Ribonuclease III-mediated processing of specific *Neisseria meningitidis* mRNAs

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Approx. 2 % of the *Neisseria meningitidis* genome consists of small DNA insertion sequences known as Correia or *nemis* elements, which feature TIRs (terminal inverted repeats) of 26–27 bp in length. Elements interspersed with coding regions are co-transcribed with flanking genes into mRNAs, processed at double-stranded RNA structures formed by TIRs. *N. meningitidis* RNase III (endoribonuclease III) is sufficient to process *nemis*$^+$ RNAs. RNA hairpins formed by *nemis* with the same termini (26/26 and 27/27 repeats) are cleaved. By contrast, bulged hairpins formed by 26/27 repeats inhibit cleavage, both *in vitro* and *in vivo*. In electrophoretic mobility shift assays, all hairpin types formed similar retarded complexes upon incubation with RNase III. The levels of corresponding *nemis*$^+$ and *nemis*$^-$ mRNAs, and the relative stabilities of RNA segments processed from *nemis*$^+$ transcripts *in vitro*, may both vary significantly.

Key words: DNA insertion sequence, pathogenic micro-organism, post-transcriptional control, RNA hairpin, segmental RNA stability.

INTRODUCTION

Neisseria meningitidis (the meningococcus) is a strictly human Gram-negative micro-organism that may either be harmless or cause life-threatening infections, especially in young adults. Meningococci colonize the nasopharynx, but may invade the epithelium, disseminate within the bloodstream and cross the blood–brain barrier, causing septicaemia and meningitis. *N. meningitidis* are classified into a few major pathogenic serogroups. In all cases, a restricted number of clonal groups, or sequence types, cause pathology [1–3]. The determination of the complete genomic sequences of the *N. meningitidis* A serogroup Z2491 [4] and B serogroup MC58 [5] strains have provided data relevant to the molecular characterization of meningococci [6,7]. In parallel, *N. meningitidis* genes essential for the development of bacteraemic disease were identified by functional screening of signature-tagged mutants *in vivo* [8].

Whole-sequence data have also provided clues about the unique organization of Neisserial chromosomes. *N. meningitidis* genomes host an unexpected variety of intergenic DNA repeats, which vary in length and relative abundance, and are found both in isolation and in arrays of 200–2700 bp [4]. *Neisseriae* are micro-organisms that are naturally competent for DNA transformation, and their genome undergoes constant remodelling [9–11]. It is plausible to hypothesize that intergenic DNA repeats may promote recombination by increasing the rate of horizontal gene transfer at flanking loci [4].

A major class of DNA repeats found in Neisserial genomes is represented by short ISs (insertion sequences) which feature 26–27 bp long TIRs (terminal inverted repeats), known as Correia elements [12] or *nemis* (for neisseria miniature insertion sequences; [13]). The genomic insertion of these repeats is accompanied by the duplication of the TA dinucleotide [13–15].

Nemis account for ≈ 2 % of the genome in *N. meningitidis* strains that have been completely sequenced [4,5]. Approx. one-third of the elements are intermingled with repeated DNA sequences in intergenic arrays. The remaining members of the *nemis* family are interspersed with coding regions, and are frequently found inserted close to either the start or the end of ORFs (open reading frames) [13,15]. This unusual distribution, which is fairly well conserved among *N. meningitidis* strains of different serogroups, sequence types and geographical origins [16], allows one to hypothesize that *nemis* sequences have a role in the control of gene expression. It has been reported that individual repeats may promote transcription initiation and termination in *Escherichia coli* and *N. gonorrhoeae* respectively [17,18], and a functional site for the transcriptional activator IHF (integration host factor) [19] has been identified in full-length *nemis* copies [14]. From these observations, it could be argued that *nemis* may impinge on the expression of flanking genes by acting variably at the transcriptional level. In contrast, the finding that *N. meningitidis* mRNAs spanning *nemis* are cleaved at hairpins formed by *nemis* TIRs [13,20] leads one to hypothesize that several members of the *nemis* family influence the expression of neighbouring genes by acting at the post-transcriptional level.

In the present paper we show that the *N. meningitidis* RNase III (endoribonuclease III) expressed *in vitro* is sufficient to process *nemis* RNA hairpins. Transcripts spanning *nemis* may be either sensitive or refractory to processing, and comparative analyses show that the segmental stability of processed *nemis*$^+$ transcripts may vary significantly *in vitro*, as *in vivo*.

EXPERIMENTAL

Bacterial strains and extracts

N. meningitidis and *N. lactamica* strains have been described previously [16,20], and were grown in GC broth supplemented with 1 % Polyvitox (Bio-Merieux). The preparation of whole-cell extracts from *E. coli* and *N. lactamica* has been described in [20].

Expression of *N. meningitidis* RNase III

To clone the *N. meningitidis rnc* gene encoding RNase III, two 31-mers (RNase up, 5′-GAAAGTTGCTGCAGACGATGTTTT-GAAACAG-3′; RNase down, 5′-CGAATCAAGCTTGCCGCC-TCATTTCTTTTTC-3′; underlined residues were deduced from

Abbreviations used: IS, insertion sequence; ORF, open reading frame; REP, repetitive extragenic palindrome; TIR, terminal inverted repeat.

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Table 1 Oligonucleotides used to construct the mini-*nemis* templates

All oligonucleotides are shown in the 5′ to 3′ direction. Residues corresponding to *nemis* TIRs are underlined.

Oligonucleotide	Sequence
Mini-26/26a	GGGAGACAAGAATAAACGCTGAACTATAGTGGATTAACAAAAATCAGGACAAG
	ATGCGCCGTACTGGTTTTTGTTAATCCACTATACACGAACTCAACGCTGTAAC
Mini-26/27	GGGAGACAAGAATAAACGCTGAACTATAGTGGATTAACAAAAATCAGGACAAG
	ATGCGCCGTACTGGTTTAAATTTAATCCACTATACACGAACTCAACGCTGTAAC
Mini-26/26b	GGGAGACAAGAATAAACGCTGAACTGTAAATGAAGTACAAAAATCAGGACAAG
	ATGCGCCGTACTGGTTTTTGTACTTCATTTACACACGAACTCAACGCTGTAAC
Mini-26/26c	GGGAGACAAGAATAAACGCTGAACTATAGTGGATTAACAATACAAGAGACAAG
	ATGCGCCGTATCTTGTGTTGTTAATCCACTATACACGAACTCAACGCTGTAAC
Mini-27/27	GGGAGACAAGAATAAACGCTGAACTATAGTGGATTAAATTTAAATCAGGACAAG
	ATGCGCCGTACTGGTTTAAATTTAATCCACTATACACGAACTCACGCTGTAAC
Mini-for	TAATACGACTCACTATAGGGAGACAAGAATAAACGCT
Mini-rev	GGTACATATTTAGGTTTGTTACAGCGTTGAGTTCGTG

the DNA sequence of *N. meningitidis* strain MC58) were used as primers to amplify the *rnc* coding region from *N. meningitidis* strain B1940. The amplified region extends from residue 711 321 to residue 712 039 in MC58 DNA. Amplimers were cleaved with *Pst*I and *Hin*dIII and inserted downstream from the T7 phage A10 promoter between the *Pst*I and *Hin*dIII sites of the pRSETB vector (Invitrogen), to direct the synthesis of transcripts eventually translated by reticulocyte cell extracts (T&T Kit; Promega).

RNA processing assays

Uniformly ^{32}P-labelled RNAs were obtained by transcribing *in vitro* linear DNA templates with either T7 or Sp6 RNA polymerase as described in [20]. Templates were obtained by PCR amplification of *N. meningitidis* DNA with 50-mers that included either T7 or Sp6 RNA polymerase promoters in their 5′ half, and residues complementary to DNA sequences of interest in their 3′ half. Mini-*nemis* DNA templates were obtained by annealing either 106- or 107-mers containing *nemis* TIRs to the mini-for oligonucleotide (oligomers used are listed in Table 1). Annealed moieties were made double-stranded by the Klenow enzyme, and amplified by PCR by using as primers the oligonucleotides mini-for and mini-rev (see Table 1). Amplimers obtained were checked by DNA sequence analysis. The T7 promoter sequence included in the mini-rev oligonucleotide allowed *in vitro* synthesis of RNA substrates for processing assays. Degradation assays with either bacterial or reticulocyte cell extracts expressing *N. meningitidis* RNase III were carried out as reported in [20]. In processing assays carried out under low salt conditions, both KCl and NH_4Cl were omitted from the reaction buffer.

RNA analyses

Total bacterial RNA was purified on an RNeasy column (Qiagen). Primer extension analyses were performed as reported in [21]. For RNase protection assays, 5 μg of total RNA was mixed with ^{32}P-labelled antisense RNA probes in 30 μl of hybridization buffer (75 % formamide, 20 mM Tris, pH 7.5, 1 mM EDTA, 0.4 M NaCl, 0.1 % SDS) and kept at 45 °C for 16 h. After a 1 h incubation at 33 °C with RNase T_1 (2 μg/ml), samples were treated with proteinase K (50 μg/ml) for 15 min at 37 °C, extracted once with phenol, precipitated with ethanol, resuspended in 80 % formamide and loaded on to 6 % (w/v) polyacrylamide/8 M urea gels.

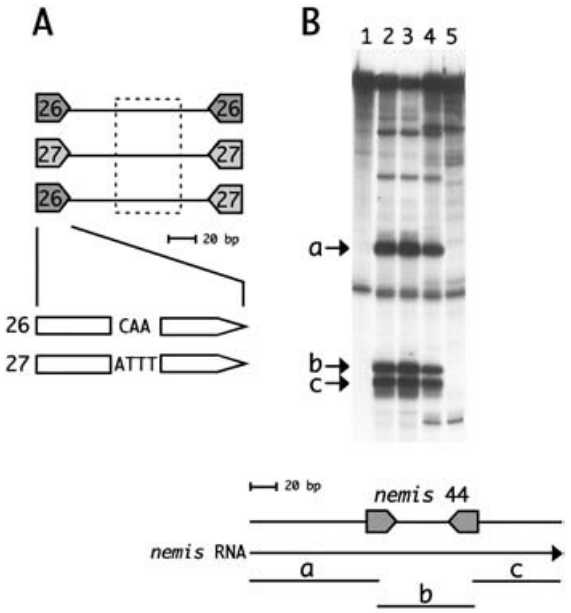

Figure 1 Organization of *nemis* repeats and cleavage of *nemis*$^+$ transcripts

(**A**) *Nemis* feature TIRs that measure, including the TA dinucleotide target duplicated upon genomic insertion, either 26 or 27 bp. TIR types differ in the presence of alternative central DNA stretches. The 50 bp central region found only in long elements is highlighted. (**B**) Approx. 50 000 c.p.m. of radiolabelled RNA spanning *nemis* 44 was incubated for 20 min at 37 °C with approx. 2 μg of two different preparations of crude *N. lactamica* cell extracts (lanes 2 and 3) or *N. meningitidis* RNase III expressed *in vitro* (lane 4). As control, the RNA probe was incubated either alone (lane 1) or with the same amount of reticulocyte extract used to express the recombinant protein (lane 5). Reaction products were separated on a 6 % (w/v) polyacrylamide/8 M urea gel. Bands a–c indicate processed RNA species.

^{32}P-labelled antisense RNA probes were transcribed *in vitro* by Sp6 RNA polymerase.

RNA band-shift experiments were carried out essentially as described in [22]. Aliquots (≈2000 c.p.m.) of gel-purified ^{32}P-labelled RNA (25–50 pmol) were incubated with either whole bacterial extracts or recombinant *N. meningitidis* RNase III in 15 μl reactions, in the presence of 40 mM Hepes (pH 7.2), 20 mM $(NH_4)_2SO_4$, 15 mM potassium acetate, 10 % (v/v) glycerol, 50 mg/ml yeast tRNA and 0.5 mg/ml BSA. Samples were incubated for 30 min at 4 °C and then loaded on to 5 % (w/v) polyacrylamide gels. Electrophoresis was carried out at 4 °C and 23 mA for 2 h in 0.5 × Tris/borate buffer.

RESULTS

Nemis RNA is cleaved by *N. meningitidis* RNase III synthesized *in vitro*

Members of the *nemis* family range in size from 106–108 to 156–158 bp due to the presence/absence of a 50 bp internal segment. Both long and short elements feature TIRs which, including the duplicated TA targets, measure either 26 or 27 bp. Both types of TIR, apart from a short central DNA segment, have the same sequence (Figure 1A). Elements inserted close to coding regions are co-transcribed with flanking ORFs into mRNAs processed at double-helical structures, formed by paired TIRs [13]. Cleavage assays carried out with whole *E. coli* cell extracts support the notion that RNase III is involved in the processing event [20]. The

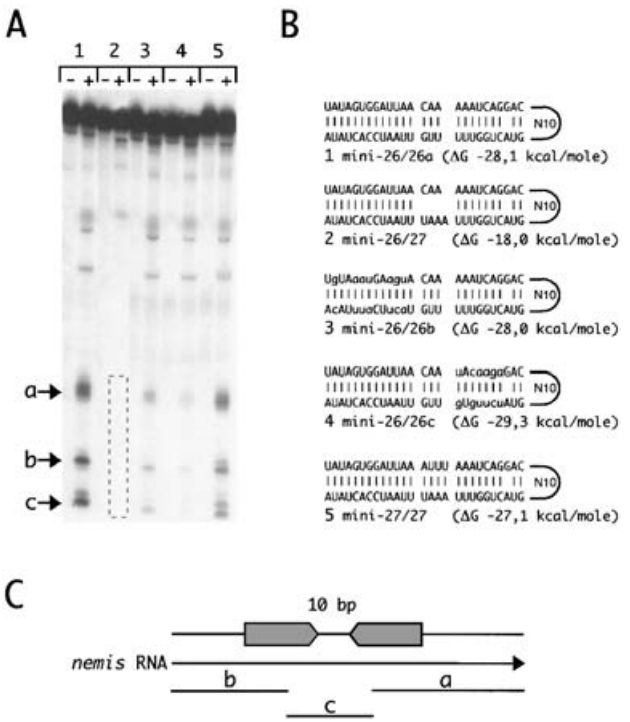

Figure 2 Cleavage of mini-*nemis* RNAs

(**A**) Radiolabelled RNAs spanning mini-*nemis* templates 1–5 carrying different types of TIRs were incubated for 20 min at 37 °C either alone or with *N. meningitidis* RNase III expressed *in vitro* (lanes − and + respectively). Reaction products were separated on a 6 % polyacrylamide/8 M urea gel. The lack of cleavage of the mini-26/27 transcripts is highlighted. (**B**) The degree of complementarity and free energies [23] of the hairpins formed by each transcript are shown (1 kcal = 4.184 kJ). Lower-case letters denote residues substituting for original *nemis* residues. (**C**) RNA species labelled a–c in (**A**) are sketched.

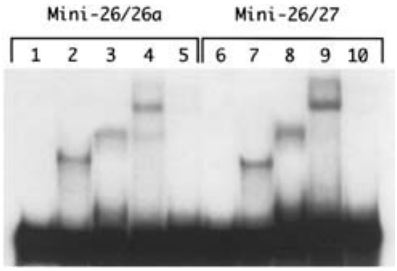

Figure 3 Electrophoretic mobility shift assays of RNA–protein complexes

Radiolabelled RNAs derived from mini-26/26a and mini-26/27 templates (approx. 3000 c.p.m.) were incubated for 20 min at 37 °C either alone (lanes 1 and 6) or with recombinant *N. meningitidis* RNase III (lanes 2 and 7) or with 2 μg of crude *N. lactamica* cell extracts (lanes 3 and 8). The same amount of probe was incubated with 2 μg of crude *E. coli* extracts from *rnc*$^+$ (lanes 4 and 9) and *rnc*$^-$ (lanes 5 and 10) cells. Reaction products were electrophoresed on non-denaturing 5 % (w/v) polyacrylamide gels.

N. meningitidis rnc gene encoding RNase III was amplified by PCR, and used to direct synthesis of the enzyme in reticulocyte cell extracts. As shown in Figure 1(B), the cleavage of *nemis*$^+$ transcripts by Neisserial whole-cell extracts (lanes 2 and 3) is fully mimicked by the recombinant *N. meningitidis* enzyme (lane 4). The low-molecular-mass band visible in lanes 4 and 5 probably results from degradation of the input RNA by nuclease activities found in the reticulocyte extract.

The data reported in Figure 1(B) demonstrate that RNase III is uniquely responsible for the processing of *nemis*$^+$ transcripts, being sufficient to both recognize and cleave *nemis* RNA targets.

The completely sequenced genomes of *N. meningitidis* strains Z2491 and MC58 [4,5] host approx. 250 *nemis* carrying TIRs at both ends. Taking into account the relative distance from neighbouring ORFs, it is plausible that about half of these elements are transcribed along with neisserial genes, and hence are targeted, at the RNA level, by RNase III. To ascertain whether transcripts with the same backbone spanning 26/26, 26/27 and 27/27 *nemis* are processed with comparable efficiency, mini-*nemis* templates were engineered by a PCR-based approach. T7 RNA polymerase-driven transcripts, in which TIR sequences of either the same or different types are separated by 10 nucleotides, were used as substrates for processing experiments (Figure 2). RNA stem–loop structures formed by pairing of either 26 or 27 nt TIRs were cleaved efficiently (Figure 2, lanes 1 and 5 respectively). In contrast, RNA hairpins formed by the pairing of 26 and 27 nt TIRs were fully refractory to RNase III-mediated cleavage (Figure 2, lane 2). The same results (not shown) were obtained by carrying out processing assays at low salt concentrations, a condition known to enhance RNase III-mediated cleavage at secondary sites [24]. In the mini-26/27 transcripts, the double-helical RNA structure formed by TIRs is partly disrupted by an asymmetrical internal loop, and this probably inhibits cleavage. These data were unexpected, since RNA stems of 12 bp are cleaved efficiently by *E. coli* RNase III *in vitro* [24,25], and mini-26/27 transcripts can form a 13 bp stem (see Figure 2). Moreover, substrates exhibiting internal loops are cleaved by the *E. coli* enzyme at one or both sides of the double-helical structure [24,25], and the mini-26/27 transcripts were actually processed by *E. coli* crude extracts (results not shown). Transcripts in which the sequence, but not the strength, of the RNA hairpin was modified were also processed, although to a different extent (Figure 2, lanes 3 and 4). Thus the crucial requirement for cleavage of *nemis*$^+$ transcripts is the degree of complementarity of TIRs.

Interactions between RNase III and *nemis* RNA hairpins

By choosing experimental conditions under which RNase III activity was inhibited, the mini-*nemis* transcripts described in Figure 2 were used as probes in electrophoretic mobility shift assays. As shown in Figure 3, RNAs susceptible and refractory to RNase III-mediated cleavage both formed retarded complexes that had the same mobility upon incubation with either recombinant RNase III (lanes 2 and 7) or neisserial whole-cell extracts (lanes 3 and 8), indicating that the binding of RNase III to *nemis*$^+$ RNA targets may occur without concomitant cleavage. As in RNase III from other micro-organisms, the catalytic and double-stranded RNA-binding domains of the *N. meningitidis* enzyme are located within the N-terminal two-thirds and the C-terminal one-third of the molecule respectively. Functional uncoupling of the two domains has been reported for the *E. coli* enzyme [26]. The sizes of the RNA–protein complexes present in lanes 2 and 7 and lanes 3 and 8 of Figure 3 suggest that RNase III activity in whole-cell extracts is found predominantly in high-molecular-mass complexes. When RNA probes were challenged with *E. coli* whole extracts, retarded electrophoretic complexes were formed with *rnc*$^+$ (Figure 3, lanes 4 and 9) but not with *rnc*$^-$ (Figure 3, lanes 5 and 10) extracts, which lack RNase III because of a Tn10 insertion in the *rnc* gene encoding the enzyme (see [26]). Data indicate that complexes are formed only in the presence of a functional RNase III, and suggest that, unless present specifically in meningococci, cellular factors other than RNase III do not interact directly with *nemis* RNA.

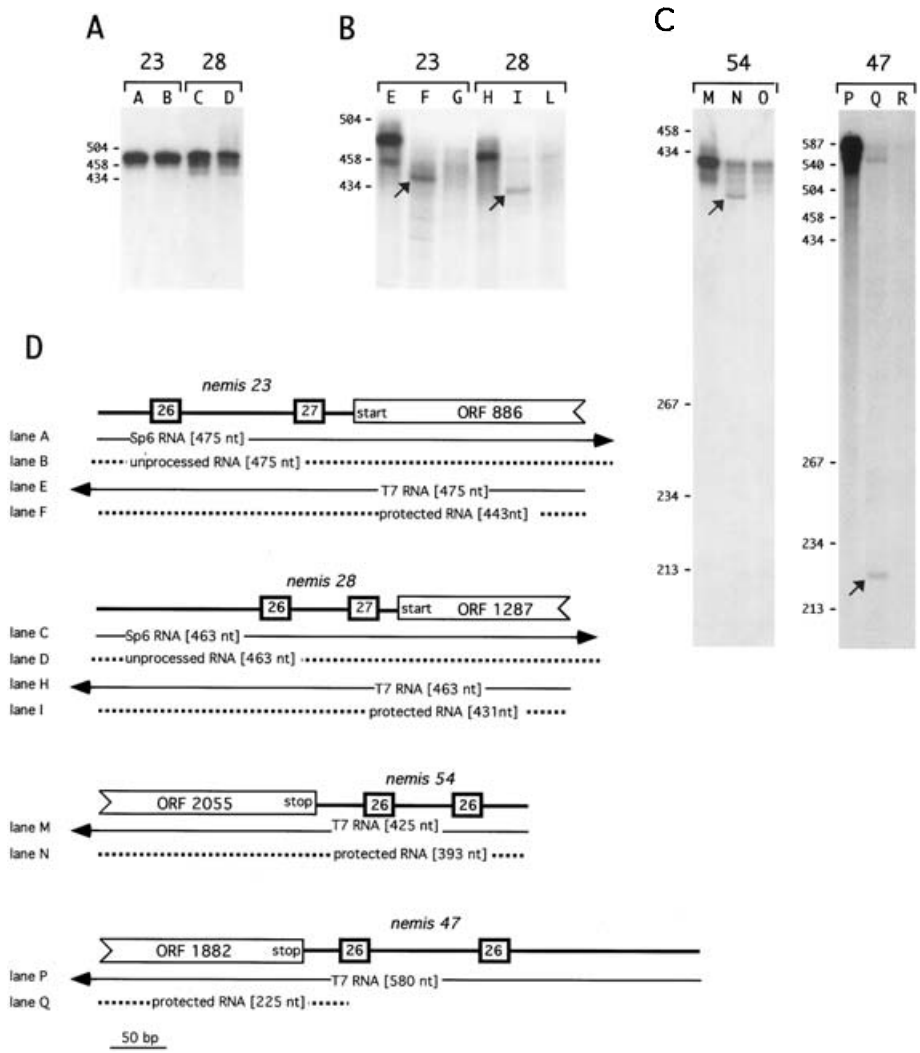

Figure 4 Cellular *nemis*$^+$ transcripts refractory to RNase III-mediated cleavage

(**A**) Uniformly ^{32}P-labelled RNAs transcribed *in vitro* by Sp6 RNA polymerase spanning *nemis* 23 and 28 were incubated either alone (lanes A and C) or with 2 μg of *N. lactamica* cell extracts (lanes B and D). Reaction products were separated on a 6 % polyacrylamide/8 M urea gel. Numbers on the left refer to the size in nucleotides of co-electrophoresed DNA molecular size markers. (**B**) Uniformly ^{32}P-labelled antisense RNAs transcribed *in vitro* by T7 RNA polymerase spanning *nemis* 23 and 28 were hybridized to 5 μg of either total RNA from *N. meningitidis* strain BL859 (lanes F and I) or yeast RNA (lanes G and L). T1 RNase-resistant hybrids were electrophoresed on a 6 % polyacrylamide/8 M urea gel, along with aliquots of untreated input probes (lanes E and H). Major reaction products are marked by arrows. DNA molecular size markers are as in (**A**). (**C**) Uniformly ^{32}P-labelled antisense RNA transcribed *in vitro* by T7 RNA polymerase spanning *nemis* 54 and 47 were hybridized to 5 μg of either total RNA from *N. meningitidis* strain BL859 (lanes N and Q) or yeast RNA (lanes O and R). T1 RNase-resistant hybrids were electrophoresed on a 6 % polyacrylamide/8 M urea gel, along with aliquots of untreated input probes (lanes M and P). (**D**) Template DNAs spanning *nemis* 23, 28, 54 and 47 and part of the neighbouring ORFs are shown. The corresponding sense and antisense transcripts (solid lines; the direction of transcription is indicated by arrows), the reaction products (dotted lines) and their sizes are shown. Transcribed Sp6 and T7 sequences in radiolabelled RNAs are highlighted. ORFs are numbered as in the *N. meningitidis* MC58 strain [5].

Nemis$^+$ mRNAs sensitive and refractory to RNase III-mediated cleavage

To verify results obtained with the mini-transcripts, chromosomal regions encompassing two 26/27 *nemis* (repeats 23 and 28) were amplified by PCR with primers that included phage promoters to allow *in vitro* synthesis of RNAs. The latter were used either as substrates for degradation assays or as antisense probes for RNase protection analyses. We were unable to detect *in vitro* processing of transcripts spanning *nemis* 23 and 28 incubated with *N. lactamica* cell extracts (Figure 4A, lanes B and D). These results contrast sharply with data reported in Figure 1(B), showing that transcripts spanning the 26/26 element 44 are processed *in vitro* by *N. lactamica* cell extracts at double-helical structures formed by paired *nemis* TIRs. Accordingly, *N. meningitidis* mRNAs spanning *nemis* 23 and 28, and ORFs 886 and 1287, located downstream of each, were found to be uncleaved when analysed by RNase T1 mapping (Figure 4B). Thus transcripts spanning 26/27 *nemis* repeats seem also to be refractory to cleavage by RNase III in their natural RNA context.

RNA hairpins formed by 26/26 *nemis* may also be resistant to RNase III-mediated cleavage. Around a dozen *N. meningitidis* mRNAs spanning 26/26 *nemis* monitored by primer extension had been found to be processed at *nemis* TIRs ([13]; see also Figure 5). However, we found that mRNAs spanning ORF 2055, which codes for a transcriptional regulator of the metR family, and *nemis* element 54 were refractory to RNase III-mediated cleavage *in vivo* (Figure 4C, left panel). As a control, RNase protection

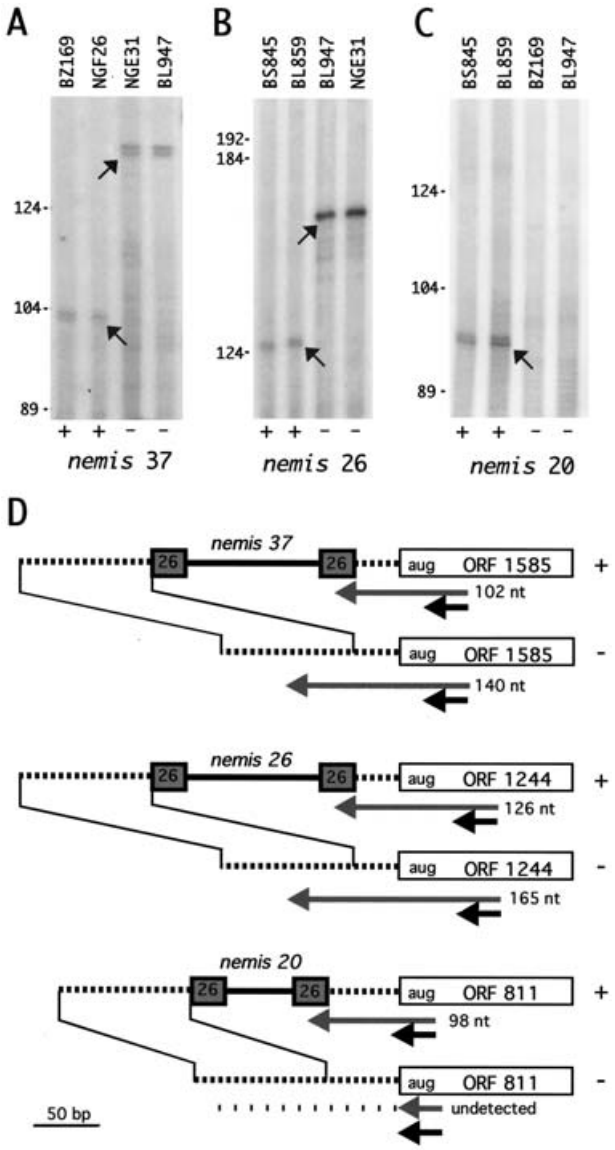

Figure 5 Analysis of corresponding *nemis*$^+$ and *nemis*$^-$ transcripts

(**A**)–(**C**) ^{32}P-5′-end-labelled primers complementary to the coding regions of ORFs 1585 (**A**) 1244 (**B**) and 811 (**C**) were hybridized to total RNA (5 μg) from *N. meningitidis* strains [16] that differ in the presence (+) or absence (−) of *nemis* 37, 26 and 20 upstream of the indicated ORFs. Annealed primer moieties were extended, in the presence of NTPs, by AMV (avian myeloblastosis virus) reverse transcriptase. Reaction products were electrophoresed on a 6 % polyacrylamide/8 M urea gel. Major reaction products are marked by arrows. Numbers to the left of each autoradiogram refer to the size in nucleotides of co-electrophoresed DNA molecular size markers, as in Figure 4. (**D**) The structures of corresponding mRNAs either containing (+) or lacking (−) *nemis* 37, 26 and 20 analysed by primer extension in (**A**)–(**C**) are shown diagrammatically. Dotted lines denote 5′ untranslated transcript regions. Primers and elongation products are denoted by black and grey arrows respectively. The sizes of the reaction products detected in the autoradiograms shown in (**A**)–(**C**) are given.

data obtained by monitoring mRNAs spanning ORF 1882 and the 26/26 *nemis* 47, which were found to be cleaved by RNase III *in vivo*, are shown in the right panel of Figure 4(C). The presence of 26 bp TIRs at the termini of *nemis* element 54 found in strain NGH26, from which RNA was isolated, was confirmed by sequence analyses, ruling out the possibility that our data may reflect changes in the structural organization of *nemis* 54. While the possibility cannot be excluded that a fraction of mRNAs spanning *nemis* 54 are processed by RNase III but cannot be

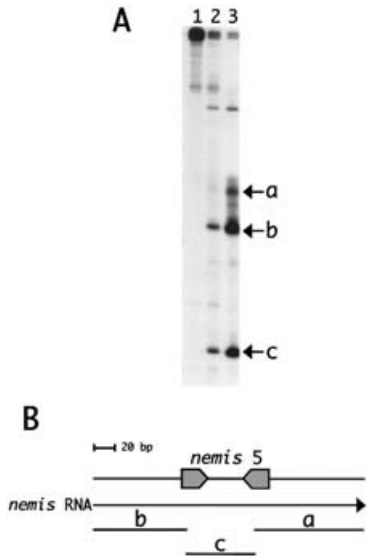

Figure 6 Different stabilities of *nemis* RNA cleavage products

(**A**) Uniformly ^{32}P-labelled RNA spanning *nemis* 5 (lane 1) was incubated either with *N. lactamica* cell extracts (lane 2) or with *N. meningitidis* RNase III expressed *in vitro* (lane 3). Reaction products were separated on a 6 % polyacrylamide/8 M urea gel. (**B**) RNA species labelled a–c in (**A**) are sketched.

detected because they are rapidly degraded, the data suggest that the overall RNA context in which *nemis* RNA hairpins are embedded may result, in some instances, in them being inhibitory for RNase III-mediated cleavage. This also means that the number of unprocessed *nemis*$^+$ mRNAs may be higher than predicted from the structure of RNA hairpins formed by the pairing of *nemis* termini.

Analysis of *nemis*$^+$ transcripts *in vivo*

The identification of *N. meningitidis* strains that lack *nemis* repeats at specific chromosomal locations [16] has enabled us to monitor the steady-state levels of corresponding *nemis*$^+$ and *nemis*$^-$ mRNAs *in vivo*. As shown by primer extension analyses, ORF 1585 transcripts accumulated at comparable levels in both *nemis*$^+$ and *nemis*$^-$ strains (Figure 5A). In contrast, the processed mRNAs encoding ribulose phosphate epimerase (ORF 1244) accumulated approximately 5 times less abundantly than the corresponding unprocessed transcripts (Figure 5B). The levels of *murB* (ORF 811) transcripts were relatively high in the *nemis*$^+$ strains BS845 and BL859, but barely detectable in the *nemis*$^-$ strains BZ169 and BL947 (Figure 5C). Although similar data were obtained by the analysis of independent strains, the possibility cannot be formally ruled out that either the level of synthesis or the overall stability of the various transcripts may vary among the strains analysed. However, quantitative differences emerging from data shown in Figure 5 epitomize the uncertainity in predicting the fate of *nemis*$^+$ mRNAs, since RNase III-mediated cleavage may increase the stability, as well as accelerate the decay, of specific transcripts [27,28].

Differences in the segmental stability of processed *nemis*$^+$ RNA species are clearly illustrated by the *in vitro* data. Transcripts spanning the 26/26 *nemis* 13 were challenged with either *N. meningitidis* RNase III or whole-cell extracts from *N. lactamica* (Figure 6). Cleavage by the recombinant enzyme yielded RNA species that accumulated at comparable levels (see bands a–c in lane 3 of Figure 6). However, when processing was carried out

with whole-cell extracts, band a was barely detectable (Figure 6, lane 2). This contrasts with data reported in Figure 1(B), in which the cleavage products of transcripts spanning the 26/26 *nemis* 44 all exhibited the same relative stability, regardless of whether cleavage was by RNase III synthesized *in vitro* or by neisserial whole-cell extracts.

DISCUSSION

nemis are a major class of repetitive DNA elements that are spread throughout the genomes of pathogenic *Neisseriae*. According to genomic surveys carried out with a representative set of repeats, *nemis* are conserved in meningococci of different sequence types and geographical origins, but are under-represented in strains of the apathogenic *N. lactamica* species [16]. However, genetic exchanges occur at high frequency among clones of the two species, which both colonize the human nasopharynx [29]. Meningococci may have a selective advantage in retaining *nemis* DNA at specific chromosomal sites, and we believe that this correlates with the possible role of several *nemis* in post-transcriptional control.

Analogous to the short *E. coli* intergenic DNA sequences called REPs (repetitive extragenic palindromes) [30–32], *nemis* can be co-transcribed with neighbouring genes. However, RNAs spanning the two classes of repeats enter different degradative pathways: REP sequences are substrates for the mRNA degradative machine [28], while *nemis* sequences are targeted by RNase III. In prokaryotes, this endoribonuclease assists in the maturation of stable RNAs such as the 16 S and 23 S rRNAs. The fate of a few specific mRNAs is also influenced by RNase III-mediated cleavage [27,28].

In order to process *nemis*$^+$ transcripts, RNase III must neither enter into a macromolecular complex nor associate with other cellular components (Figure 1B). Hairpins formed by artificial *nemis*$^+$ transcripts are all cleaved by RNase III, although to different extents (Figure 2). The distance that separates *nemis* TIRs is not relevant for processing. The data also indicate that the degree of complementarity of TIRs, rather than a specific sequence content, is crucial in whether processing occurs. Transcripts spanning elements featuring the same termini (26/26 and 27/27 *nemis*) are cleaved efficiently. In contrast, the bulge in the RNA hairpins formed by TIRs of 26/27 elements results in structures that are resistant to RNase III-mediated cleavage, both *in vitro* and *in vivo* (Figures 2 and 4 respectively). The data reflect an inherent difference between the *N. meningitidis* and the *E. coli* enzymes, since the 26/27 *nemis* RNA substrates can be processed *in vitro* by *E. coli* RNase III. In wholly sequenced neisserial genomes, the 26/27 repeats account for approximately one-quarter of the *nemis* family [13]. *nemis*$^+$ mRNAs refractory to cleavage may be more abundant than those predicted by RNA folding data emerging from *in silico* analyses. Neisserial genomes undergo frequent remodelling, and 'cleavable' 26/26 *nemis* may be replaced by 'uncleavable' 26/27 repeats at any intergenic *nemis*$^+$ site. Examples of such rearrangements are provided by *in silico* comparisons of corresponding regions in the genomes of strains Z2491 and MC58 ([13]; P. P. Di Nocera, unpublished work). Moreover, the overall RNA context in which *nemis* RNA hairpins are embedded may also influence cleavage, and transcripts spanning cleavable *nemis* targets may not be processed *in vivo* (Figure 4C).

Cleavable and uncleavable RNA hairpins formed by *nemis* TIRs both bind RNase III (Figure 3). It has been calculated that fewer than 500 molecules of the enzyme are present in *E. coli* [26]. Should this estimate also apply to *N. meningitidis*, uncleaved *nemis* RNA hairpins may help in tuning RNase III levels by enzyme titration. The hairpin-bound inactive molecules may also provide a 'road block' to 3′ exonuclease activities. Susceptibility or resistance to RNase III-mediated cleavage may either delay or facilitate the turnover of *nemis*$^+$ transcripts. This point is supported by differences both in the steady-state levels of corresponding *nemis*$^+$ and *nemis*$^-$ mRNAs among *N. meningitidis* strains *in vivo* (Figure 5) and in the relative stabilities of the cleavage products of distinct *nemis*$^+$ RNAs *in vitro* (Figures 1B and 6; see also the Experimental section). The set of mRNAs 'decorated' by *nemis* sequences also includes transcripts spanning 26/26 and 27/27 elements which are partly cleaved at *nemis* TIRs, probably due to subtle variations in the structure of RNA stems formed by *nemis* inserted in different orientations [13]. ORFs and elements located either 60–70 bp upstream or 20–30 bp downstream are likely to be co-transcribed. Accordingly, the number of *N. meningitidis* genes potentially regulated by the formation of *nemis* hairpins within their mRNAs could be approx. 100. Of these, many encode transcriptional and/or regulatory factors, and others encode proteins known or hypothesized to be involved in pathogenesis [15,16]. The hypothesis that *nemis*-mediated RNA processing may be relevant in the life cycle of meningococci as pathogenic micro-organisms is substantiated by the knowledge that RNase III, which is not required for viability, is a molecule that is crucial for the survival of meningococci in the infected host [8].

In addition to their role in assembling, re-assorting or disrupting genes, ISs can also activate the expression of neighbouring genes; this generally occurs because of the formation of novel, hybrid promoters at the junction of the IS with the interrupted gene [33]. To our knowledge, *nemis* represent the first example of ISs that are able to influence gene expression by acting as RNA elements. Future analyses should provide a comprehensive list of genes that are co-transcribed along with *nemis* in *N. meningitidis* populations, so clarifying whether the presence/absence of *nemis* repeats at specific chromosomal sites may account for the selective properties of particular pathogenic *N. meningitidis* strains.

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