RNase III Is Required for Actinomycin Production in Streptomyces antibioticus

Junghoon Lee, Emory University
Marcha L. Gatewood, Emory University
George H Jones, Emory University

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Using insertional mutagenesis, we have disrupted the RNase III gene, rnc, of the actinomycin-producing streptomycete, *Streptomyces antibioticus*. Disruption was verified by Southern blotting. The resulting strain grows more vigorously than its parent on *S. antibioticus* with the wild-type *S. coelicolor* mutant. Specifically, they reported that the production of undecylprodigiosin (encoded by the *absB* gene) was reduced to only 2% of the normally observed level and antibiotic levels were even lower in an RNase III disruptant than in C120 (11), resulting in a change of leucine to proline in the RNase III amino acid sequence (11). They demonstrated further that antibiotic levels were even lower in an RNase III disruptant than in C120 (11), a result that was recently confirmed (12). We have shown that the *absB* gene identified by Champness et al. does indeed encode a double-strand-specific endoribonuclease, viz. an RNase III (13). It was somewhat surprising to find that an RNase was involved in the regulation of antibiotic production in *S. coelicolor*. The question, then, was whether this is an isolated phenomenon, restricted to that organism, or whether RNase III can play this regulatory role more generally. To answer this question, we have examined actinomycin production in *Streptomyces antibioticus* and in a mutant strain in which the RNase III gene, *rnc*, was disrupted. Our studies show that RNase III is required for actinomycin production in *S. antibioticus*.

**MATERIALS AND METHODS**

Growth of organisms. *S. antibioticus* IMRU 3720 was grown on NZ-amime and galactose-glutamic acid (GGA) medium as described previously (14, 29). NZ-amime cultures were inoculated with spores and were grown for 36 to 48 h to obtain inocula for GGA medium. Apramycin (50 μg/ml) was added to some NZ-amime cultures containing the disrupted *rnc* gene. *E. coli* strains XL-1 Blue (Stratagene, La Jolla, CA) and ET12567/ pUZ8002 were grown on L broth containing antibiotics as necessary. Conjugation mixtures were plated on SFM agar (15). Growth of the various strains was measured as mycelial dry weight as described previously (16).

Cloning and disruption of the *S. antibioticus* RNase III (rnc) gene. A fragment bearing the *S. antibioticus* rnc gene was identified by Southern blotting of a BamHI/Smal digest of chromosomal DNA. The *S. coelicolor* RNase III gene was used as a probe for the blotting experiments. A mini-library of fragments of 0.5 to 3 kb from a large-scale BamHI/Smal digest was cloned into pBluescript SK+ (Stratagene), and a positive clone was identified by colony hybridization, again using the *S. coelicolor* RNase III gene as a probe. The resulting plasmid was designated pSE1900. Sequencing of this construct verified the presence of the *rnc* gene within the insert of pSE1900.

The 831-bp *rnc* gene itself was disrupted in the *S. antibioticus* chromosome by insertional mutagenesis (16, 17). A 724-bp internal fragment of the gene was synthesized using the PCR, with primers 5′-CTCGAGCCGC AAAGAATTTCGGCGGACAA-3′ (forward) and 5′-GCGGCCGCTTGCCG AATTCGCGGCCTCG-3′ (reverse). That fragment, with EcoRI sites at its
5' and 3' ends (underlined in the primer sequences above), was digested with EcoRI and cloned into EcoRI-digested pKC1132 (18). The resulting construct, designated pJSE1980, was used to transform E. coli ET12567/pUZ8002 and was then transferred to S. antibioticus by conjugation as described previously (16). pKC1132 bears an apramycin resistance gene and cannot replicate in S. coelicolor. Thus, apramycin resistance can only be acquired by exconjugants via homologous recombination with the insert borne by the vector. Disruption of the rnc gene by single crossover of pJSE1980 with the chromosome was verified by Southern blotting using the S. antibioticus rnc gene as a probe. A PCR fragment representing the rnc gene was synthesized using the primers 5'-AGGTCGTACGCCGC ATATGAGAGGC-3' (forward) and 5'-CTGTGCGGATCCTCGTCAGG CGGA-3' (reverse) and pJSE1900 as the template. This fragment contained restriction sites for NdeI and BamHI, underlined in the primer sequences shown above.

To complement the disrupted rnc gene, a wild-type copy of the S. antibioticus rnc gene, cloned in the Streptomyces expression vector pIJ8600 to yield pJSE1995, was transferred to the rnc disruptant strain, JSE1980, by conjugation from E. coli. pIJ8600 bears the thiostrepton-inducible tipA promoter, situated upstream of a multiple-cloning site (19). The multiple-cloning sites include restriction sites for NdeI and BamHI, which allowed the cloning of the S. antibioticus rnc gene from the PCR product described above into pIJ8600. As a control, pIJ8600 was transferred to the rnc disruptant strain by conjugation from E. coli. GGA cultures of strains containing pJSE1995 and pIJ8600 were grown in the presence of 15 μg/ml of thiostrepton to induce the tipA promoter.

Measurement of mRNA half-lives. Half-life measurements were performed essentially as described previously (20). Briefly, cultures of S. antibioticus IMRU3720 and its derivatives were grown for 12 h on GGA medium, when 20-ml portions were removed and incubated for 5 min with 75 μg/ml of [3H]uridine (31.9 Ci/mmol) (DuPont NEN). At the end of this incubation, rifampin was added to the cultures to a final concentration of 500 μg/ml and incubation was continued. Duplicate 1-ml samples were removed and placed into 0.2 ml of 50% trichloroacetic acid (TCA) at 0, 2.5, 5, 7.5, and 45 min following rifampin addition. TCA samples were collected as described previously (20), and the numbers of cpm in mRNA were calculated by subtracting the cpm values obtained at 45 min, which represent stable RNAs, from the values obtained at the earlier time points (21). Half-lives were determined by regression analysis of the decay data.

Miscellaneous procedures. Southern blotting was performed as described previously using the cloned rnc gene as a probe (16). Mycelial extracts were prepared by sonication, and Western blotting was performed using the Bio-Rad Opti-4CN kit (Bio-Rad, Hercules, CA), with a 1:5,000 dilution of affinity-purified polyclonal antibody to S. coelicolor RNase III. Actinomycin concentrations were estimated by extraction of culture medium with ethyl acetate. The absorbance of the extracts was then determined at 443 nm, and actinomycin concentrations were calculated using an extinction coefficient of 24,800 (22).

Nucleotide sequence accession number. The rnc sequence has been deposited in GenBank under accession no. KF479205.

RESULTS AND DISCUSSION

Cloning and disruption of the RNase III (rnc) gene of S. antibioticus. To examine the possibility that RNase III regulates antibiotic production in Streptomyces species other than S. coelicolor, we chose an organism that produces an antibiotic that is unrelated to those synthesized by S. coelicolor, viz., actinomycin produced by S. antibioticus. The actinomycins are chromopeptide antibiotics whose peptide chains are synthesized by nonribosomal peptide synthetases (23). S. antibioticus does not produce antibiotics related to the four major antimicrobial compounds synthesized by S. coelicolor, nor does S. coelicolor produce actinomycins. Moreover, a recent phylogenetic study of the genus Streptomyces indicates that S. coelicolor and S. antibioticus are not closely related species within the genus (24).

We cloned a BamHI/SmaI minilibrary of S. antibioticus chromosomal DNA into pBluescript SK+ and identified a clone containing the rnc gene by colony hybridization using the S. coelicolor rnc gene as a probe. Analysis of the insert from the relevant recombinant plasmid identified a 1.2-kb fragment. DNA sequencing confirmed that the entire 831-bp S. antibioticus rnc gene was contained within that fragment. Comparison of the S. antibioticus RNase III protein sequence with the corresponding proteins from the sequenced genomes of other Streptomyces species revealed a high degree of sequence identity between those proteins. In particular, the nucleotide sequence of S. antibioticus RNase III is 96% identical to its counterpart from S. coelicolor and the two proteins are 91% identical and 94% similar. Most of the sequence variation between the two proteins is confined to the C-terminal ends.

The S. antibioticus rnc gene was disrupted in the S. antibioticus chromosome by insertional mutagenesis (16, 17). A 724-bp internal fragment of the gene (Fig. 1) was synthesized using the PCR, and that fragment, with EcoRI sites at its 5' and 3' ends, was cloned into EcoRI-digested pKC1132 (18). The resulting construct, designated pJSE1980, was transferred to S. antibioticus by conjugation from Escherichia coli, as described previously (16). pKC1132 cannot replicate in Streptomyces (18), so the isolation of apramycin-resistant exconjugants in this experiment resulted from single crossover of the marked rnc fragment into the S. antbioticus chromosome.

Disruption of the rnc gene by single crossover was verified by Southern blotting as shown in Fig. 2. In these experiments, we examined disruptant cultures grown on NZ-amine in the presence or absence of apramycin to determine the stability of the disruption in the absence of selection. GGA cultures did not contain apramycin. We also examined DNA from cultures grown for various lengths of time: viz. 19 h when cultures were growing vegetatively and not producing actinomycin and 60 h after actinomycin production had begun. As shown in Fig. 2, a hybridizing band was observed in a BclI digest of DNA from the parental strain, S. antibioticus IMRU3720, while a larger band was obtained from disruptant strains grown under various conditions. The larger band reflects the incorporation of pJSE1980 into the chromosome of S. antibioticus by homologous recombination. Figure 2 shows that

FIG 1 Schematic representation of the rnc gene of S. antibioticus, showing several restriction sites in the gene. The line shown below the schematic of the gene represents the internal fragment used for insertional mutagenesis. The diagram is drawn to scale.

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the integration of the recombinant plasmid was stable over several days of growth in liquid medium and in the presence or absence of apramycin in the NZ-amide growth medium.

**Measurement of growth and antibiotic production in disrupted and control strains.** Mycelial growth and actinomycin production for JSE1980 are shown in Fig. 3 in comparison with the parental strain. In these experiments, JSE1980 was cultured on NZ-amide medium, in the presence of apramycin, prior to the inoculation of production medium (GGA medium) as described previously (16). Strains were grown from NZ-amide inocula on GGA medium. The NZ-amide cultures were grown without apramycin. Strains JSE1980/pJSE1995 and JSE1980/pIJ8600 were grown in the presence of 15 μg/ml thioestrepton to induce the tipA promoter.

Although the sequence of the *S. antibioticus* genome has not yet been determined, in *S. coelicolor* rnc is a part of an operon that includes the gene encoding ribosomal protein L32 (sco5571) and two genes of unknown function (12). The gene downstream of the *S. coelicolor* rnc gene is transcribed in the same direction as the rnc operon. Thus, it was essential to demonstrate that the decrease in actinomycin production and the other changes in phenotype that accompanied disruption of *rnc* in *S. antibioticus* were due to the loss of RNase III rather than to polar effects on downstream genes or effects on other genes in the operon. To this end, the wild-type *S. antibioticus* rnc gene was cloned in the overexpression vector, pIJ8600, as described in Materials and Methods. Complementation of JSE1980 with pJSE1995 restored actinomycin production to nearly wild-type levels (Fig. 3B). In contrast, the JSE1980 derivative containing pIJ8600 produced actinomycin at levels essentially identical to those observed for the disruptant strain. In experiments not shown here, we also attempted to complement the *rnc* mutation in *S. antibioticus* with the wild-type *S. coelicolor* rnc gene and with a mutant form of the gene, which produces a form of RNase III that can bind RNA substrates but cannot cleave them (25). Like the *S. antibioticus* rnc gene, the wild-type *S. coelicolor* gene restored actinomycin production to nearly wild-type levels, while the mutant form was unable to do so (data not shown). This last observation indicates that, as is the case in *S. coelicolor*, the endonuclease activity of RNase III is required for it to exert an effect on antibiotic production in *S. antibioticus*.

We are at this point unable to explain the observation that JSE1980, JSE1980/pJSE1995, and JSE1980/pIJ8600 grew to higher mycelial densities than did the wild-type strain. However, the fact that we observed robust growth of these strains on actinomycin production medium makes it highly unlikely that any defect in...
growth properties is responsible for the failure of JSE1980 and JSE1980/pJSE8600 to produce normal levels of actinomycin.

**Western blotting of mycelial extracts.** The results in Fig. 3 indicate that RNase III is required for the production of normal levels of actinomycin by *S. antibioticus*. The fact that JSE1980 produces some actinomycin might be explained if low levels of RNase III were present in the cultures at the later time points shown in Fig. 3. Low levels of RNase III activity might be produced in either of two ways. The insertion mutagenesis strategy produces two truncated *rnc* genes in the chromosome of the disruptant (17). Intrachromosomal recombination between those two copies could produce an intact and presumably functional copy of the gene. However, Fig. 2 shows that even after 60 h of incubation in actinomycin production medium, when JSE1980 had already started to produce the antibiotic (Fig. 3B), the hybridization pattern was identical to that observed at earlier times. There is no evidence for the formation of an intact RNase III gene in actinomycin-producing cultures of JSE1980.

An alternative explanation for actinomycin production by JSE1980 would involve the formation of partially functional RNase III molecules as a result of the translation of one of the truncated *rnc* derivatives present in the disrupted chromosome (e.g., the 3′ truncation). If this is the case, we would expect to detect a truncated protein at later times in growing cultures of JSE1980. To examine this possibility, Western blotting was performed using polyclonal antibody to *S. coelicolor* RNase III and mycelial extracts of IMRU3720 and JSE1980. The results of this analysis are shown in Fig. 4. Lane 1 shows the reaction of the antibody with authentic *S. coelicolor* RNase III. Lanes 2 to 5 show the reaction with mycelial extracts of IMRU3720 grown on actinomycin production medium for 12, 24, 51, and 72 h after inoculation. The first interesting observation from this analysis is that the expression of *rnc* is temporally regulated in *S. antibioticus*. RNase III was easily detectable in extracts of 12-h mycelium (lane 2) but was present at a considerably lower level in extracts of 24-h mycelium (lane 3) and was undetectable in the mycelium from the 51- and 72-h cultures (lanes 4 and 5). It is noteworthy that our Western blotting data are consistent with results reported by Sello and Buttner for *S. coelicolor*. These authors found that the *rnc* gene was only transcribed during the exponential phase of growth in that organism (12). Thus, in both *S. antibioticus* and *S. coelicolor*, it is possible that whatever role RNase III plays in antibiotic production, that role occurs early on, perhaps even before antibiotic production is initiated.

Lanes 6 to 8 of Fig. 4 show the blotting results for extracts of JSE1980 at 12, 51, and 72 h postinoculation. Except for a very faint band migrating more rapidly than authentic RNase III (lane 6), observed only in the 12-h extract from JSE1980 mycelium (lane 6), there was no evidence of any proteins in JSE1980 with immunological similarity to RNase III.

Given these observations, the most likely interpretation of the results in Fig. 3B is that in older cultures of JSE1980, an RNase III-independent mechanism becomes operative that supports the production of very low levels of actinomycin, even though RNase III is absent. Our results do show clearly that in wild-type IMRU3720, RNase III is normally required for actinomycin production. In a recent study, we have identified a number of genes whose expression is affected by the absence of RNase III. We have proposed a model in which antibiotic production in *S. coelicolor* is affected because of the sheer number of transcripts that are cleaved by RNase III (26). A similar situation may obtain in *S. antibioticus*.

**Measurement of mRNA half-lives.** Babitzke et al. examined the half-life of bulk mRNA in an *rnc* mutant of *E. coli* and found no significant differences in half-life compared with that of the wild-type strain (27). It was of interest to determine whether the same situation obtained in *S. antibioticus*. To this end, the half-lives of pulse-labeled mRNAs were measured in *S. antibioticus* IMRU3720, the *rnc* mutant, JSE1980, and the complemented mutant, JSE1980/pJSE1995, all grown in GGA medium from NZ-amine cultures that were prepared in the presence of apramycin. The results of this analysis are shown in Fig. 5, and it is apparent that there is no significant difference in the half-life measured for the wild-type and *rnc* mutant strains. This result is not surprising given the results from the *E. coli* system and given our recent analysis of the transcriptome of an *rnc* mutant of *S. coelicolor*. That analysis suggests that no more than ca. 10% of the transcripts synthesized during vegetative growth of *S. coelicolor* are substrates for RNase III (26). Thus, the absence of RNase III might not be expected to have a dramatic effect on the half-life of bulk mRNA. Nevertheless, the studies reported here and those from *S. coelicolor* clearly indicate that one or more transcripts, required for
antibiotic production, are processed or degraded by pathways involving RNase III.

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