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Reviews

Transcriptional organization and regulation of the nosiheptide resistance gene in *Streptomyces actuosus*

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SUMMARY

The nosiheptide resistance gene (*nshR*) and a putative regulatory gene (*nshA*) are found together on a 2326 bp *Bam*HI-*Pst*I DNA fragment isolated from *Streptomyces actuosus* ATCC 25421. The putative regulatory gene, *nshA*, situated upstream from the nosiheptide resistance gene in the 2326 bp DNA fragment, contains apparent DNA-binding and RNA-binding domains. Interruption of *nshA* in the chromosome of *S. actuosus* alters nosiheptide production, suggesting that *nshA* is involved in regulation of nosiheptide biosynthesis. Two transcription initiation sites were found upstream of *nshA* as demonstrated by high-resolution S1 nuclease mapping. A weak transcription start site for *nshR* was found which initiated transcription from the first nucleotide of the open reading frame. Although a stem-loop structure with apparent termination activity was found between *nshA* and *nshR*, readthrough of transcription between *nshA* and *nshR* was demonstrated by S1 nuclease mapping of the 3′ terminus of the *nshA* transcript. Time-course S1 experiments of the three promoters (*nshA-p1*, *nshA-p2*, *nshR-p*) indicated highly regulated differential expression of the promoters. *nshA-p2* is a strong, constitutive promoter whereas *nshA-p1* being regulated temporally with maximal activity at 96 h. Approximately 30% of the total *nshA-p1/p2* transcript reads through the terminator and into the *nshR* gene, accounting for more than half of the total steady-state *nshR* transcript. The implications of the regulation of *nshA* and *nshR* gene expression, as well as the expression of two other linked genes, are presented.

INTRODUCTION

One method to study the expression of streptomycete antibiotic biosynthesis genes is to analyze the self-resistance of the organisms to the antibiotics they produce, since production of antibiotics in the absence of self-resistance mechanisms would be lethal [19]. Since resistance genes are inherently easy to clone by direct selection, several of these genes have been cloned and sequenced. Antibiotic resistance genes are typically clustered with the genes encoding the biosynthesis of that antibiotic, which has enabled investigators to isolate antibiotic biosynthesis genes by cloning large DNA fragments and selecting for resistance to the antibiotic [12,15,23,52,56,64]. Here we describe our work on the structure and transcriptional regulation of the nosiheptide resistance gene (*nshR*) and the potential functions of genes clustered with it.

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NOSIHEPTIDE AND RESISTANCE TO THIOPEPTIDES

Nosiheptide (Fig. 1) is a thiopeptide antibiotic of the 'thiostrepton group', which includes thiostrepton, siomycin, sporangiomycin, and thiopeptin [57]. Nosiheptide, produced by *Streptomyces actuosus* ATCC 25421, *Streptomyces* sp. ATCC 31463, and *Streptomyces glaucogriseus* NRRL LL-BP 189, is a coccidiostat that is used as an animal feed additive to promote weight gain [5]. Thiostrepton, produced by *Streptomyces azureus* ATCC 14921, *Streptomyces laurentii* ATCC 31255, and *Streptomyces hawaiiensis* ATCC 12236, is a veterinary topical antibacterial medicine [67]. Both of these antibiotics inhibit protein synthesis by binding to the 23S rRNA and ribosomal protein L-11 to inhibit the activities of elongation factors EF-Tu and EF-G [20]. These antibiotics

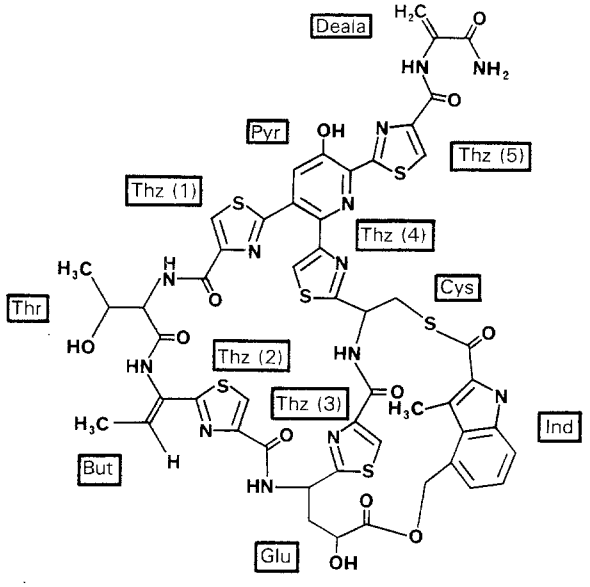

Fig. 1. Structure of the thiopeptide antibiotic, nosiheptide [27,57]. Abbreviations: deala, dehydroalanine residue; pyr, pyridine; thz 1–5, thiazole rings 1–5; thr, threonine; but, dehydrobutyrine residue; glu, γ-hydroxyglutamate moiety; and ind, indolic acid moiety.

also interfere with the synthesis of the stringent response factor, ppGpp [55]. Expression of the thiostrepton resistance gene (*tsr*), encoding 23S rRNA methylase, prevents thiostrepton from inhibiting ppGpp formation [55]. Both *S. actuosus* and *S. azureus* become resistant to thiopeptide antibiotics by methylating an adenosine residue of 23S rRNA to produce 2′-*O*-methyladenosine [20].

The thiostrepton resistance gene from *S. azureus* has been isolated [66] and sequenced [7], and the structure of its promoters has been delineated by high resolution S1 mapping and promoter probe experiments [40,41]. The *tsr* gene from *S. azureus* is a selectable marker in many of the plasmid vectors used in streptomycete cloning [32].

RIBOSOMAL vs. ENZYMATIC BIOSYNTHESIS OF NOSIHEPTIDE

Peptide antibiotics are synthesized either by normal peptide synthesis mechanisms using a messenger RNA template or by a specialized enzyme complex known as a peptide synthetase. Peptide antibiotics synthesized ribosomally include nisin [43], epidermin [61], and subtilin [4]. Peptide antibiotics synthesized by specialized peptide synthases include gramicidin S [45], tyrocidine [51], bacitracin [39], and cyclosporin [10]. It is not yet known whether thiopeptide antibiotics (e.g., nosiheptide, thiostrepton, siomycin, sporangiomycin, thiopeptin) are synthesized ribosomally or via peptide synthetases. Preliminary experiments have indicated that the biosynthesis of nosiheptide is not as sensitive to chloramphenicol inhibition as protein synthesis, suggesting that nosiheptide is produced via a peptide synthetase system [27,70]. Moreover, we were unable to find hybridization, even at low stringencies, to high G + C-biased, degenerate oligonucleotide probes containing nucleotide (nt) sequences corresponding to the putative amino acid sequences of the precursor peptides of thiostrepton (H_2N-L-ile-L-ala-ser-L-ala-ser-cys-L-thr-thr-D-cys-L-ile-cys-L-thr-cys-ser-cys-ser-ser-ser-???-COOH) and nosiheptide (H_2N-ser-cys-L-thr-thr-cys-L-glu-cys-L-cys-cys-ser-cys-ser-ser-???-COOH) [70]. By our predictions, the biosynthesis of nosiheptide by a peptide synthetase followed by modifications of the incorporated amino acids could theoretically involve as many as 16 to 32 enzymes (data not shown). The biosynthesis of nosiheptide, particularly modification of the amino acids incorporated into the peptide, has been described in detail elsewhere [27,37].

CLONING, SEQUENCING, AND ANALYSIS OF NOSIHEPTIDE GENES

We recently isolated an 8.5 kbp *Bam*HI DNA fragment from the nosiheptide producer, *S. actuosus* ATCC 25421 which conferred resistance to both nosiheptide and thiostrepton on *Streptomyces lividans* 1326 [25] (Fig. 2). We sequenced and analyzed a 2326 bp *Bam*HI-*Pst*I fragment, subcloned from the 8.5 kbp *Bam*HI fragment, that carried the nosiheptide resistance determinant [48]. Two open reading frames (ORFs) were found in this sequence, an ORF containing 699 nt (*nshA*; putative regulatory gene) and an ORF of 822 nt (*nshR*; nosiheptide resistance gene), both reading in the same direction, separated by a stem-loop structure (Fig. 2; designated 'T'). The *nshR* gene has 72% DNA sequence identity with the *tsr* gene of *S. azureus* and the deduced proteins encoded by these genes have a 74% amino acid identity (79% when 13 additional conservative substitutions are taken into account [48]).

We have also recently sequenced a 1.1 kbp DNA fragment downstream of *nshR* which contains two additional open reading frames, labeled *orfB* and *orfC* (Fig. 2), that fit the coding preferences typically found in streptomycete genes. The functions of these potential genes are not yet known. Other investigators also have cloned nosiheptide resistance genes from *S. actuosus* [18,21]. The 10.5 kb *Sac*I DNA fragment cloned by Dary et al. [21] significantly overlaps the 8.5 kbp *Bam*HI DNA fragment we cloned; it is not certain if the DNA fragment cloned by Cho et al. [18] is the same or a different fragment [25].

To determine the range of genes displaying homology

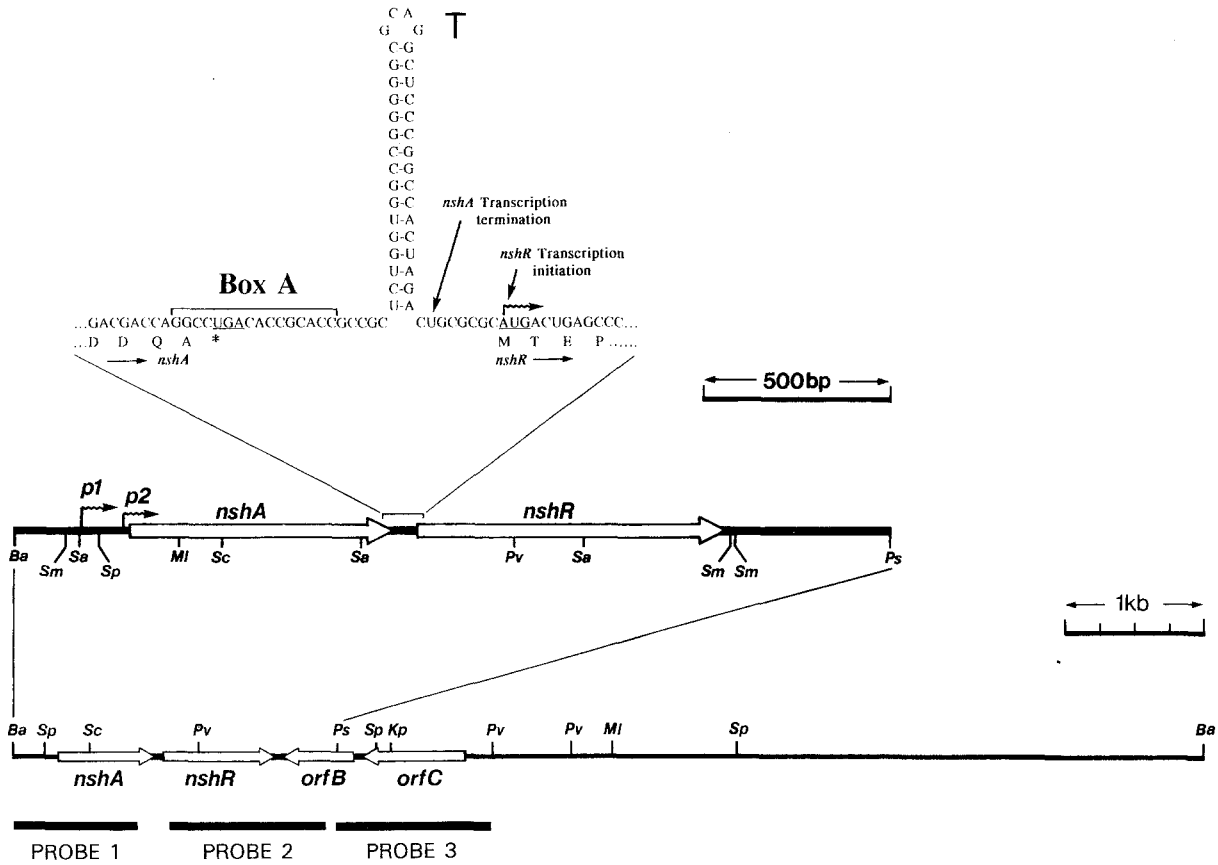

Fig. 2. Restriction map of 8.5 kbp DNA fragment, containing the nosiheptide resistance gene, isolated from *S. actuosus*. The region from the left-hand *Bam*HI site to the *Pvu*II site directly after *orfC* has been sequenced. The sequence of the 2326 bp fragment containing *nshA*, *nshR*, and a part of *orfB* has been published elsewhere [48]. The sequence of the 3′ end of *nshA*, the Box A region identified as a potential antiterminator binding site, the terminator 'T', the *nshA* transcription termination site, and the *nshR* combined transcription and translation initiation sites are identified in the top inset. The probes for hybridization of nosiheptide related genes from *S. actuosus* to DNA from other streptomycetes are identified by bars at the bottom of the figure. Transcriptional initiation sites (*nshA-p1*, *nshA-p2*) are indicated by wavy arrows. Abbreviations for restriction endonuclease recognition sites shown: *Ba*, *Bam*HI; *Sm*, *Sma*I: *Sa*, *Sal*I; *Sp*, *Sph*I; *Ml*, *Mlu*I; *Sc*, *Sac*I; *Pv*, *Pvu*II; *Ps*, *Pst*I; *Kp*, *Kpn*I.

to the ORFs surrounding *nshR*, DNA probes of *nshA* (Probe 1; Fig. 2), *nshR/orfB* (probe 2; Fig. 2), and *orfB/orfC* (Probe 3; Fig. 2) from *S. actuosus* were used to hybridize DNA isolated from other nosiheptide-producing streptomycetes, streptomycetes that produce thiopeptides other than nosiheptide, and streptomycetes that do not produce thiopeptide antibiotics (Table 1). The data indicated that all of the thiopeptide producers contained DNA that hybridized to the *nshR/orfB* gene probe (Probe 2), only the nosiheptide producers contained DNA that hybridized to the *orfB/orfC* probe (Probe 3), and 27 out of 33 streptomycetes tested, most of which do not produce thiopeptides, contained DNA that hybridized to the *nshA* probe (Probe 1). These data suggest that the thiopeptide resistance genes are relatively similar, as already demonstrated by the similarities between *nshR* and *tsr* [48], and that *orfB* and *orfC* may be genes specifically involved in nosiheptide biosynthesis (but not required for the biosynthesis of other thiopeptides). The data also suggest that *nshA* may be a gene common to most streptomycetes (Table 1), although this must be confirmed with additional hybridizations, since Probe 1 contains both the ORF and the upstream promoter region. The significance of this final inference is discussed later in the section concerning the possible function(s) of *nshA*.

UNUSUAL GENE STRUCTURE OF *nshR*

The *nshR* gene from *S. actuosus* has several unusual features: (i) transcription from *nshR-p* and translation of *nshR* appear to be initiated from the same nt (Figs. 2, 3);

TABLE 1

Homology of genes from streptomycetes to probes derived from *nshA*, *nshR/orfB*, and *orfB/orfC* genes isolated from *S. actuosus*[a]

Streptomycete strain	Antibiotic produced	Homology to probes		
		Probe 1 (*nshA*)	Probe 2 (*nshR/orfB*)	Probe 3 (*orfB/orfC*)
S. actuosus	nosiheptide	+[b]	+	+
Streptomyces 31463	nosiheptide	+	+	+
S. glaucogriseus	nosiheptide	+	+	+
S. azureus	thiostrepton[c]	+	+	−
S. hawaiiensis	thiostrepton[c]	+	+	−
S. laurentii	thiostrepton[c]	+	+	−
S. sioyaensis	siomycin[c]	+	+	−
S. coelicolor A3[2]	actinorhodin[d]	+	−	−
S. lividans TK24	actinorhodin	+	−	−
S. peucetius	daunomycin	+	−	−
Other streptomycetes	non-thiopeptide antibiotics[e]	19/27[f]	0/27[f]	nd
Escherichia coli	np	−	−	−
Pseudomonas aeruginosa	np	−	−	−

[a] See Fig. 2 for the DNA covered by each probe.

[b] Symbols and abbreviations: +, hybridization occurred at a stringency allowing for ca. 80% homology; −, no hybridization observed at a stringency allowing for ca. 80% homology (stringency conditions were two washes for 30 min each at 68 °C with 0.5 × SSC and 0.2% sodium dodecyl sulfate). nd, not done; np, none produced.

[c] Non-nosiheptide, thiopeptide antibiotics.

[d] Also produces the antibiotics undecylprodigiosin, methylenemycin, and a Ca^{2+}-induced antibiotic.

[e] Several strains were tested, of which none produced thiopeptide antibiotics.

[f] Number of strains showing hybridization at stringency designed to allow ca. 80% homology/number of strains tested. DNA from an additional four of the negative strains showed homology to Probe 1 at a lower stringency.

(ii) upstream from *nshR* is an open reading frame of 699 nt (*nshA*), transcribed in the same direction, that has amino acid sequences resembling DNA and RNA binding sites; (iii) transcription of *nshR* occurs not only from its own promoter, but also likely from two promoters upstream of *nshA*, which makes *nshR* the only antibiotic resistance

#	Gene	Initiation codon	Protein purified?	Reference
1.	*nshR*	5′-CGC AUG ACU GAG CCC GCC AUC AUC ACG-3′	no	[48]
2.	*aacC7*	5′-UCC AUG GAC GAA CUC GCC UUG CUC AAG-3′	no	[49]
3.	*rph*	5′-ACC AUG GAA AGC ACG UUG CGC CGG ACA-3′	yes	[37]
4.	*aph-p1*	5′-CCC AUG GAC GAC AGC ACG UUG CGC CGG-3′	yes	[42]
5.	*afsA*	5′-ACU AUG GAC GCG GAG GCC GAG GUG GUG-3′	no	[36]
6.	*sta*	5′-GUC AUG ACC ACG ACC CAT GGC AGC ACG-3′	yes	[34]
7.	*ermE-p1*	5′-GCG GUG AGC UCG GAC GAG CAG CCG CGC-3′	no	[9]

Fig. 3. N-terminal nucleotide sequences of streptomycete genes which are proposed to be transcribed and translated from the same initiating nucleotide. Rows: 1. *nshR-p*, promoter of nosiheptide resistance gene [48]; 2. *aacC7-p*, promoter for aminocyclitol acetyltransferase from *S. rimosus* forma *paromomycinus* [49]; 3. *rph-p*, promoter of ribostamycin phosphotransferase from *S. ribosidificus* [36]; 4. *aph-p1*, the *p1* promoter of the aminoglycoside phosphotransferase gene from *S. fradiae* [8]; 5. *afsA-p*, promoter for *afsA* transcription from *S. griseus* [35]; 6. *sta-p*, promoter for streptothricin acetyltransferase from *S. lavendulae* [33]; and 7. *ermE-p1*, promoter *p1* of the erythromycin resistance gene from *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*) [9]. Underlined nucleotides designate site of transcription initiation. If 'yes' is stated for protein purified, then analysis of the N-terminal amino acid sequences have confirmed the translation initiation site.

gene (of which the authors are familiar) in streptomycetes shown to be transcribed on a polycistronic message [48]; (iv) a hairpin loop separates *nshR* from *nshA* (Fig. 2), although substantial readthrough of this structure occurs throughout most of the growth cycle (Fig. 4); and (v) *nshA-p1* is active only between 44 h and 140 h (with maximal activity at 96 h), indicating a temporal regulation of this promoter (Fig. 4).

S1 nuclease protection experiments indicate that the transcription of the *nshR* is initiated at the first nt of the AUG which initiates translation (Fig. 3). The initiation of transcription at, or very near, the translational initiation codon has been observed with six other streptomycete genes: streptothricin acetyltransferase (*sta-p*) from *S. lavendulae* [33], promoter *p1* of the erythromycin resistance gene (*ermE-p1*) from *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*) [9], the *p1* promoter of the aminoglycoside phosphotransferase gene (*aph-p1*) from *S. fradiae* [8], ribostamycin phosphotransferase (*rph-p*) from *S. ribosidificus* [36], aminocyclitol acetyltransferase (*aacC7-p*) from *S. rimosus* forma *paromomycinus* [49], and *afsA* from *S. griseus* [35] (Fig. 3). The transcription initiation sites were characterized using S1 nuclease protection experiments and (or) in vivo promoter probe analyses (Fig. 3). Additionally, in vitro run-off transcription analyses of *aph-p1* [42] and *ermE-p1* [9] confirmed that those transcripts were not the result of mRNA degradation. Edman degradation of the purified *rph* [36] and *sta* [33] gene products also confirmed the N-terminal amino acid sequence as that which was deduced from the nt sequences. Other prokaryotic genes in which transcription and translation are initiated from the same or nearby nts include transcription of the lambda *cI* gene from the *prm* promoter [59], the *tetR* gene in transposon Tn*1721* [44], and the bacteriorhodopsin (*bop*); [26], halo-opsin (*hop*); [11], and bacteriorhodopsin-related (*brp*) [6] genes in *Halobacterium halobium*. Recently, translation initiation-stimulating regions were found at nts + 15 to + 25 downstream of the AUG in the bacteriophage T7 gene 0.3, further indicating the plausibility of this unusual gene structure [63]. This type of gene structure displays an unusual relationship between transcription and translation which is strikingly different from the common promoter-ribosome binding site-translation initiation sequence of most prokaryotic genes [28].

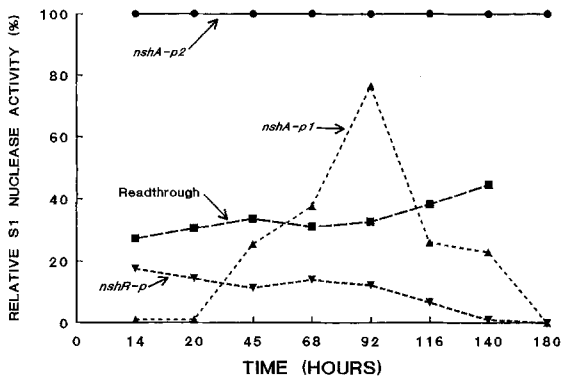

Fig. 4. Time course experiments demonstrating the relative transcription activities of *nshA-p1*, *nshA-p2*, *nshR-p*, and readthrough of the terminator, T, from S1 nuclease protection experiments. The S1 nuclease protection experiments were carried out by the methods of Murray [53], which virtually eliminate DNA-DNA hybrids. The *nshA-p1* and *nshA-p2* activities were compared to the readthrough and *nshR* activities by comparing data on the 3′-termination of *nshA* and the 5′-transcription of *nshR* on the same gels. Autoradiograms of each gel were scanned using the DELL Microsoft System 310 program with the Microscan 100 gel analysis system. Activities are designated as follows: *nshA-p2* activity (●) was designated as the 100% activity to which the other activities were compared; the activity of *nshA-p1* (▲) increased with time, peaked at 96 h, and then decreased; the level of transcript which was detected as readthrough of the terminator T (■); and the activity of *nshR-p* (▼).

The unusual structure of these six streptomycete genes suggests a specialized regulation for them, especially interesting in light of the fact that all of these genes are involved in secondary metabolism, i.e., antibiotic resistance or differentiation (as in the case of *afsA* [35]). Furthermore, the best potential ribosome binding site within the first 100 nt of the *nshR* open reading frame, a GAGC sequence, would normally be considered to be a poor ribosome binding site based on the sequence of the 3′ end of *S. lividans* 16S ribosomal RNA [48]. The *sta* [33], *rph* [36], and *aacC7* [49] genes also lack typical ribosome binding sites. The *aph* [42] and *rph* [36] genes are strongly expressed in *S. lividans*, however, indicating that *S. lividans* can effectively translate this unusual gene structure.

The organization of the sequences upstream of *nshR* [48] is similar to the organization of the 5′-regions of *sta* [33] and *aacC7* [49]. All three genes are preceded by stem-loop structures, and by another unknown open reading frame reading in the same direction. In the case of *nshR*, at least some of the transcription originates from the *nshAp1/nshAp2* promoters, which may be important physiologically due to the apparently poor ribosome binding site in the 5′ region of *nshR*. Furthermore, *nshA* and *nshR* are separated by only 62 nt, suggesting that the major promoter activity driving the expression of *nshR* may be from the P1/P2 promoter regions of *nshA*, with readthrough to *nshR* (Fig. 4). Experiments carried out to determine readthrough of the transcription termination site of *nshA* indicated that a significant amount of readthrough occurs, even though a stem-loop with a large

$-\Delta G$ (-24 kcal/mol) was present between the genes [48]. This would suggest that an antitermination mechanism may play an important role in the regulation of *nshR* expression in vivo. Distler et al. [24] have previously proposed that antitermination may play a role in the regulation of expression of the *S. griseus* streptomycin resistance gene (*aphD*), a gene which is also preceded by a stem-loop structure [58]. They suggest [58] a model in which the product of the gene preceding *aphD* may act as an antiterminator to allow transcription through the stem-loop preceding the resistance gene. The *sta* [33] and *aacC7* [49] genes, on the other hand, were transcribed from their own promoters in *S. lividans* without the requirement of their respective preceding genes. No readthrough from the upstream gene was observed in S1 nuclease experiments on the *aacC7* gene [49], indicating that even though these generalized gene structures are similar, the transcription and regulation patterns may be different. Comparison of the deduced amino acid sequences of *nshA* (Fig. 1; [48]), the gene preceding *sta*, *orf372* [33], and the gene preceding *aacC7*, *orf357* [49], indicates that the proteins do not have significant similarities (data not shown).

It thus appears that *nshR* [48], as well as *sta* [33], *ermE* [9], *aph* [42], *rph* [36], *aacC7* [49], and *afsA* [35], may form a unique class of streptomycete genes that have the following general characteristics in common: (i) transcription and translation are initiated from the same nt (with at least one of their promoters); (ii) most of them are preceded by stem-loop structures and by open reading frames that may encode putative regulatory proteins; (iii) they are involved in secondary metabolism; and (iv) they typically have very poor ribosome binding sites that are within the translated regions of the transcript, a structural feature not considered normal gene structure.

TRANSCRIPTIONAL ANALYSES

One feature of streptomycete genes that has been found repeatedly is the presence of multiple promoters for a single gene which differentially directs its transcription [13,17,31]. Data generated thus far with cloned *Streptomyces* genes suggest that there is a consensus for '*E. coli*-like' promoters, in which two relatively conserved hexameric sequences are separated by 18 nt (TTGaca-18 nt-tAGgaT-6-8 nt-N [17,31]). Of 69 streptomycete promoters recently surveyed, 24 had promoter sequences falling into this class [2]. Nevertheless, a wide variety of other promoter structures (i.e., 45/69) also was observed [2], which suggests the existence of several different 'classes' of promoters in streptomycetes. Two transcription initiation sites were found upstream of *nshA* by high-resolution S1 nuclease mapping [48]. One transcription initiation site was 27 nt upstream from the ORF which yielded a strong S1 signal and the other was 126 nt upstream of the ORF yielding a weaker signal [48]. Upstream from both of these transcription start sites were sequences that were unusual when compared to the typical '*E. coli*-like' promoter regions of several streptomycete genes [48].

The -25 to -45 region of weaker promoter, *nshA-p1*, shares sequence homology (gGACGg) with promoters *sph-p1* and *sphORF1-p1* of *S. glaucescens* [68] (Fig. 5a). The promoters for the hygromycin B phosphotransferase gene (*hyg-p*) of *S. hygroscopicus* [60], *sapA*, a gene encoding a spore-associated peptide of *S. coelicolor* [29], and

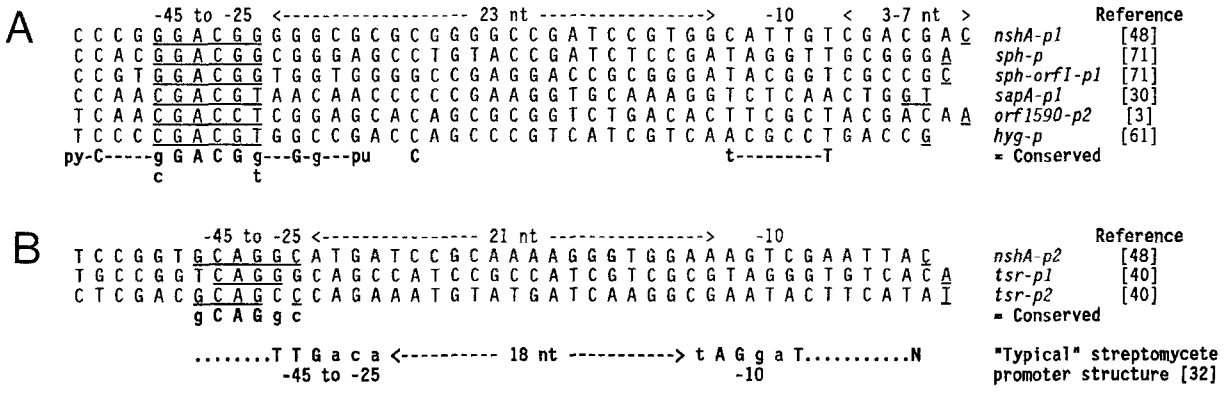

Fig. 5. Comparison of promoters upstream of *nshA* with similar promoters described in the literature. The structure of typical *Escherichia coli*-like streptomycete promoters [31], containing -25 to -45 (TTGaca) and -10 (tAGgaT) regions separated by 18 nt, is shown at the bottom for comparison. A. Structure of promoters *nshA-p1* [48], *sph-p* [68], *sph-orf1-p1* [68], *sapA-p1* [29], *orf1590-p2* [2,3], and *hyg-p* [60]. The -45 to -25 regions of these promoters are highly conserved and may lend to the regulated timing noted for some of these promoters. B. Structure of promoters *nshA-p2* [48], *tsr-p1* [40], and *tsr-p2* [40]. Note the similarities in the -25 to -45 regions and the dissimilarities in the -10 region.

orf1590, a gene involved in differentiation of *S. griseus* [3], also may belong to this group of promoters. The −25 to −45 conserved regions of these promoters are separated from the −10 region by 23 nt (Fig. 5a). The stronger promoter, *nshA-p2*, has significant homology only to the two *tsr* promoters, *tsr-p1* and *tsr-p2* [40], but only in the −25 to −45 regions (gCAGgc; Fig. 5b). The −10 region of *nshA-p2* is different from any other streptomycete promoter yet analyzed [48]. A strong potential ribosome binding site, AGGAGGAGG, immediately follows *nshA-p2*.

S1 nuclease protection experiments for the 3′-end of *nshA* showed that termination occurred at uracil-1061, which was 2 nt after the hairpin loop structure, now designated as terminator T (Fig. 2). In both *nshA* 3′-transcription termination experiments and *nshR* 5′-transcription initiation experiments, probe-length RNase H-sensitive bands were observed that indicated readthrough from a promoter upstream of the 5′ end of the DNA fragment used in the experiment (probably from either *nshA-p1* and/or *-p2* [48]). Additionally, the nt sequence directly upstream of T has strong homology with other 'Box A' sequences of *E. coli* and *Bacillus subtilis* [22,62] known to be sites for the binding of antiterminators (Fig. 7). This strongly suggests that the transcription of *nshR* from *nshA-p1/p2* is actively modulated by an antiterminator protein.

Temporal promoter activities of nshA-p1, nshA-p2, and nshR-p

Time-course S1 experiments were carried out to determine the relative strengths and timing of expression of the two promoters driving *nshA* transcription, readthrough of T, and *nshR-p* activity (Fig. 4). Transcription from *nshA-p2* was observed within 16 h and was strong throughout the course of the experiment. Under the same conditions, transcription from *nshA-p1*, the weaker of the two promoters, was observed only during the period between 44 and 140 h (with a maximum activity at 96 h) and was never as strong as that originating from *nshA-p2* (Fig. 4). This is the time period when *S. actuosus* produces nosiheptide. Thus it is possible that this promoter is part of a regulatory cascade which may be important for secondary metabolic functions such as sporulation and antibiotic production. The promoter of a *S. coelicolor* gene that is turned on at this same relative time, *SAP-p1* [29], contains a similar −25 to −45 region (Fig. 5A).

Transcription from *nshR-p* began immediately in exponential phase and declined steadily thereafter (Fig. 4). Readthrough of T from *nshA-p1/p2* occurred at all times tested, and increased in stationary phase cultures (Fig. 4). The total state level of *nshR* transcript, originating from both *nshR-p* and readthrough of T, was approximately 45% of the transcript level arising from the constitutive promoter, *nshA-p2*. The combined data suggest a high steady-state level of *nshA* transcripts from *nshA-p1* and *nshA-p2*, particularly during the period in which antibiotic biosynthesis occurs (between ca. 68 h and 116 h), and a considerably lower steady state level of *nshR* transcripts from both readthrough and *nshR-p* (Fig. 4).

Analysis of transcription from nshR-p in S. lividans

A plasmid which contained a 1463 bp fragment comprising a small portion of the 3′ end of *nshA* and all of *nshR* conferred resistance to 50 μg/ml of nosiheptide on *S. lividans*, indicating that *nshR* encodes nosiheptide resistance. S1 nuclease protection analysis of this *nshR-p* under these conditions indicated that transcription began at adenosine-1068 (data not shown), as it had in *S. actuosus* [48]. In another experiment, cloning a DNA fragment containing only the 3′ end of *nshA*, *nshR-p*, and the 5′ end of *nshR* upstream of the promoterless *aph* (aminoglycoside phosphotransferase) gene in the promoter-probe vector, pIJ487 [69], yielded high-level neomycin resistance (data not shown). Also, a DNA fragment containing the entire *nshA* gene, both its promoters, and the 5′ end of *nshR* were cloned upstream of *aph* in pIJ486. This construct conferred neomycin resistance on *S. lividans* and strong aminoglycoside phosphotransferase activity was demonstrated, indicating that *aph* was being driven by either *nshA-p1/p2* or *nshR-p* (data not shown). S1 nuclease protection experiments of the 5′-end of *nshR*, carried out to determine the location of transcription initiation sites, indicated that transcription from *nshR-p* in *S. lividans* under these conditions also initiated at adenosine-1068 (Fig. 6). Under all conditions tested, termination occurred at the same nt (uracil-1061) as it did in *S. actuosus*; no full-length mRNA-protected probe was found (Fig. 6), indicating that there was no detectable readthrough from the *nshA* promoters in these experiments (Fig. 6). This suggests either that *S. lividans* is devoid of some factor, present in *S. actuosus*, which assists in or accomplishes antitermination at T between *nshA* and *nshR*, or that *S. lividans* possesses a factor (absent in *S. actuosus*) which causes termination and does not allow readthrough.

ANALYSIS AND POTENTIAL FUNCTION(S) OF NshA.

Regulatory genes that control the activity of antibiotic biosynthesis genes in streptomycetes generally fall into two categories, pathway-specific genes which are clustered with the biosynthetic genes that they regulate (e.g., *act*II [30], *milb* II [38], *redD* [54], *mmy* [15], *brpA* [1], *strR* [24], and *dnrR1/dnrR2* (C.R. Hutchinson, personal com-

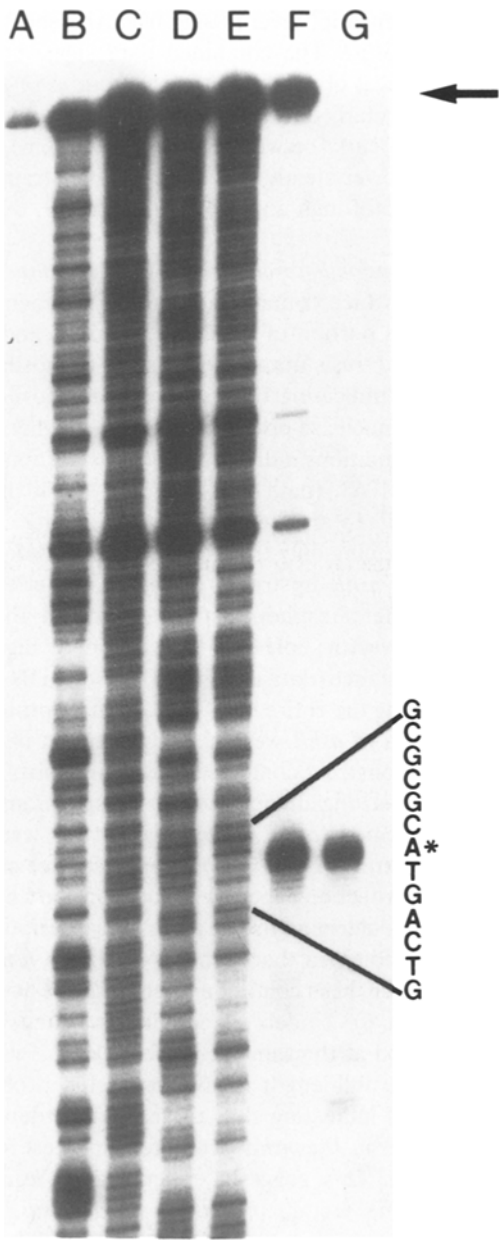

Fig. 6. S1 nuclease protection analysis, using the procedures of Murray [53], to map the 5′ transcription initiations site of *nshR* in *S. actuosus* vs. *S. lividans*. The labeled DNA fragment used to protect the transcribed mRNA was a 432 nt fragment spanning from nt 945 to an *Xma*III site at nt 1377 [48]. The DNA was sequenced by the method of Maxam and Gilbert [50] to identify the location of the transcriptional start site(s). Lanes: A, probe control; B, Maxam-Gilbert C sequence reactions; C, Maxam-Gilbert T + C sequence reactions; D, Maxam-Gilbert G + A sequence reactions; E, Maxam-Gilbert G sequence reactions; F, DNA fragment protected by RNA isolated from *S. actuosus* ATCC 14921 (72 h of growth); G, DNA fragment protected by RNA isolated from *S. lividans* TK24(pANT415) (72 h of growth). Plasmid pANT415 consists of the entire *nshA* gene, the terminator, T, and the 5′ end of *nshR*. Note that transcription of *nshR* begins at the first nucleotide of the ATG translation initiation codon in both organisms. The arrow indicates the approximate probe-length RNA-DNA hybrid formed as a result of readthrough of the termination site of *nshA* in *S. actuosus*; this readthrough transcript was not found in S1 nuclease protection analyses of *nshR* transcription in *S. lividans*.

munication), and 'pleiotropic' genes which are usually not clustered with antibiotic biosynthesis genes (e.g., *afsA* [35], *afsB* [34], *afsR* [65], *whiG* [16], *bldA* [46]). The position of *nshA* directly upstream from *nshR* suggests for it a role in nosiheptide biosynthesis, since it has been widely found in streptomycetes that antibiotic resistance genes are located within antibiotic biosynthesis gene clusters (for that antibiotic). The amino acid sequence deduced from the nt sequence of *nshA* predicts a basic protein (p*I*, 8.92) containing 233 amino acids with a M_r of 25006. A striking feature of *nshA* is the presence of a potential α-helix-β-turn-α-helix structure in the middle region of the gene [48]. NshA also has an arginine-rich sequence of amino acids that is remarkably similar to a recently described prokaryote RNA-binding motif found in prokaryotic proteins [47] (L V R K H E R R A R T (conserved amino acids are underlined)). The significance of both of these possible regulation-related domains, however, remains clouded until experiments can be carried out to prove the potential function of NshA.

We recently generated a mutated form of *nshA* in a pIJ101-derivative plasmid that became integrated into the genome of *S. actuosus* after protoplast-curing of the plasmid. Southern blot analysis of strains altered phenotypes confirmed that we had generated the desired deletion in the chromosomal *nshA* gene of two candidate mutants. The *nshA* mutants (*S. actuosus-nshA*$^-$) initially overproduced nosiheptide and upon complementation with wild type *nshA*, normal nosiheptide production levels were regained. After about five transfers, the *nshA*$^-$ strains reverted to low or no nosiheptide production. Restriction mapping of these revertants indicated a stable mutant genotype, suggesting that an intergenic second-site mutation (suppressor mutation) was responsible for the reversion. The combination of the p*I* of NshA, its location, its partial co-transcription with the *nshR*, the presence of a putative DNA binding site, and the unusual data obtained by its inactivation appear to suggest that this protein may be a negative regulator of nosiheptide biosynthesis that, when inactivated, causes an unstable genetic condition that results in a suppressor mutation. Chater pointed out recently that most genes involved in regulating antibiotic biosynthesis operate via activation mechanisms, not negative regulatory mechanisms [14]. Nevertheless, the

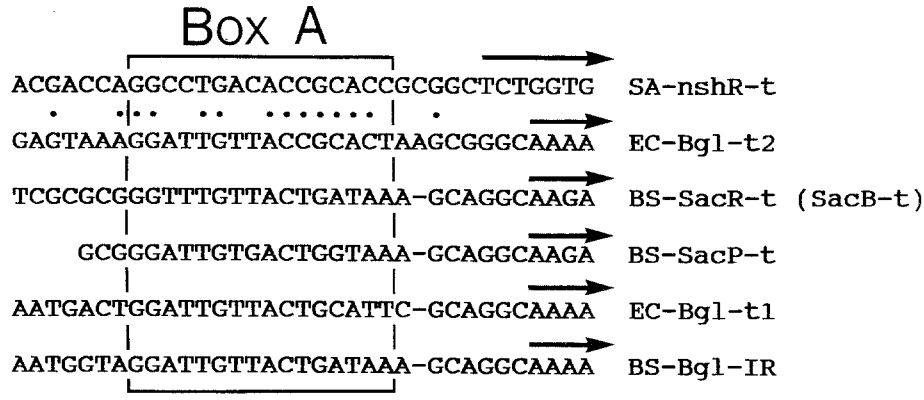

Fig. 7. Nucleotide sequences of the regions upstream from terminator stem-loop structures indicating the conserved 'Box A' region thought to be the binding site for antiterminator proteins [22,63]. Abbreviations: SA-nshR-t, *S. actuosus nshR* terminator (see also Fig. 2); EC-Bgl-t2, *Escherichia coli bgl* operon, terminator t2 [63]; BS-SacR-t, *Bacillus subtilis SacR* terminator [63], also recently designated as *SacB* terminator [22]; BS-SacP-t, *B. subtilis SacP* terminator [22]; EC-Bgl-t1, *E. coli Bgl* operon terminator t1 [63]; and BS-Bgl-IR, *B. subtilis Bgl* operon inverted repeat sequence [63]. The SA-nshR-t Box A has the highest homology with EC-Bgl-t2 Box A (11/16 nt matching) as indicated by the dots. The arrows indicate the beginning of the inverted repeat sequences which can form secondary RNA terminator stem loop structures.

nshA gene probe hybridized with DNA isolated from 29 out of 37 streptomycetes tested at high stringency (> 80% homology; Table 1), and from an additional four strains when the stringency was lowered to that allowable for hybridization at ca. 70% homology (two washes for 30 min each at 37 °C in 0.5 × SSC and 0.1% SDS; [70]).

In control experiments, the *nshR* gene probe was found to hybridize only to DNA isolated from *S. actuosus* and other organisms which produce thiopeptide antibiotics. Although inactivation of *nshA* clearly affects nosiheptide biosynthesis, these preliminary hybridization data suggest that *nshA*-homologs are genes common to streptomycetes in general, rather than being a gene specific to nosiheptide biosynthesis. Recently we have cloned the *nshA* homolog from the thiostrepton producer, *S. azureus*. Future experiments with *nshA* and its homologs will be aimed at determining the potential regulatory role that these genes may play in antibiotic biosynthesis in streptomycetes.

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NOTE ADDED IN PROOF

Two additional streptomycete genes have been described in which transcription and translation are initiated at the same nt. These are the *cat* gene of *Streptomyces acrimycini* (Murray et al. 1989; Gene 85: 283–291) and *korB* of plasmid pIJ101 (Stein et al., 1989; J. Bacteriol. 171: 5768–5775). These two examples were accidentally ommitted from the text.

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