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The abundant class of *nemis* repeats provides RNA substrates for ribonuclease III in *Neisseriae*

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Abstract

About 2% of the *Neisseria meningitidis* genome is made up by *nemis*, short DNA sequences which feature long terminal inverted repeats (TIRs). Most *nemis* are interspersed with single-copy DNA and are found at close distance from cellular genes. In this work, we demonstrate than RNAs spanning *nemis* of different length and sequence compositions are specifically cleaved at hairpins formed by *nemis* termini by total cellular lysates derived from both *Escherichia coli* and *Neisseria lactamica* strains. The use of cellular extracts from *E. coli* strains impaired in the activity of known ribonucleases let to establish that cleavage at *nemis* TIRs is specifically mediated by the endoribonuclease RNase III. Data set the base for the identification of all of the neisserial genes that are regulated by RNase III because of their physical association with *nemis* DNA. © 2002 Published by Elsevier Science B.V.

Keywords: Repeated DNA family; Terminal inverted repeat; Pathogenic bacteria; RNA hairpin; RNA processing

1. Introduction

Neisseria meningitidis (or meningococcus) and *Neisseria gonorrhoeae* (or gonococcus) are two strictly human pathogens which belong to the same genospecies. Though closely related, the two bacterial species colonize different epithelia and cause notably different diseases. *N. gonorrhoeae* is generally responsible for localized inflammation of the urogenital tract, *N. meningitidis* for meningitis and septicaemia. To rapidly identify potential vaccine candidates, the whole genome sequences of *N. meningitidis* A and B serogroup strains have been recently determined [1,2]. The sequence of a pathogenic *N. gonorrhoeae* strain has also been recently determined (http://dna1.chem.ou.edu/gono.html). Whole genome data provided insights on the unique organization of genetic material in *Neisseriae*. The meningococcus hosts an extraordinary number of different repeated DNA sequences. Of these, many are variously combined in intergenic repeat arrays plausibly involved in recombination processes frequently occurring in *Neisseriae* [1]. Other DNA repeats, as the so-called Correia [3] or *nemis* [4] sequences, are predominantly found as individual units. *Nemis* are miniature DNA insertion sequences (80–160 bp), which feature 26–27 bp long terminal inverted repeats (TIRs). The presence of target site duplications at *nemis* ends, and the identification in sequenced strains of homologous chromosomal regions containing or lacking *nemis* DNA, both indicate that *nemis* are (or have been) mobile genetic elements [4]. Both transposition and recombination events contributed to the genomic spread of this unusual class of DNA repeat family, which is organized in a few major structural subsets and includes, both in A and B serogroup meningococci, ~300 members. Of these, about two-thirds are interspersed with single-copy DNA, and inserted at close distance from coding regions [4]. Both the abundance and the peculiar pattern of interspersion suggest that *nemis* may have regulatory functions. The hypothesis that these repeats may influence gene expression is supported by the finding that in 7/7 *N. meningitidis* genes analyzed, the 5′ termini of the gene transcripts lye within flanking *nemis* sequences [4].

In this work, we provide evidence that *nemis* TIRs fold into hairpin structures, and RNAs encompassing *nemis* sequences are invariably cleaved by cellular lysates from

Abbreviations: bp, base pairs; nt, nucleotide(s); cpm, counts per minute

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either *Escherichia coli* or *Neisseria lactamica* strains at hairpins formed by folding of *nemis* TIRs. Cleavage is specifically mediated by RNase III.

2. Materials and methods

2.1. Bacterial strains and extracts

The *E. coli* strains FB1 (Δ*hisGDCBHAFI-gnd*, *rhaA*), HT115 (*rnc*14::ΔTn10), SK5006 (*thr*, *leu*, pDK39, Cm^r *rnb*500) and its derivatives SK5003 (*pnp*7, *rnb*500) and SK5695 (*rne*1) are described in Ref. [5]. The *N. lactamica* strain 4627 was obtained from the Pasteur Institute. Cellular extracts (S30 and S100) were prepared according to Zubay [6]. *E. coli* strains were grown in LB medium, the *N. lactamica* strain 4627 in GC broth supplemented with 1% Polyvitox (Bio-Merieux).

2.2. Plasmid construction

Plasmids pGEM-378 and pGEM-417 contain *Neisseria* DNA flanking a full-length and an internally rearranged *nemis*, respectively. The pGEM-378 and pGEM-417 inserts are homologous to the intervals 1796373 to 1796751 and 383037 to 383420 of the *N. meningitidis* serogroup B strain MC58 [2], respectively. DNA inserts, obtained by amplifying genomic DNA from the *N. meningitidis* 1055 strain via PCR by using appropriate oligonucleotides as primers, were cloned in the *Sma*I site of the vector pGEM4Z to obtain complementary RNA transcripts, since the vector carries Sp6 and T7 promoters next to the cloning region. Clones were checked by DNA sequencing [7].

2.3. RNA substrates and in vitro cleavage analyses

Uniformly ^{32}P-labeled RNAs were obtained by transcribing in vitro linearized pGEM-378 and pGEM-417 DNAs with either T7 or SP6 RNA polymerase, in reaction mixtures containing ATP, CTP and GTP (500 μM each), UTP (250 μM) and 25 μCi (alpha-^{32}P)-UTP (400 Ci $mmol^{-1}$). Samples aliquots (25,000–30,000 cpm) were incubated with either S30 or S100 bacterial extracts as previously described [5]. Primer extension assays were performed essentially as described [8]. The sequences of the oligomers used as primers in the experiments reported in Fig. 4 are given below:

oligomer **bo** 5′-GCCTTAGCTCAAAGAGAACGATTC-TCTAAG-3′
oligomer **fo** 5′-CAGACAGTACAGACAGATAGTACG-GAACCG-3′
oligomer **lo** 5′-GGACAGGAAGAACACAGCGTTTT-CATCTGA-3′

3. Results

3.1. Transcripts encompassing nemis sequences are cleaved E. coli cellular lysates at specific sites

The DNA fragment cloned in the plasmid pGEM-378 spans the 5′ end region of the *N. meningitidis* gene encoding the orf NM1970 [1] and a flanking, full-length *nemis* element (Fig. 1). The plasmid was used as template to obtain in vitro radiolabeled RNAs of known length directed by either the Sp6 or the T7 polymerase. The incubation of both T7- and Sp6-driven transcripts with *E. coli* S30 cellular extracts resulted in the accumulation of three major classes of RNAs (Fig. 1, lanes 2 to 5). Cleaved RNA species resulted quite stable over incubation times from 20 to 60 min at 37 °C (data not shown). The sizes of the bands marked a to f in Fig. 1 nicely fit with the hypothesis, stemming from RNA extension analyses carried out in vivo [4], that transcripts encompassing *nemis* are cleaved at RNA hairpins formed by *nemis* TIRs. The cleavage pattern was due to soluble activities present in the lysate, since *E. coli*

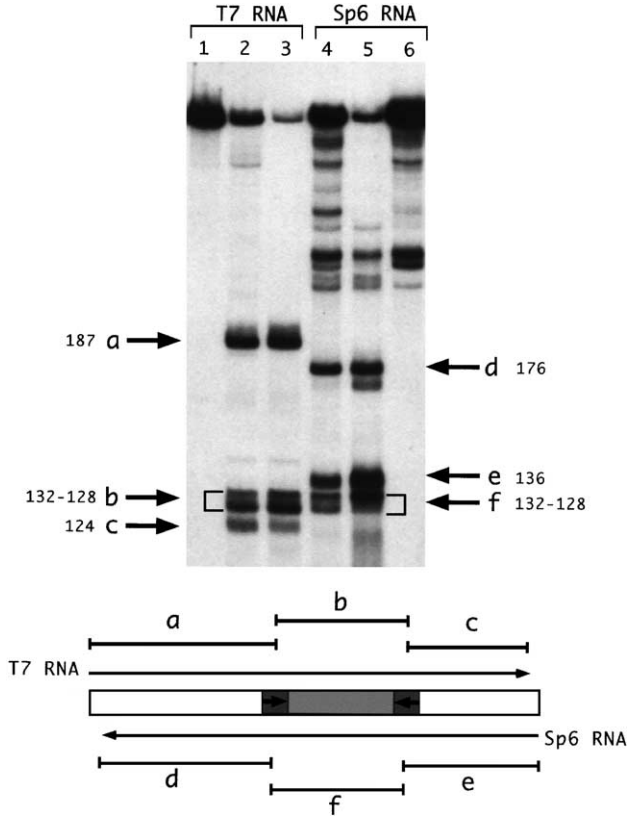

Fig. 1. Specific cleavage occurs at *nemis* RNA. Radiolabeled RNA obtained by transcribing pGEM-378 DNA with either T7 or Sp6 encoded RNA polymerase (lanes 1–3 and 4–6, respectively) were incubated with either 0.5 (lanes 2 and 4) or 1 (lanes 3 and 5) μg of S30 crude cell extracts from the *E. coli* wild-type strain FB1 for 20 min at 37 °C. Reaction products were separated onto a 6% acrylamide-8 M urea gel. Numbers next to bands a to f refer to their length in nt, calculated by co-electrophoresing the reaction products with RNA MW markers not shown in the autoradiogram. Boxed arrows in the diagram at the bottom denote *nemis* TIRs.

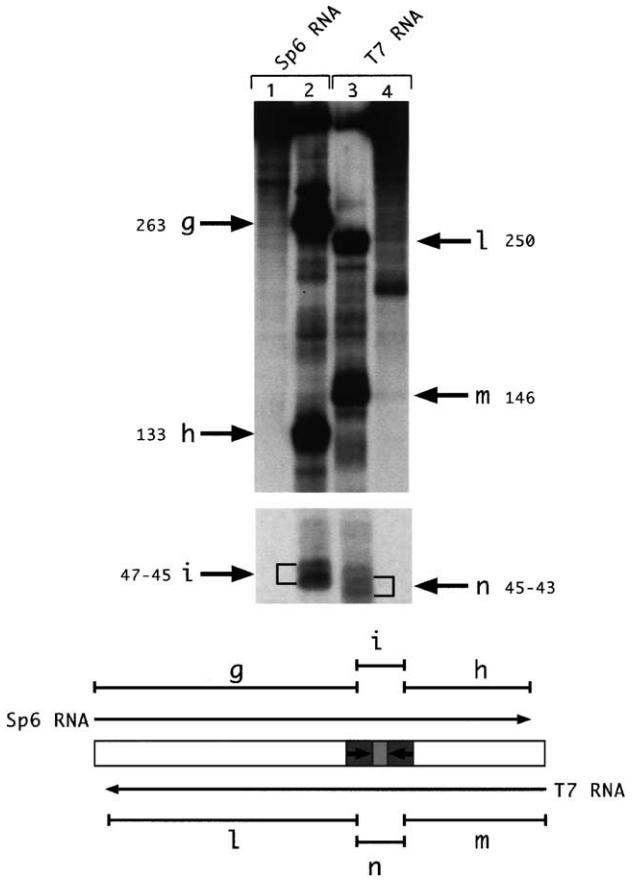

Fig. 2. Cleavage of pGEM-417 transcripts. Radiolabeled pGEM-417 RNA (lanes 1 and 4) were incubated with 0.5 μg of *E. coli* S100 extracts (lanes 2 and 3). The size of bands g to n were determined as in Fig. 1.

S100 cellular extracts produced the same RNA species shown in Fig. 1 (data not shown). Subsequent experiments were therefore carried out with S100 cellular fractions.

The full-length *nemis* cloned in pGEM-378 features 26-bp-long TIRs. Several *nemis* are rearranged because of the loss of a 50-bp-long internal segment. Moreover, elements featuring 27 bp long TIRs, which partly differ in sequence from 26 bp long TIRs, also occur [4]. Different members of the nemis family could generate alternative RNA structure, and RNA cleavage could be influenced by either the sequence content of *nemis* and/or the distance between TIRs. To verify the issue, transcripts from pGEM-417, a plasmid which contains an internally rearranged, 81 bp long *nemis* featuring 27 bp long TIRs, were also challenged with the *E. coli* cellular extracts. Bands marked g to i and l to n in Fig. 2 have the size expected for RNA moieties produced by cleavage of the pGEM-417 substrate at *nemis* TIRs. We also set up competition experiments in which radiolabeled pGEM-417 transcripts were challenged with cellular lysates in the presence of two to five hundred-fold excesses of cold RNA. Cleavage of radiolabeled pGEM-417 transcripts was competed at comparable levels by cold excess of pGEM-417 and pGEM-378 transcripts (data not shown). On the whole, data indicate that transcripts encompassing *nemis* elements, which differ either in size or sequence content, are processed, at least in vitro, with the same efficiency.

3.2. A cellular lysate derived from N. lactamica cleaves nemis RNA

Challenging pGEM-378 transcripts with cellular extracts from a wild type strain of the apathogenic *N. lactamica* species gave essentially the same cleavage pattern produced by the *E. coli* extracts (Fig. 3). The size of the RNA species measured in the experiments shown in Figs. 1–3 is not definitive proof that cleavages occur at *nemis* inverted repeats. Such hypothesis was confirmed by primer extension analyses shown in Fig. 4. The experiments were performed by incubating first cold *nemis* RNAs (either pGEM-378 or pGEM-417) with either *E. coli* or *N. lactamica* cellular lysates. Specific oligonucleotides were subsequently annealed to the in vitro processed b (**bo** oligomer) f (**fo** oligomer), l and n+l (**lo** oligomer) RNA species (see also Fig. 1), and extended with the reverse transcriptase to map cleavage sites. Products of extensions on pGEM-378 transcripts, driven by either the T7 (bands I, panel A, top) or the Sp6 promoter (bands II, panel A, bottom), were obtained in separate experiments. In panel B, bands III and IV denote

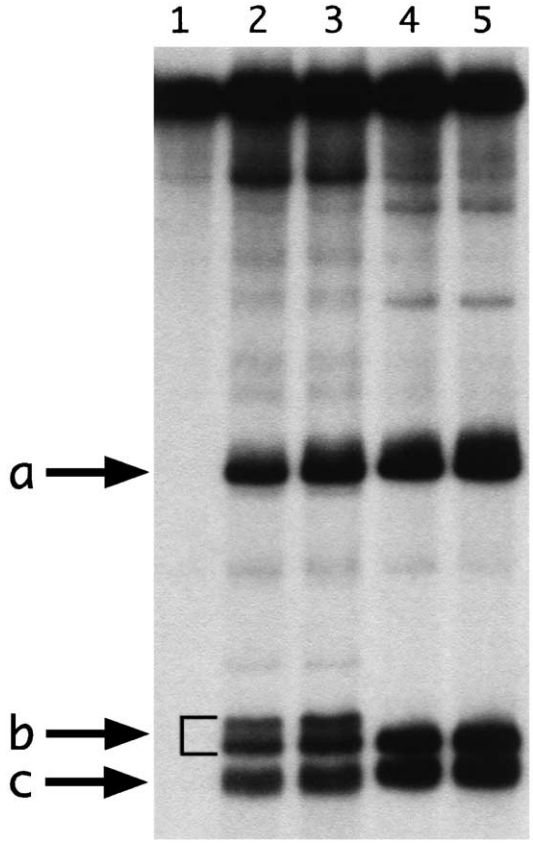

Fig. 3. *N. lactamica* lysates cleave *nemis* RNA. pGEM-378 transcripts (lane 1) were incubated with either 0.5 (lanes 2 and 4) or 1 μg (lanes 3 and 5) of crude S100 extracts from either *E. coli* (lanes 2 and 3) or *N. lactamica* (lanes 4 and 5).

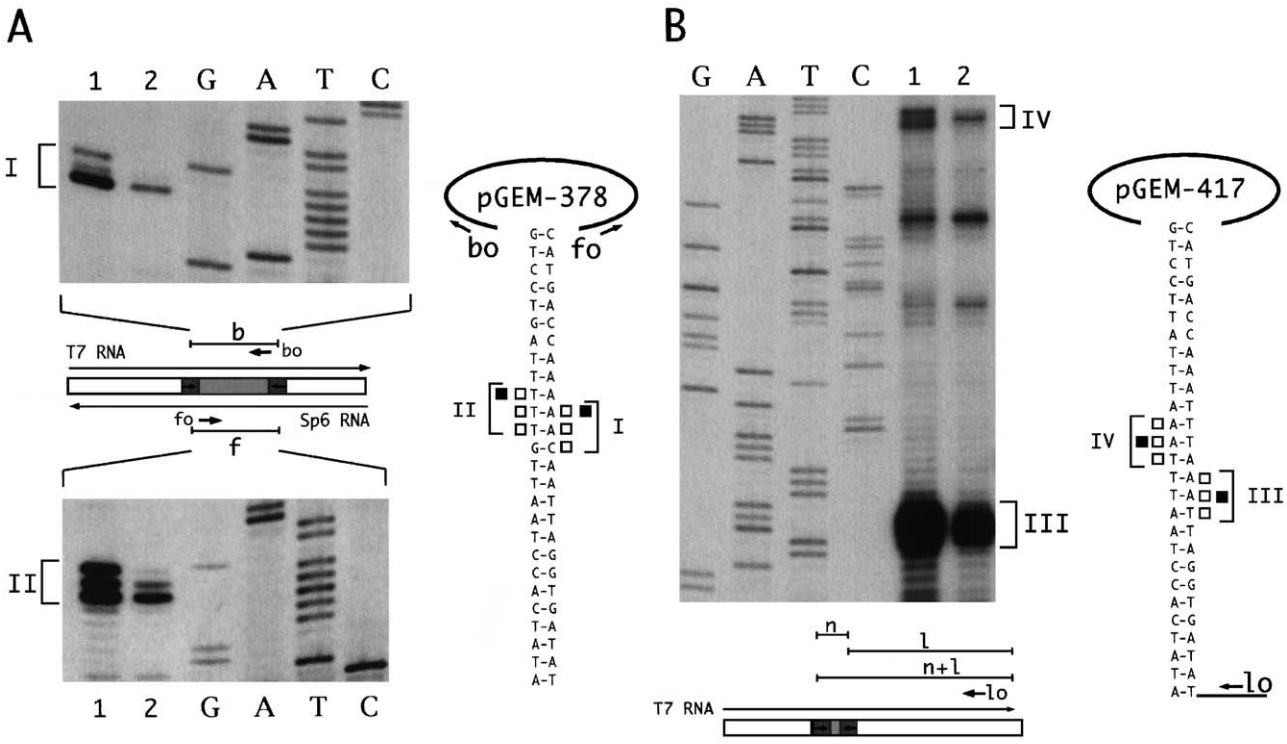

Fig. 4. Cleavage sites at *nemis* hairpins. (A) Cold RNA substrates, obtained by transcribing pGEM-378 with either T7 (upper panel) or Sp6 polymerase (lower panel), were challenged with either *E. coli* (lanes 1) or *N. lactamica* (lanes 2) lysates. Processed RNA species were subsequently mixed with the ^{32}P-5′-end-labeled DNA oligomers **bo** and **fo**. Annealed primer moieties were elongated, in the presence of deoxynucleosides triphosphates, by the SuperScript II reverse transcriptase. Reaction products were separated onto a 8% acrylamide-8 M urea gel. (B) pGEM-417 transcripts directed by the T7 polymerase were challenged with either *E. coli* or *N. lactamica* lysates (lanes 1 and 2, respectively). Samples were subsequently mixed with the ^{32}P-5′-end-labeled DNA oligomer **lo**, and processed as in panel A. Sequencing ladders of the plasmids pGEM-378 and pGEM-417 were obtained by the dideoxy chain termination method by using the **bo**, **fo** and **lo** oligomers as primers. Bands I to IV denote cleavage sites within *nemis* TIRs. Empty and filled squares mark cleavage sites mapped with *E. coli* and *N. lactamica* lysates, respectively.

the elongation products obtained with processed pGEM-417 transcripts driven by the T7 promoter. The additional bands in lanes 1 and 2 of panel B of Fig. 4 may denote either premature stops of the reverse transcriptase or elongation products of minor processed species of pGEM-417 RNA. Slight differences in the cleavage specificity of the *E. coli* and *N. lactamica* processing activities are highlighted in Fig. 4.

3.3. Nemis RNA hairpins are targeted by RNase III

Radiolabeled pGEM-378 transcripts were incubated with S100 cellular extracts derived from *E. coli* strains harboring mutant alleles for specific ribonucleases. Only extracts from HT115, a strain carrying a *rnc* allele encoding an inactive endoribonuclease RNase III [9], were totally unable to cleave the RNA substrate (Fig. 5, lane 5). The prominent band immediately below the input RNA band in lane 5 of Fig. 5 likely corresponds to uncleaved substrate moieties in which the formation of secondary structures caused a block to 3′ exonuclease activities. This conclusion is supported by the finding that the same RNA species is relatively less abundant in samples incubated with cellular extracts from the strain SK5003, in which a mutation in the *pnp* gene inactivates the 3′ exonuclease activity of the phosphonucleotide phosphorylase (Fig. 5, lanes 3 and 4). It is of interest to note that the mRNA-specific RNase E has no role in the process of cleaving *nemis* RNA (Fig. 5, lanes 6 and 7).

4. Discussion

Bacterial cells possess several endoribonuclease activities, which function both in the processing of stable RNAs and the mRNA decay [10–14]. Intergenic sequences may play an important role in the control of RNA enzymatic decay. Both in *E. coli* and *S. typhimurium* REPs, short DNA repeats inserted within operons, protect mRNAs from 3′–5′ exonuclease decay, by forming stem-loop structures able to suppress the action of 3′ endoribonucleases [15–17]. *Nemis*, an abundant class of DNA repeats uniquely found in *Neisseriae*, represent an additional example of intergenic sequences which may influence the expression of neighbouring genes by acting at the RNA level. We recently reported that the 5′ end termini of several *N. meningitidis* transcripts mapped within *nemis* TIRs [4]. Here, we showed

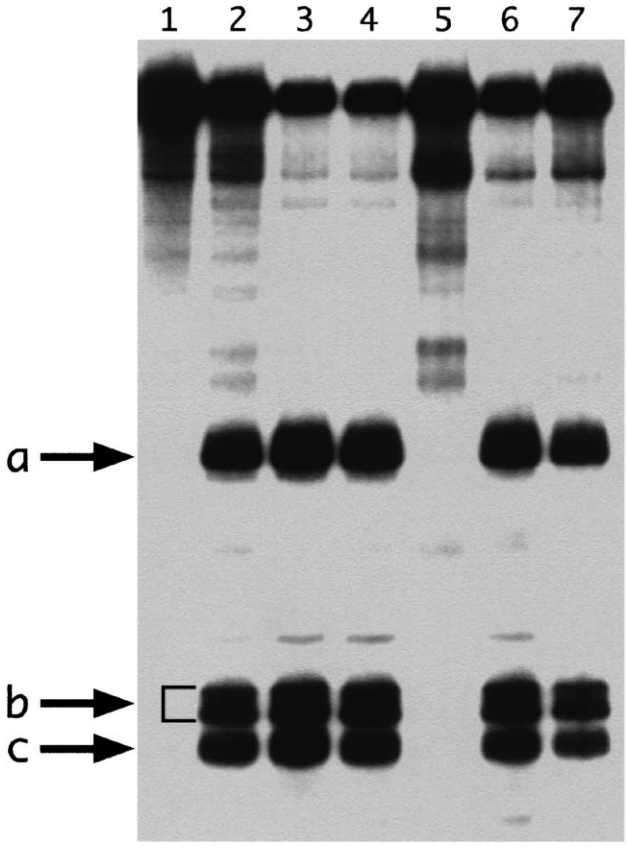

Fig. 5. The endonucleolytic activity responsible for *nemis* RNA cleavage is RNase III. PGEM-378 RNA transcribed by the T7 promoter was incubated for 20 min at 37 °C either alone (lane 1) or with 0.5 μg of S100 cellular lysates from the *E. coli* strains FB1 (lane 2), SK5003 (lanes 3, 4) HT115 (lane 5), SK5695 (lanes 6, 7) grown at 32 °C. Extracts in lanes 4 and 7 derived from SK5003 and SK5695 cells grown to early logarithmic phase at 32 °C and shifted to 44 °C for 45 min before harvesting, to inactivate RNases II and E encoded by the *rne*1 and the *rneB*500 alleles, respectively. Reaction products were separated onto a 6% acrylamide-8 M urea gel.

that cellular lysates from either *E. coli* or *N. lactamica* strains specifically cleave transcripts encompassing *nemis* sequences at hairpins formed by complementary *nemis* TIRs (Figs. 1–3). Double helical RNA structures formed by the folding of *nemis* termini are cleaved at either site, the location of scissile bonds varying in hairpins of different sequence composition (Fig. 4). Cleaved RNA species are rather stable. Plausibly, processed RNA moieties are still kept by hydrogen bonds in robust secondary structures, and hence are protected by exonucleolytic attack. By assaying extracts from *E. coli* strains carrying mutant alleles of genes encoding specific ribonucleases, we settled that the processing activity corresponds to RNase III (Fig. 5).

The *N. meningitidis* genome encodes a 239-aminoacid-long protein homologous to ribonucleases III [1,2] exhibiting 49% of homology (66.7% similarity) to the *E. coli rnc* gene product. RNases III include two functional modules, one responsible for recognition and binding to the target (RIBOc domain), the other for endonucleolytic activity (Double Stranded Recognition Motif or DSRM; see Ref. [18]). By using the SMART program [19], we found that both motifs are conserved in the *Neisseria rnc* gene product, the RIBOc domain starting at position 26 and ending at position 154, the DSRM domain starting at position 161 and ending at position 229 (not shown).

In *E. coli*, bulk mRNA is processed by RNase E [11,14]. The preeminent role of RNase III is to cleave the primary transcript of the *rrn* operons, to provide the immediate precursors to the mature 16S and 23S ribosomal RNA species. Nevertheless, RNase III is known to cleave a few mRNAs, either increasing the stability or accelerating the decay of specific phage and cellular transcripts [10,13,14,20]. Similarly, cleavage of mRNAs reading through *nemis* may provide a mechanism controlling at the post-transcriptional level the half-life of a large number of specific mRNAs in *Neisseriae*. To our knowledge, this is the first evidence that members of an abundant repetitive DNA family provide a series of RNA substrates which are recognized and cleaved by RNase III in prokaryotes. Both the abundance and the genomic distribution of *nemis*, which are preferentially inserted near to cellular genes, suggest that RNase III may act as global regulator in *Neisseriae*. The hypothesis that cleavage of *nemis* RNA plays an important regulatory role is supported by in silico surveys indicating that several transcriptional units are conserved in both pathogenic *N. gonorrhoeae* and *N. meningitidis* strains, but contain *nemis* sequences in the meningococcal strains only [[4]; P.P. Di Nocera, unpublished results]. Thus, intriguingly, post-transcriptional processes involving *nemis* sequences may contribute to the differential expression of homologous genes in different species of the genus *Neisseria*. It will be of interest to ascertain whether specific *nemis* RNA hairpins, either per se or because of the RNA context in which they are embedded, may be refractory to cleavage by RNase III, as the enzyme domain endowed with endonucleolytic activity is not activated in all dsRNA–RNase III interactions [18]. The presence/absence of *nemis* at specific chromosomal locations might as well account for differences in the intracellular levels of specific mRNAs among pathogenic and non-pathogenic meningococcal strains, and future investigations are aimed at clarifying this important issue.

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References

[1] J. Parkhill, M. Achtman, K.D. James, S.D. Bentley, C. Churcher, S.R. Klee, G. Morelli, D. Basham, D. Brown, T. Chillingworth, R.M. Davies, P. Davis, K. Devlin, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, S. Leather, S. Moule, K. Mungall, M.A. Quail, M.-A. Rajandream, K.M. Rutherford, M. Simmonds, J. Skelton, S. Whitehead, B.G. Spratt, B.G. Barrell, Complete DNA sequence of a serogroup

A strain of *Neisseria meningitidis* Z2491, Nature 404 (2000) 502–506.

[2] H. Tettelin, N.J. Saunders, J. Heidelberg, A.C. Jeffries, K.E. Nelson, J.A. Eisen, K.A. Ketchum, D.W. Hood, J.F. Peden, R.J. Dodson, W.C. Nelson, M.L. Gwinn, R. DeBoy, J.D. Peterson, E.K. Hickey, D.H. Haft, S.L. Salzberg, O. White, R.D. Fleischmann, B.A. Dougherty, T. Mason, A. Ciecko, D.S. Parksey, E. Blair, H. Cittone, E.B. Clark, M.D. Cotton, T.R. Utterback, H. Khouri, H. Qin, J. Vamathevan, J. Gill, V. Scarlato, V. Masignani, M. Pizza, G. Grandi, L. Sun, H.O. Smith, C.M. Fraser, E.R. Moxon, R. Rappuoli, J.C. Venter, Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58, Science 287 (2000) 1809–1815.

[3] F.F. Correia, S. Inouye, M. Inouye, A 26-base-pair repetitive sequence specific for *Neisseria gonorrhoeae* and *Neisseria meningitidis* genomic DNA, J. Bacteriol. 167 (1986) 1009–1015.

[4] M. Mazzone, E. De Gregorio, A. Lavitola, C. Pagliarulo, P. Alifano, P.P. Di Nocera, Whole-genome organization and functional properties of miniature DNA insertion sequences conserved in pathogenic *Neisseriae*, Gene 278 (2001) 211–222.

[5] P. Alifano, F. Rivellini, C. Piscitelli, C.M. Arraiano, C.B. Bruni, M.S. Carlomagno, Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA, Genes Dev. 8 (1994) 3021–3031.

[6] G. Zubay, In vitro synthesis of protein in microbial systems, Annu. Rev. Genet. 7 (1973) 267–287.

[7] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

[8] E. De Gregorio, L. Chiariotti, P.P. Di Nocera, The overlap of Inr and TATA elements sets the use of alternative transcriptional start sites in the mouse galectin-1 gene promoter, Gene 268 (2001) 215–223.

[9] H.E. Takiff, S.M. Chen, D.L. Court, Genetic analysis of the *rnc* operon of *Escherichia coli*, J. Bacteriol. 171 (1989) 2581–2590.

[10] D.L. Court, RNA processing and degradation by RNase III, in: G.J. Belasco, G. Brawerman (Eds.), Control of Messenger RNA Stability, Academic Press, San Diego, CA, 1993, pp. 71–116.

[11] A.J. Carpousis, G. Van Houwe, C. Ehretsmann, H.M. Krisch, Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation, Cell 76 (1994) 889–900.

[12] S. Naureckiene, B.E. Uhlin, In vitro analysis of mRNA processing by RNase E in the *pap* operon of *Escherichia coli*, Mol. Microbiol. 21 (1996) 55–68.

[13] A.W. Nicholson, Structure, reactivity and biology of double-stranded RNA, Prog. Nucleic Acid Res. Mol. Biol. 52 (1996) 1–65.

[14] G.A. Coburn, G.A. Mackie, Degradation of mRNA in *Escherichia coli*: an old problem with some new twists, Prog. Nucleic Acid Res. Mol. Biol. 62 (1999) 55–108.

[15] S.F. Newbury, N.H. Smith, E.C Robinson, I.D. Hiles, C.F. Higgins, Stabilization of translationally active mRNA by prokaryotic REP sequences, Cell 48 (1987) 297–310.

[16] S.F. Newbury, N.H. Smith, C.F. Higgins, Differential mRNA stability controls relative gene expression within a polycistronic operon, Cell 51 (1987) 1131–1143.

[17] R.S. McLaren, S.F. Newbury, G.S. Dance, H.C. Causton, C.F. Higgins, mRNA degradation by processive 3′–5′ exoribonucleases in vitro and the implications for prokaryotic mRNA decay in vivo, J. Mol. Biol. 221 (1991) 81–95.

[18] S. Dasgupta, L. Fernandez, L. Kameyama, T. Inada, Y. Nakamura, A. Pappas, D.L. Court, Genetic uncoupling of the dsRNA-binding and RNA cleavage activities of the *Escherichia coli* endoribonuclease RNase III—the effect of dsRNA binding on gene expression, Mol. Microbiol. 28 (1998) 629–640.

[19] J. Schultz, R.R. Copley, T. Doerks, C.P. Ponting, P. Bork, SMART: a web-based tool for the study of genetically mobile domains, Nucleic Acids Res. 28 (2000) 231–234.

[20] P. Regnier, M. Grunberg-Manago, RNase III cleavages in non-coding leaders of *Escherichia coli* transcripts control mRNA stability and genetic expression, Biochimie 72 (1990) 825–834.