The EMBO Journal vol.14 no.18 pp.4569–4577, 1995

Activation of alpha-toxin translation in *Staphylococcus aureus* by the *trans*-encoded antisense RNA, RNAIII

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The synthesis of virulence factors in *Staphylococcus aureus* is controlled by a regulatory RNA molecule, RNAIII, encoded by the *agr* locus. Transcription of genes coding for secreted toxins and enzymes is stimulated, while transcription of cell-surface protein genes is repressed by RNAIII. In the case of staphylococcal alpha-toxin, RNAIII also seems to stimulate translation by an independent mechanism. In this report we show that in a mutant lacking RNAIII the rate of alpha-toxin (*hla*) production relative to the cellular concentration of *hla* mRNA was reduced 10-fold as compared with the wild-type strain. A 75% complementarity between the 5′ end of RNAIII and the 5′ untranslated region of the *hla* transcript suggests a direct interaction between the RNAs. A complex of RNAIII and *hla* mRNA was demonstrated in extracts of total RNA from the wild-type strain, and also with *in vitro* synthesized RNAs. Ribonuclease T1 digestion experiments revealed that the ribosome binding site of the *hla* transcript is blocked by intramolecular base-pairing. Hybridization with RNAIII prevents this intramolecular base-pairing and makes the *hla* mRNA accessible for translation initiation. This is, to our knowledge, the first example of an 'antisense RNA' that stimulates translation of the target mRNA.

Keywords: *agr* locus/alpha-toxin/antisense RNA/*Staphylococcus aureus*/translation control

Introduction

The Gram-positive bacterium *Staphylococcus aureus* is a major pathogen, causing abscess formation and severe tissue damage as well as toxin related diseases such as food poisoning and toxic shock syndrome. Virulence has been ascribed to a large number of extracellular toxins and enzymes (i.e. alpha-toxin, hemolysins, leucocidin, proteases, lipase, nuclease and staphylokinase) and to several cell-surface proteins (e.g. protein A, coagulase and fibronectin receptor).

In vitro, the production of the above mentioned exoproteins and cell-surface proteins is regulated such that the former are preferentially synthesized during the post-exponential phase of growth, whereas the latter are synthesized during the exponential phase (Björklind and Arvidson, 1980; Janzon *et al.*, 1986; Recsei *et al.*, 1986; Morfeldt *et al.*, 1988). The primary mediator of this regulation appears to be a polycistronic locus known as the accessory gene regulator, *agr* (Recsei *et al.*, 1986). Recently two distinct loci, *sar* and *xpr*, have been identified, which seem to modulate the activity of the *agr* locus and therefore have a similar effect on exoprotein synthesis to *agr* (Cheung *et al.*, 1992; Smeltzer *et al.*, 1993; Cheung and Projan, 1994). In *agr*$^-$ mutants the production of extracellular toxins and enzymes is reduced to a relatively low constitutive level, whereas the production of cell wall-associated proteins is increased (Recsei *et al.*, 1986; Morfeldt *et al.*, 1988). The effector of this positive and negative regulation appears to be a 0.5 kb RNA molecule, RNAIII or *hld* RNA, encoded by the *agr* locus (Janzon and Arvidson, 1990; Novick *et al.*, 1993). In addition to its function as a regulator, RNAIII is also an mRNA coding for delta-hemolysin (*hld*) (Janzon and Arvidson, 1990). The designation RNAIII will be used henceforth to avoid confusion.

A diagram of the *agr* locus is presented in Figure 1. The locus consists of two divergent transcription units, one coding for the above-mentioned RNAIII and another coding for a 3 kb polycistronic RNA, RNAII (Janzon *et al.*, 1989; Kornblum *et al.*, 1990). Two of the predicted products from RNAII, AgrC and AgrA, correspond to the sensory transducer and response regulator respectively of the classical two-component regulatory systems in bacteria (Stock *et al.*, 1989; Parkinson and Kofoid, 1992). The main function of this signal transduction system seems to be to regulate the synthesis of RNAIII. In wild-type strains the *agr* system is activated in late exponential phase and maximum amounts of RNAIII are seen in the post-exponential phase of growth when synthesis of exoproteins is also maximal (Janzon *et al.*, 1989; Vandenesch *et al.*, 1991). Though the nature of the activation signal of the *agr* system remains obscure, several reports show that activation is inhibited by acid or alkaline pH (Regassa and Betley, 1992; Regassa *et al.*, 1992). Recently, it has been shown that *agr* is autoinduced by a proteinaceous factor produced and secreted by the bacteria (Balaban and Novick, 1995).

The role of RNAIII as the regulating molecule was first suggested by the finding that an insertion in the RNAIII region, that did not impair delta-hemolysin production, resulted in a typical *agr*$^-$ phenotype (Arvidson *et al.*, 1989). This was later confirmed by experiments showing that the cloned RNAIII determinant, defective in delta-hemolysin production, restored both the positive and the negative effects on the expression of virulence genes in an RNAIII deletion mutant (Janzon and Arvidson, 1990), and in an *agr*-null strain (Novick *et al.*, 1993).

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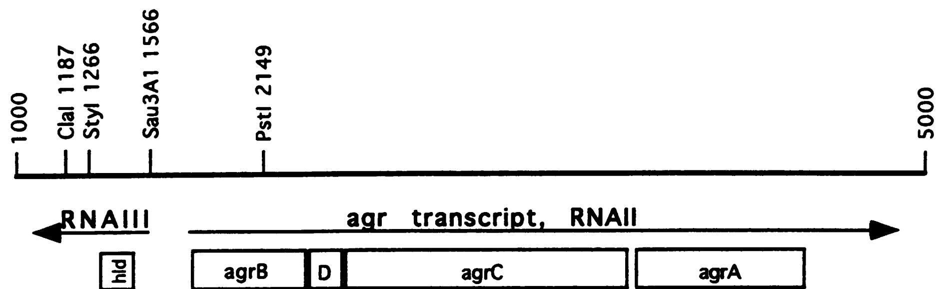

Fig. 1. Organization of the *agr* locus. Schematic map of the *agr* locus showing the major transcripts, RNAII and RNAIII (arrows) and the open reading frames indicated by boxes. The positions of relevant restriction sites are indicated by vertical lines [numbering system according to Kornblum *et al.* (1990)].

RNAIII seems to act primarily on the initiation of transcription of virulence genes, as indicated by transcriptional and translational fusion experiments localizing the *agr*-responsiveness to the promoter regions of target genes (Patel *et al.*, 1992; Novick *et al.*, 1993). Surprisingly, RNAIII also seems to have a positive effect on the translation of some target genes (Novick *et al.*, 1993). A cloned RNAIII that had been deleted in either the 5′ or the 3′ region could still activate transcription of the alpha-toxin gene (*hla*) in an *agr*-null mutant, but translation of the *hla* transcript was totally impaired. This may suggest that RNAIII has two independent functions, one as an activator of transcription and another as an activator of translation. The effect on translation, which is probably due to a direct interaction between RNAIII and the mRNAs of the regulated genes, is the subject of this study.

Here we report that translation of alpha-toxin (*hla*) mRNA is impaired in a mutant lacking RNAIII. In the absence of RNAIII a stem–loop structure is formed within the *hla* mRNA such that the ribosome binding site is blocked. Furthermore we provide evidence that RNAIII binds to the *hla* transcript, both *in vitro* and *in vivo*, in a way that uncovers the ribosome binding site of the *hla* transcript. A model for positive translational control by a *trans*-encoded antisense RNA is presented.

Results

Translational control of alpha-toxin synthesis

It has recently been suggested (Novick *et al.*, 1993) that RNAIII, in addition to its role as a transcriptional activator, also affects translation of the alpha-toxin gene, *hla*, in a positive way. To study the effect of RNAIII on translation of *hla* in more detail we have used a mutant of *S.aureus* (K6812-1) that lacks RNAIII (Morfeldt *et al.*, 1988), but still produces significant amounts of *hla* transcript. The reason why transcription of *hla* is less repressed in this mutant than in the prototype *agr* mutant from strain 8325-4 is not known.

Analyses of *hla* mRNA concentrations and alpha-toxin production in the mutant, *S.aureus* K6812-1, and the corresponding wild-type strain, *S.aureus* V8, at hourly intervals during growth revealed a significant difference in the efficiency of translation of *hla* mRNA between the strains. The differential rate of alpha-toxin synthesis was determined by plotting the toxin concentration versus bacterial density. As seen in Figure 2, a 70-fold difference in toxin production rate was found by comparing the slopes of the two curves. In contrast, measurements of the intracellular concentrations of alpha-toxin mRNA during the period when the difference in the toxin production rate was maximal, revealed a modest 5- to 10-fold difference between the two strains. Since nucleotide sequencing of the 5′ UTR (untranslated region) and the first part of the *hla* gene in the mutant and the wild type showed a complete identity we conclude that translation of the *hla* message was significantly reduced in the mutant due to lack of RNAIII.

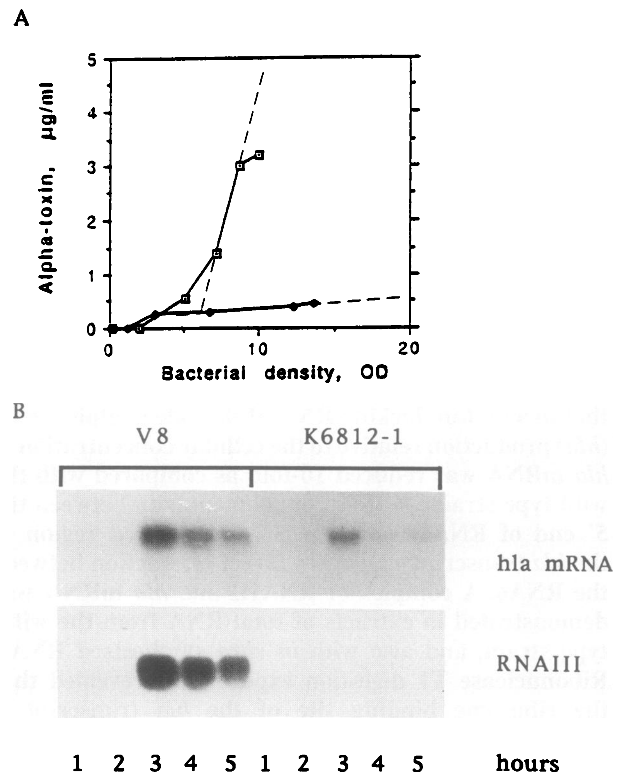

Fig. 2. Alpha-toxin production (**A**) and concentrations of alpha-toxin mRNA (**B**) in *S.aureus* V8 (wild type) and an RNAIII deficient mutant (K6812-1), grown in brain heart infusion broth. (A) Alpha-toxin and bacterial density were measured at hourly intervals and plotted against each other. The slopes of the curves (inserted dotted lines) represent the differential rates of alpha-toxin synthesis. ⊡: V8; ◆: K6812-1. (B) Total RNA was extracted from cells taken at 1, 2, 3, 4 and 5 h and analysed for alpha-toxin mRNA by Northern blot hybridization. Ten micrograms of RNA were loaded in each well. Radioactivity (c.p.m.) was detected by a radioisotope imaging system (Autograph, Oxford Positron Systems Ltd, UK).

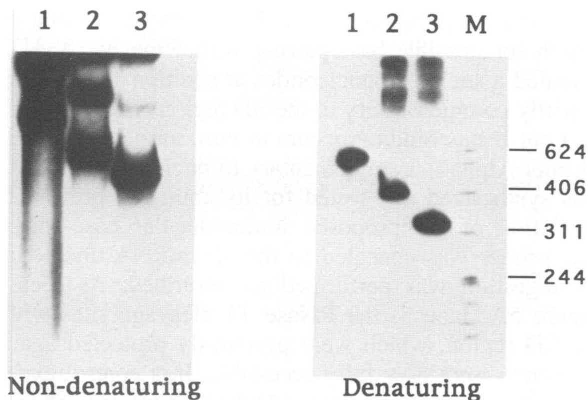

Fig. 3. Analysis of *in vitro* transcribed RNAIII by polyacrylamide gel electrophoresis under non-denaturing and denaturing conditions. Lane 1, full-length RNAIII; lane 2, RNAIII truncated at the *Cla*I site; lane 3, RNAIII truncated at the *Sty*I site. M, size marker, *Hpa*II digested pBR322.

Complex formation of RNAIII and hla transcript in vitro

The most likely way by which RNAIII could affect translation of the *hla* gene would be by direct interaction with the corresponding mRNA. The alpha-toxin mRNA has an unusually long untranslated 5′-region (5′ UTR) of 330 nucleotides, which has been suggested to be the target for RNAIII interaction based on partial complementarity (Novick *et al.*, 1993). For this reason we decided to look for such an interaction *in vitro*. Both RNA species, or sections thereof, were synthesized *in vitro*, using T7 promoter fusion constructs (for construction see Materials and methods). The RNA species were produced by 'run-off' transcription, using templates which were cleaved with appropriate restriction enzymes (see Materials and methods and legend to Figure 1).

When analysed by electrophoresis under non-denaturing conditions RNAIII migrated as two bands, whereas in a denaturing gel it appeared as one band of the expected size (Figure 3). Similar results were obtained with two truncated forms of RNAIII, which were generated by cleavage of the template at the *Sty*I or *Cla*I site respectively (Figure 3). By mixing full-length and truncated RNAIII we could show that two RNAIII molecules can bind to each other and that the slow migrating bands in Figure 3 represent RNAIII dimers (data not shown).

The 5′ UTR of the *hla* gene was subcloned from pDU1148 and fused to a T7 promoter. The sequence of the subcloned fragment was identical to that of the corresponding fragment from *S.aureus* V8. The subcloned fragment contained the entire 5′ UTR, starting at position +1, plus 210 bp of the coding sequence. This construct was used as template in an *in vitro* transcription reaction together with RNAIII templates, cleaved at either the *Sty*I or the *Cla*I site (indicated in Figure 1). When analysed by electrophoresis under denaturing conditions only two bands of the expected sizes were seen in both cases. However, under non-denaturing conditions two new bands were seen in addition to the expected bands (Figure 4A). All bands from the non-denaturing gel were cut out and subsequently analysed by denaturing gel electrophoresis. As seen in Figure 4B, the two upper bands (a and b) contained both RNAIII and alpha-toxin mRNA, indicating

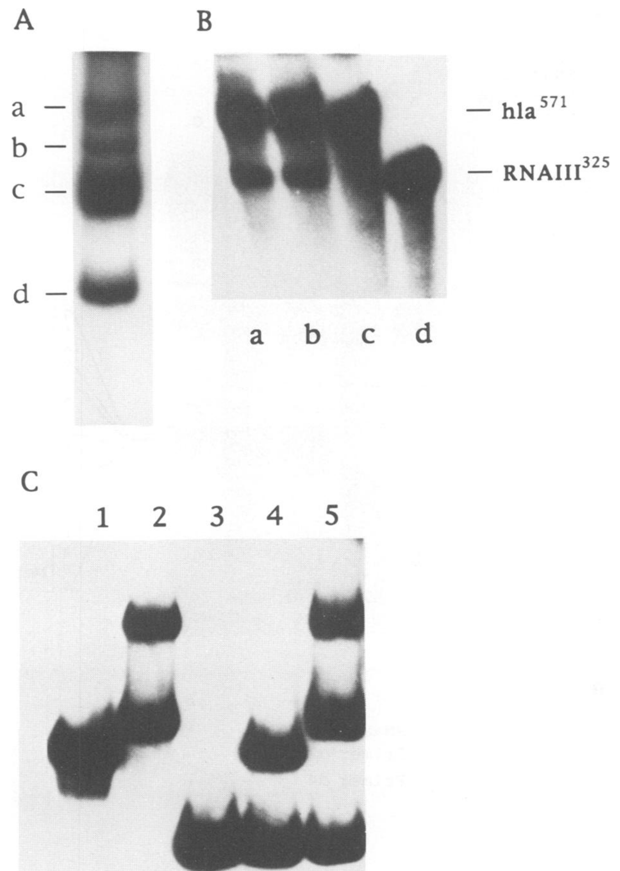

Fig. 4. Demonstration of complex formation between RNAIII and the 5′ untranslated region (UTR) of the alpha-toxin mRNA (*hla*) *in vitro*. RNAIII truncated at the *Sty*I site and the 5′ UTR of *hla* mRNA, using pHLA540 as template, were co-synthesized and analysed by gel electrophoresis under non-denaturing conditions (**A**). The bands marked a–d were excised, the RNA eluted and analysed under denaturing conditions (**B**). The RNAIII and *hla* mRNA species are indicated. As a control RNAIII was synthesized together with *E.coli ompA* mRNA and analysed on a non-denaturing gel (**C**). Lane 1, RNAIII truncated at the *Sty*I site; lane 2, RNAIII truncated at the *Cla*I site; lane 3, 5′ UTR of *ompA* mRNA; lane 4, RNAIII (*Sty*I) plus *ompA* mRNA; lane 5, RNAIII (*Cla*I) plus *ompA* mRNA.

the formation of a complex between the two RNA species, while bands c and d contained one RNA species each. Complex formation was also obtained with the full-length alpha-toxin transcript and the different RNAIII run-off transcripts (data not shown). As a negative control we used the *Escherichia coli* mRNA coding for the outer membrane protein A (*ompA*) (Figure 4C).

We do not understand why the heterodimer of RNAIII and *hla* mRNA migrated as two bands. This could either be due to different conformations of the complex with different electrophoretic mobilities, or that both the monomer and the dimer of RNAIII can bind to the alpha-toxin mRNA.

A stem–loop involving the hla Shine–Dalgarno sequence is released by RNAIII binding.

Based on the ability of ribonuclease T1 to cleave single-stranded RNA at the 3′ side of G nucleotides, base-paired regions (inaccessible to cleavage) of the *hla* leader transcript were mapped. *In vitro* transcribed *hla* leader transcript was digested with RNase T1 under conditions

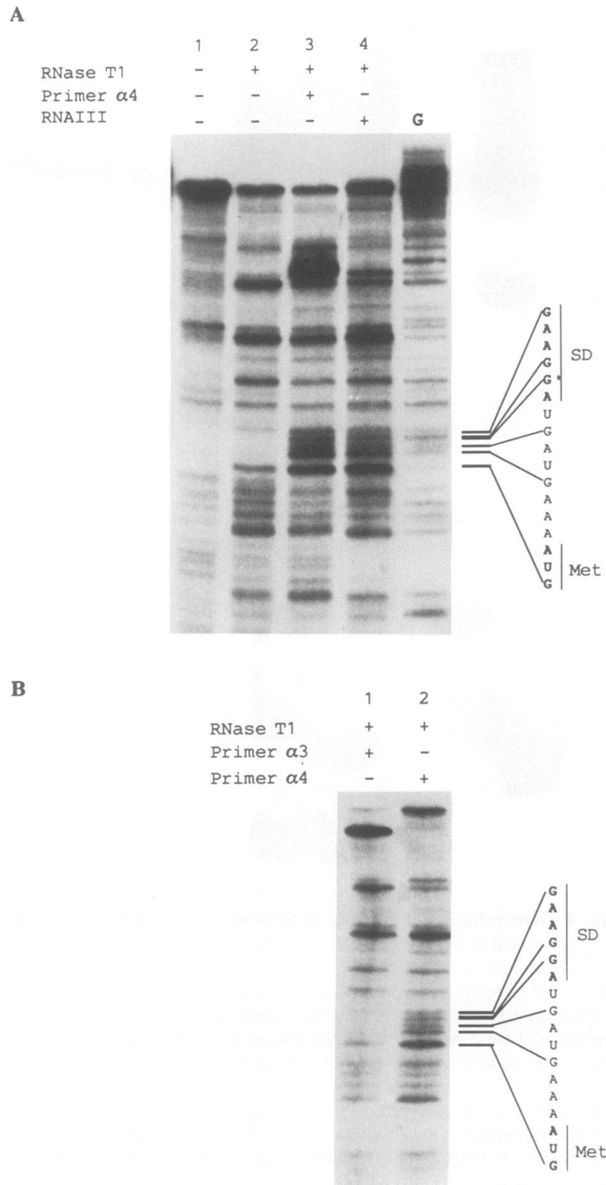

Fig. 5. Partial ribonuclease T1 cleavages of *hla* leader transcript identified by reverse transcription. (**A**) *hla* leader transcript alone (lane 2), in complex with primer Alpha4 (lane 3), RNAIII (lane 4), or (**B**) primer Alpha3 (lane 1) was cleaved with nuclease T1, as described in Material and methods. Cleaved transcripts were probed by reverse transcription using primer Alpha7, and analysed by polyacrylamide gel electrophoresis. A sequencing reaction, obtained with the same primer, is shown in the lane marked with G. (A) Lane 1 shows the reverse transcripts of untreated *hla* leader transcript. G nucleotides within the region of the SD sequence and the start codon are indicated.

where most of the molecules were cleaved only once. The 5′ ends of the T1 cleavage products were mapped by primer extension reactions. The run-off products were compared with those of a sequencing reaction obtained with the same primer. As seen in Figure 5A (lane 2) the G residues at positions 320, 321, 324 and 327, which are part of the Shine–Dalgarno (SD) sequence and the region immediately upstream of the start codon, were inaccessible to the enzyme cleavage, suggesting that this region of the *hla* transcript is base-paired. As seen in lane 1 (Figure 5A) reverse transcriptase generated some shortened transcripts even without previous RNase T1 treatment. A computer search for possible base-pairing within the *hla* 5′ UTR, revealed a stretch of nucleotides at position 179–198 that is partly complementary to the SD region (Figure 6A). To find out if this binding occurs *in vitro* an oligonucleotide (primer Alpha4), complementary to nucleotides 173–198, was synthesized and tested for its ability to prevent the formation of the proposed intramolecular base-pairing. The primer was annealed to the *hla* mRNA and RNase T1 digestion was performed as described. As seen in Figure 5A (lane 3) the RNase T1 cleavage sites within the SD region, which were previously protected against digestion, were now fully accessible. In a control experiment, when another primer (Alpha3), complementary to nucleotides 62–82, was annealed to the *hla* transcript, the SD sequence remained protected against RNase T1 cleavage (Figure 5B).

In a set of similar experiments *hla* leader transcript was mixed with different concentrations of RNAIII before RNase T1 digestion. As seen in Figure 5A (lane 4) the G residues within the ribosome binding region of the *hla* transcript were rendered susceptible to the action of the enzyme. This is consistent with the 5′ end of RNAIII being complementary (77%) with nucleotides 163–198 of the *hla* transcript (Figure 6B), which means that base-pairing between the two RNA molecules prevents the formation of the intramolecular structure encompassing the ribosome binding site.

Complex of RNAIII and the hla transcript in growing bacteria

To demonstrate the presence of a complex between RNAIII and alpha-toxin mRNA in growing bacteria, total RNA was prepared from the wild-type (agr^+) *S.aureus* strain (8325-4) by a method that does not involve hot phenol or other denaturing agents (Kornblum *et al.*, 1988). Samples taken at different times during growth were separated in a non-denaturing gel and analysed by Northern blot hybridization for RNAIII and alpha-toxin mRNA. Multiple bands appeared with both probes. Comparison of the autoradiographs of a filter strip that was first probed for alpha-toxin mRNA, and then for RNAIII, revealed a band indicated by arrowheads in Figure 7, that seemed to react with both probes. To confirm that the two RNA molecules really migrated in a single band, the same sample of total RNA was analysed by a two dimensional electrophoresis procedure, as described in Materials and methods. After electrophoresis the gel was blotted to a nylon filter and probed for RNAIII and alpha-toxin mRNA. As seen in Figure 8, the upper band (same as in Figure 7) in the first dimension was resolved into RNAIII and alpha-toxin mRNA in the second dimension, indicating that these two RNA species bind to each other also *in vivo*.

It should also be noticed that all bands hybridizing with the RNAIII probe after electrophoresis in the first dimension (non-denaturing) had the same mobility in the second dimension (denaturing). This indicates that they were all full-length transcripts with different mobility in the first dimension, due to interaction with different target RNA species, dimer formation, conformational differences or complex formation with proteins that were resistant to proteinase K treatment.

To confirm that the major slow moving band seen in

A

1 AUCGAUUACA UUUUUAAAUC AAACAAAUCA UUAGUUUUAA AGAAAUUAAU CAAUAGAAUU AGCUAUGUCU UUUCCUUGUU UCAUAAAGAA UUUAUUCAAC

Alpha3

101 UUUGACUAAC CCUCGAAAUU GAAAUGCUUC CUUUCAAAUU UUAAAUAAAA GUUAAAAACA UAUUCUUAAA AUAAUCACUC AUCAUCACUC AGUAAUUUAU

Alpha4

201 CAGUUGCUAU AUUAAAUAUA CUAAUAUAAA CGAUUAUAAA UAUUUGAUAU GUCUCAACUG CAAAUAUUCU AAAUUGACAU AUUGAUUAUU GUUUCCUCAA

301 AUCGUUCAAA AAAAUAGAAG GAUGAUGAAA AUGAAAA

SD Met

B

RNAIII

SD Met

15 UGAUGGAAAAUAGUUGAUGAGUUGUUUAAUUUUAAGAGU..........UUUUAUCUUAAUUAAGGAAGGAGUGAUUUCAAUG 87

UUUAAUGACUCACUACUACUCACUAA..UAAAAUUCUUAUACAAAAAUUGAAAAUAAAUUUUAAACUUUCCUUCGUAAAGUUAA 117

AAAAAAUAGAAGGAUGAUGAA AAUGAAAACACGUAUAGUCAG 350

SD Met

hla mRNA

Fig. 6. Intramolecular base-pairing of the *hla* leader transcript and its putative duplex formation with RNAIII (**B**). The SD sequences and start codons of *hla* (alpha-toxin) and *hld* (delta-lysin) respectively are indicated. Numbering of sequences according to the EMBL database (X01645), and Janzon *et al.* (1989). Complementary regions were identified by the sequence analysis software package from the Wisconsin Genetics Computer Group (Deveraux *et al.*, 1984) and the alignments completed manually. (**A**) shows the nucleotide sequence of the *hla* leader transcript with the G nucleotides that were inaccessible to RNase T1 cleavage (see Figure 5) indicated by arrows. Boxes underneath the sequence indicate the primers, Alpha3 and Alpha4, which were used in the RNase T1 digestion experiments.

Figure 7 represents a complex of *hla* mRNA and RNAIII, we also analysed the mutant strain K6812-1 (Figure 9A), which lacks RNAIII. Samples of total RNA, extracted from the mutant and the wild-type strain at hourly intervals during growth, were separated by non-denaturing electrophoresis and probed for RNAIII and alpha-toxin mRNA. As seen in Figure 9B the banding pattern of alpha-toxin mRNA was significantly changed in the mutant as compared with the wild type. Particularly, the upper band in the wild-type strain was absent in the mutant. This is consistent with the lack of RNAIII in the mutant strain, and confirms that the retardation of the upper band in the wild-type strain is due to a complex between *hla* mRNA and RNAIII.

Discussion

In this report we have shown that in a *S.aureus* mutant (K6812-1) lacking the regulator RNAIII, the steady state level of alpha-toxin mRNA was reduced 5- to 10-fold while the differential rate of alpha-toxin production was reduced 70-fold, as compared with the corresponding wild-type strain (V8). These results strongly support the conclusion of a previous study (Novick *et al.*, 1993), that RNAIII activates *hla* gene expression both at the transcriptional and translational level. In the case of the translational activation our data suggest that it is achieved by a direct interaction between RNAIII and the target mRNA.

A complex of RNAIII and the *hla* transcript could be demonstrated both *in vitro* and *in vivo*. The basis for this interaction is the partial complementarity (~75%) between the 5′ end of RNAIII and a stretch of ~80 base pairs within the SD region of the *hla* mRNA, which seems to result in ordinary base-pairing between the two molecules. As a consequence, in the absence of RNAIII, the alpha-toxin mRNA should be in a non-translatable configuration, and binding of RNAIII would result in a conformational change that allows translation to occur. As seen in Figure 6, a stretch of eight nucleotides at position 179–186 can form an intramolecular hybrid with the SD sequence (position 321–328) in such a way that it would prevent initiation of translation. The results from digestions with RNase T1 of native *hla* leader transcript confirm that the G residues in the SD region are base-paired with the proposed upstream region (nucleotides 179–187). Since only base-paired G residues could be detected by the RNase T1 digestion experiments, we cannot exclude the fact that other secondary structures are present which help to stabilize the non-translatable conformation of the molecule. Although the intramolecular base-pairing occurred *in vitro* without any added proteins, protein factors may also be needed to stabilize the translation impairing structure under *in vivo* conditions, as proposed for translational repressor proteins (Gold, 1988; McCarthy and Gualerzi, 1990).

Since RNAIII is complementary to the anti-SD region (nucleotides 179–186) of the *hla* transcript, hybridization

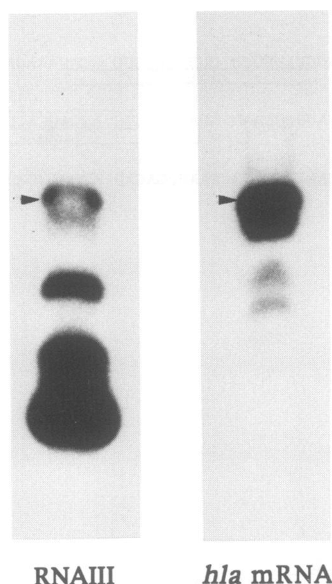

Fig. 7. Northern blot analysis of non-denatured total RNA from *S.aureus* 8325-4 (agr^+). RNA prepared according to Kornblum *et al.* (1988) was separated on a 1% agarose gel in TBE buffer. After *in situ* denaturation and blotting as described in Materials and methods, the filter was probed for RNAIII and after stripping and extensive washing, for *hla* mRNA.

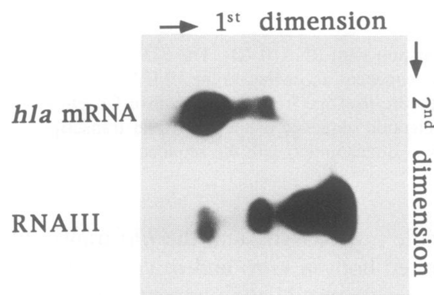

Fig. 8. Northern blot analysis of total RNA from *S.aureus*, 8325-4 (agr^+), separated by two dimensional gel electrophoresis. The first dimension of electrophoresis was under non-denaturing conditions as described in Figure 7. One lane from the first dimension was excised, denatured and applied at the top of an agarose–formaldehyde gel as described in Materials and methods. After electrophoresis the gel was analysed by Northern blot hybridization. The filter was first probed for RNAIII, exposed to film, probe removed and the blot subsequently probed for *hla* mRNA, and re-exposed.

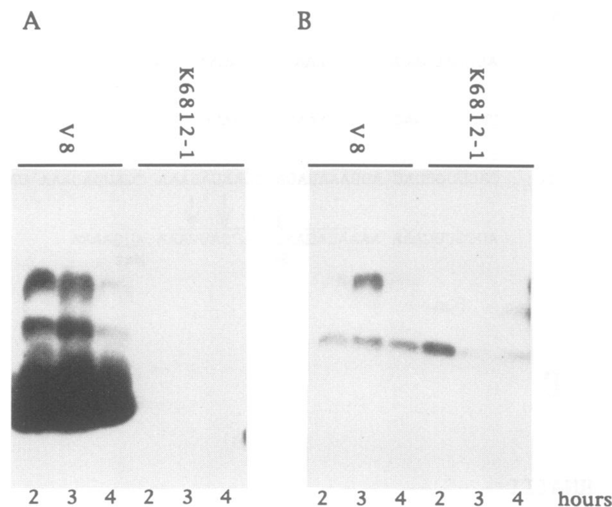

Fig. 9. Northern blot analysis of non-denatured *hla* mRNA in a mutant lacking RNAIII. RNA prepared according to Kornblum *et al.* (1988) from cultures of *S.aureus* strains V8 (agr^+) and K6812-1 (agr^-) at 2, 3 and 4 h was separated on a 1% agarose gel in TBE buffer. After denaturation and blotting as described in Materials and methods, the filter was probed for RNAIII (**A**) and after extensive washing for *hla* mRNA (**B**).

between the two molecules would disrupt the intramolecular base-pairing and make the SD region accessible to ribosome binding as proposed in Figure 10. This model was strongly supported by the finding that in the presence of RNAIII the G residues in the SD region were rendered susceptible to RNase T1 cleavage (Figure 5A). The model is also supported by results presented by Novick *et al.* (1993), showing that deletion of the first 95 nucleotides of RNAIII blocked translation of alpha-toxin. Thus RNAIII seems to be an example of an antisense RNA that stimulates translation of the target transcript, in contrast to the known antisense RNA species which block translation by interfering with the binding of ribosomes to the target mRNA (Simons and Kleckner, 1988). RNAIII belongs to the class of *trans*-encoded antisense RNAs, which are encoded by genes that are located at a genetic loci other than those of their target genes (Delihas, 1995). Like other *trans*-encoded antisense RNAs, RNAIII displays only partial complementarity to its target RNA. However, structural probing of the *micF/ompF* mRNA duplex suggested that non-canonical base pairs may participate in the RNA/RNA interaction (Delihas, 1995). Furthermore, the interaction of sense and antisense RNA has been shown to be not just simple hybridization between complementary sequences, but is initiated by interaction of loops between specific stem–loop structures in both RNA species (called a kissing complex) (Blomberg *et al.*, 1990; Eguchi *et al.*, 1991; Nehen and Lichtenstein, 1993).

The appearance of the *in vivo* produced RNAIII as multiple bands on non-denaturing electrophoresis gels (Figures 7 and 9) indicates that it may bind to several different target mRNA species. Preliminary experiments have demonstrated a complex between RNAIII and *tst* (toxic shock syndrome toxin-1) mRNA (E.Morfeldt, D.Taylor and S.Arvidson, unpublished). This is consistent with the observation by Novick *et al.* (1993) that RNAIII appears to stimulate translation of several *agr*-regulated exoproteins. However, as pointed out by these authors, the leader regions of other *agr*-dependent exoprotein genes do not show significant complementarity with the 5′ end of RNAIII. Furthermore, we have not found any significant intramolecular base-pairing of the kind demonstrated in *hla* mRNA that could block translation of other exoproteins. Another mechanism of regulation may be considered in those cases. However, a minimal codon–anticodon interaction has been found to regulate translation of the aminoacyl-tRNA synthetase genes in *Bacillus subtilis* (Putzer *et al.*, 1992; Gendron *et al.*, 1994).

One puzzling aspect of the dual function of RNAIII is the question, why does expression of alpha-toxin need to be regulated both at the level of transcription and translation? One explanation could be that *hla* mRNA has a relatively long half-life of 10–15 min (E.Morfeldt, D.Taylor and S.Arvidson, unpublished data), so in order to decrease the production of alpha-toxin quickly in

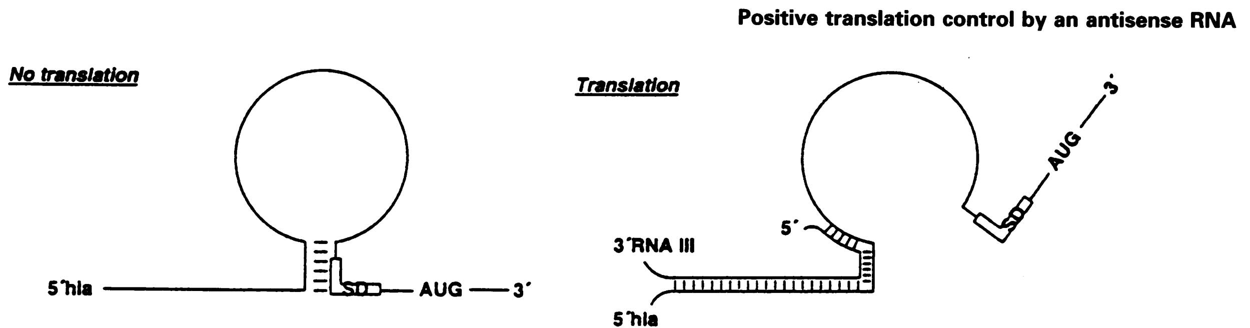

Fig. 10. Model for the translational control of alpha-toxin. The figure shows how the intramolecular base pairing in *hla* mRNA blocks the SD, and thereby translation, and how this is released by the interaction with RNAIII.

response to environmental signals the bacterium needs to down-regulate translation of preformed mRNA although transcription has been silenced. As a consequence of this, one must assume that RNAIII can alter between an active and an inactive form, as a result of interaction with an accessory factor (protein). Considering that RNAIII is also mRNA coding for delta-lysin one would expect translation to interfere with its regulatory functions. As seen in Figure 6B, the proposed base-pairing between RNAIII and *hla* mRNA blocks also the SD sequence of the *hld* gene, suggesting that an RNAIII molecule which stimulates translation of alpha-toxin cannot be translated itself.

In summary, RNAIII turns out to be unique in many respects; it seems to regulate both transcription and translation by independent mechanisms, it appears to be the first regulating RNA that activates transcription (Novick *et al.*, 1993), and it is also the first example of an antisense RNA where interaction with the target mRNA leads to stimulation of translation rather than inhibition.

Materials and methods

Bacterial strains

Staphylococcus aureus strains used in this study were 8325-4 (Novick, 1967), V8 and K6812-1, an exoprotein deficient mutant derived from V8 (Björklind and Arvidson, 1980). *Escherichia coli* strains TG1 (Gill *et al.*, 1986), JM 101 (Messing, 1979) and MC 1061 (Casadaban and Cohen, 1980) were used as hosts in cloning experiments.

Cultivation conditions

For exoprotein determinations and RNA extractions, *S.aureus* strains were grown overnight in tryptic soy broth (TSB, Difco). Bacteria from the precultures were collected by centrifugation and used to inoculate 10 vol of brain heart infusion broth (BHI, Difco) in baffled flasks. Cultures were grown on a rotary shaker (120 r.p.m.) at 37°C, and growth was monitored by measuring optical density at 600 nm (OD_{600}).

Alpha-toxin determination

The concentration of alpha-toxin in culture supernatants was determined by a double sandwich enzyme-linked immunosorbent assay (ELISA) as described by Söderquist *et al.* (1993). A standard curve obtained with purified alpha-toxin (SMI, Stockholm, Sweden) was used to calculate concentrations in µg/ml. The haemolytic activity against rabbit erythrocytes was determined as described by Kanclerski and Möllby (1987).

DNA sequencing

The 5′-UTR of the alpha-toxin gene was amplified from *S.aureus* strains V8 and K6812-1 by PCR (Saiki *et al.*, 1988) using the primers, 5′-ATCTAGATCGATTACATTTTTAAATC-3′, (nucleotides 1–20, with addition of six nucleotides at the beginning of the gene) and 5′-AGGATTCATCAATATGGAAC-3′ (nucleotides 377–396), (Gray and Kehoe, 1984). The sequences were determined by terminator cycle sequencing using fluorescence labelled dideoxynucleotides (Taq Dye-Deoxy Terminator Sequencing kit, Applied Biosystems) and the reactions were analysed on an Applied Biosystems 373A DNA sequencer.

Construction of plasmids

Competent cells of *E.coli* were prepared and transformed as described by Sambrook *et al.* (1989). Transformants were selected on LB plates containing 50 µg/ml ampicillin. Plasmid DNA was extracted using a kit for DNA purification (Wizard Minipreps, Promega, USA).

pEX100. To be able to synthesize RNAIII *in vitro* by run-off transcription, a *Hpa*I site was created downstream of the RNAIII terminator in pEX7 (Janzon *et al.*, 1989). A *Sau*3A fragment (nucleotides 1–1650, Janzon *et al.*, 1989) containing the entire RNAIII sequence, was cloned into pBS(–) (Yanisch-Peron *et al.*, 1985). The primer 5′-CATAATGATG-GTTAACTCATC-3′ (nucleotides 360–380, Janzon *et al.*, 1989) with a base-substitution from G to A (shown in bold), was used for *in vitro* mutagenesis, using a kit (Oligonucleotide-directed *in vitro* mutagenesis system, Version 2, Amersham) based on the method developed by Taylor *et al.* (1985). The mutation was confirmed by terminator cycle sequencing using fluorescence labelled dideoxynucleotides as described above.

pHLA540. A *Cla*I fragment from pDU1148, nucleotides 1–537 (Gray and Kehoe, 1984) comprising the untranslated 5′-end and the beginning of the coding sequence of the alpha-toxin gene of *S.aureus* strain Wood 46 was cloned into the *Acc*I site in pBS(–) (Yanisch-Peron *et al.*, 1985) generating the plasmid pHLA540.

pHLA1450. The whole alpha-toxin gene was amplified from pDU1148 (Gray and Kehoe, 1984) by PCR (Saiki *et al.*, 1988) using the primers, 5′-ATCTAGATCGATTACATTTTTAAATC-3′, (nucleotides 1–20, with addition of six nucleotides generating a *Cla*I site at the beginning of the gene) and 3′-CATGAACTAAACGAAAGGACGTCAT-5′, (nucleotides 1425–1443, with addition of five nucleotides generating a *Pst*I site at the end of the gene), and cloned into pGEM-T (Promega, WI, USA).

In vitro synthesis of RNA

pEX100 was used as template for synthesis of RNAIII. The plasmid was digested with *Hpa*I, *Cla*I or *Sty*I to generate full-length or truncated RNAIII products. pHLA540 digested with *Cla*I was used as template for synthesis of the 5′-untranslated region of the alpha-toxin mRNA, and pHLA1450 digested with *Pst*I was used for synthesis of full-length alpha-toxin mRNA. pUH101 (Sohlberg *et al.*, 1993) digested with *Nru*I was used as template for synthesis of outer membrane protein A (OmpA) mRNA from *E.coli*.

The various templates were linearized with the desired restriction nucleases and tested by agarose gel electrophoresis for complete digestion. The linearized DNA was transcribed in a 50 µl reaction volume containing 40 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 40 U RNase inhibitor (Boehringer), 10 mM DTT, 300 mM rNTPs and 0.5 mM [α-^{32}P]CTP (NEN). The reaction was started by addition of 1 U T7 RNA polymerase (Boehringer) to the reaction mixture, followed by 1 h of incubation at 37°C. The DNA template was digested with RNase-free DNase I, for 20 min at 37°C. The products were analysed by electrophoresis under denaturing conditions on 6% polyacrylamide gels containing 7 M urea in TBE buffer (45 mM Tris-borate, 1 mM EDTA).

Polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis. Aliquots (1–10 µl) from transcription reactions were added to loading buffer [10× loading buffer: 10 mM Tris–HCl, pH7.5, 1 mM EDTA, 50 mM NaCl, 5% (v/v) glycerol] to a final volume of 20 µl and kept on ice. Electrophoresis was performed at 4°C on a 5% polyacrylamide gel in running buffer containing 6.7 mM Tris–HCl, pH 7.5, 3.3 mM Na-acetate and 1 mM EDTA (Hennighausen and Lubon, 1987) at 20 mA.

Denaturing polyacrylamide gel electrophoresis. Aliquots (1–10 μl) of transcription reactions or RNA eluted from non-denaturing polyacrylamide gels, were added to loading buffer containing formamide to a final volume of 20 μl and electrophoresed on a 6% polyacrylamide gel containing 7 M urea in TBE buffer at 20 mA. After electrophoresis gels were dried and exposed to Fuji RX film overnight.

Elution of RNA from a non-denaturing gel. The gel was kept wet and autographed on Fuji RX film for 5 min. The radioactive bands were cut out, put in 500 μl elution buffer (500 mM NH_4-acetate, 10 mM $MgCl_2$, 1 mM EDTA and 0.1% (w/v) SDS) and incubated at 37°C overnight. After extraction several times with phenol, the RNA was precipitated with ethanol and dissolved in water. The radioactivity was measured in a scintillation counter (LKB, Sweden).

RNA preparation

For standard Northern blot hybridization, total *S.aureus* RNA was prepared by extraction of lysostaphin-treated bacteria with hot phenol as previously described (Janzon *et al.*, 1986). Lysostaphin was from Applied Microbiology Inc., New York. Concentrations of RNA were determined spectrophotometrically at 260 nm. Electrophoresis of RNA, transfer to Biodyne A nylon membrane (Pall Ultrafine Filtration Corp., NY, USA) and hybridization were carried out as described (Janzon *et al.*, 1986). RNA to be analysed under non-denaturing conditions was prepared by the method of Kornblum *et al.* (1988). In this method proteins are removed by extensive digestion with proteinase K instead of phenol extraction. RNA samples were separated on a 0.8% agarose gel. After electrophoresis the gel was soaked for 30 min in warm (65°C) denaturing buffer containing 2.2 M formaldehyde, 0.2 M MOPS, pH 7; 50 mM Na-acetate and 1 mM EDTA. Blotting and hybridization was carried out as above. The probes, a *Cla*I fragment (nucleotides 536–1258; Gray and Kehoe, 1984) from the *hla* gene and a *Eco*RV–*Cla*I fragment (nucleotides 528–813; Janzon *et al.*, 1989) from RNAIII, were radiolabelled to a specific activity of $1–5 \times 10^8$ c.p.m./mg with [α-^{32}P]dCTP purchased from NEN (Du Pont, USA) using a 'random primed labelling kit' from Boehringer Mannheim (Mannheim, Germany).

Two-dimensional agarose gel analysis

RNA was prepared according to the method of Kornblum *et al.* (1988). The cell extracts were run on a 1% agarose gel. After electrophoresis one full lane was excized and the gel slice denatured in warm buffer as described above. The slice of gel was then put in a horizontal gel apparatus and a solution containing 1.2% agarose in 2.2 M formaldehyde, 0.2 M MOPS, pH 7, 50 mM Na-acetate and 1 mM EDTA was poured on top. The native gel slice was thereby fixed at the cathode end of the denaturing gel. Electrophoresis was carried out in 1× running buffer (2.2 M formaldehyde, 0.2 M MOPS, pH 7, 50 mM Na-acetate and 1 mM EDTA). Transfer to a nylon membrane and hybridization was carried out as described above.

Detection of RNase T1 cleavage products

Partial ribonuclease T1 digestion of *hla* transcripts was performed in principle as described by Öhman and Wagner (1989). A digestion reaction contained 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 μg/μl tRNA in 6 μl. 0.025 U RNase T1 (Pharmacia) was used for partial digestion of 0.1 pmol transcript. When a complex of *hla* transcript and primer, or RNAIII, was to be digested, the molecules were annealed before digestion by heating the mixture at 95°C for 30 s, at 65°C for 3 min and at 42°C for 5 min. The reaction mix was incubated at 37°C for 10 min and the digestion stopped with 20 μl of TE-equilibrated phenol. The samples were then diluted in TE buffer (10 mM Tris–HCl pH 7.9, 1 mM Na_2EDTA) to 100 μl and extracted with an equal volume of phenol. The RNA was precipitated with ethanol together with 6 μg of carrier tRNA at 22°C. The pellet was resuspended in water. For detection of the cleavage sites by reverse transcription, 1 pmol of labelled primer (Alpha7) was annealed (see above) to the digested transcripts in RT buffer (Boehringer: 25 mM Tris–HCl, pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT). The primer extension was carried out in RT buffer with 125 mM of each dNTP and 1.25 U AMV reverse transcriptase (Boehringer) in a total volume of 8 μl. The reaction mixture was incubated for 45 min at 42°C. The reaction was stopped by the addition of 16 μl of stop mix (80% formamide, 10 mM NaOH, 1 mM Na_2EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). After boiling, aliquots were electrophoresed on a 6% sequencing gel. A size marker was synthesized using plasmid pDU1148 as template in a sequencing reaction. The reaction was performed with the Sequenase kit (United States Biochemical) using the same primer (Alpha7) as in the reverse transcription.

Oligodeoxyribonucleotides

The primers used in RNase T1 digestion experiments were Alpha 3: 5′-GAAACAAGGAAAAGACATAGC-3′, (nucleotides 62–82, Gray and Kehoe, 1984), Alpha 4: 5′-TAAATTACTGAGATGATGATGTGAGATT-3′, (nucleotides 173–200, Gray and Kehoe, 1984). The primer used in reverse transcription experiments, Alpha 7: 5′-GTATTGCTTCCAATAT-CTGTAG-3′, (nucleotides 440–461, Gray and Kehoe, 1984), was labelled with [γ-^{32}P]ATP (Amersham) using polynucleotide kinase (Boehringer).

Acknowledgements

We thank Agneta Wahlquist for skilful technical assistance, and Patricia Colque-Navarro for alpha-toxin determinations. This work was supported by grant no. 4513 from the Swedish Medical Research Council to S.A. and by grants from the Austrian Science Foundation (FWF) and the Austrian Federal Department of Science to A.v.G.

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Received on October 13, 1994; revised on May 24, 1995