78.3
DT21	(pTGIS21)	3.5	-	26.3	88.1
DT12	(pTGIS12)	-	3.5	24.7	87.6

The densitometric values were expressed in integrational units (see material and methods).

Termination of transcription by the IRS

The precise site of transcription termination was determined by the S1 mapping procedure. It was clear that termination occurs at or around a C 63 bp downstream from the nonsense TGA codon (Figs 1 and 5). This site is located immediately 3' of the presumptive stem-loop of the transcripts.

DISCUSSION

The important role that transcription terminators play in bacterial gene expression is well known (1,2,3,4). The existence of inverted repeat sequences (IRS), which could give rise to stem-loop structures at the end of several *Streptomyces* genes suggested that they may have important regulatory functions in gene expression in these bacteria (18,19,20). In the present work we have examined in *S. lividans* the effect on the levels of transcription and size of transcripts of a DNA fragment containing the IRS from the *aph* gene of *S. fradiae* on the expression of the hIFN α2 gene, as directed by the *Streptomyces aph* or *hyg* gene promoters. The analysis of the mRNAs for hIFN α2 synthesized in vivo from different plasmid constructions strongly suggested that the *aph* IRS determines transcription

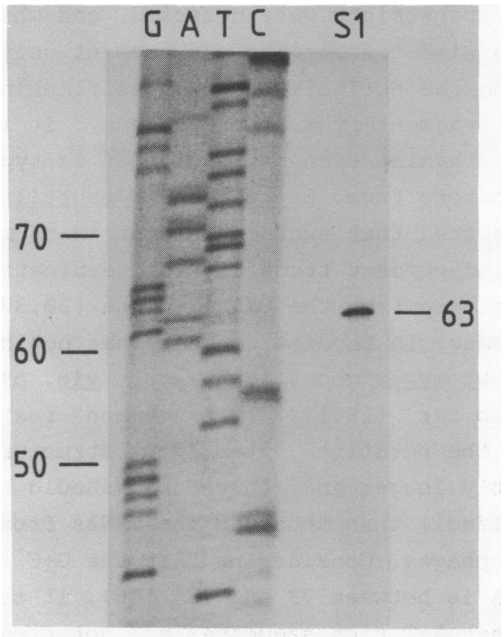

Figure 5. S1 mapping of the transcription end points 3' to hIFN 2 fused to IRS. RNA was isolated from S. lividans clone DT19, harbouring pTNIS19. A 160 bp XbaI-KpnI DNA fragment from pTNIS19, end-labeled by Klenow treatment of the XbaI site (using $\alpha(^{32}P)$dCTP only) was used as probe. Lanes labeled G, A, T and C contain standards from sequencing reactions (see Materials and Methods). Numbers on the left indicate the number of bases of the standard and that on the right the size of the length of DNA fragment protected from the action of S1 nuclease.

termination efficiently (~90%). Moreover, the presence of this IRS at the end of the mRNAs increases considerably the abundance of hIFN α2, a finding that may reflect an increase in the stability of the transcripts. A similar stabilizing effect has been found with several transcription terminators in E. coli and the bacteriophage lambda (9,36,37). As an alternative explanation of the results presented in Fig 3, one may consider that transcription termination takes place well beyond the secondary structure, followed by 3'⟶ 5' exonucleolytic degradation back to the IRS. This possibility has been documented in several reports with E. coli (4). However we think it is unlikely, because no intermediary forms between the

long and small transcripts were detected and when the IRS is absent, the long size transcripts are present only.

Concerning the nucleotide sequences flanking the IRS in the cloned DNA fragment from the aph gene, it is remarkable that it lacks a thymine rich sequence that is typically found after the terminators from E. coli and B. subtilis (1,2,3,4). It has been suggested that such enrichment in thymine residues favors the rho-independent transcription termination process by allowing mRNA release from the DNA template (38,39). Nevertheless, such increase in thymine content has not been found in any of the Streptomyces genes (aph, tsr, vio, hyg) that have been examined so far (18,19,20). A common feature of these genes is that the putative "stem-loop" structures of their mRNAs, are notably longer and, therefore, should be more stable (-30 to -80 kcal/mol) than those in the mRNAs from E. coli and several bacteriophages. Considering that the G+C content of the Streptomyces DNA is between 73 and 75% (16), it seems reasonable to expect that A+T rich sequences are not frequent in their genes. Indeed, Streptomyces promoter sequences are richer in G+C than the E. coli promoters (2,17). Therefore, thymine-rich sequences may be scarce after the transcription terminators of Streptomyces genes. This can be balanced with long and strong stem-loop structures that increased the efficiency of transcription termination. An "in vitro" modification that results in either increase or reduction of the stem-loop size of E. coli IRSs determines a parallel effect on the efficiency of transcription termination (39,40,41). It should also be noted that, as suggested for E. coli (39,42) the transcription termination process may be modulated by the secondary structure of the nucleotide sequences close to Streptomyces terminators. In this respect, transcription termination in vivo at the hyg gene from S. hygroscopicus takes place prior to the first of the two succesive dyads which are present at its 3' terminus (20). Similar double putative stem-loop structures have not been found in any of the other Streptomyces genes that have been sequenced so far. Although the first (smaller) IRS could induce transcription termination (i.e., the RNA polymerase would unfold the DNA during polymerization and allow the small

hairpin to be formed in the DNA strand thereby stopping transcription) an alternative hypothesis could be that transcription termination at the hyq gene is dependent upon a protein factor. Whichever the case, it appears that transcription termination in Streptomyces has some different features than what is known for other bacteria.

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