

# Insertion of a homing endonuclease creates a genes-in-pieces ribonucleotide reductase that retains function

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In bacterial and phage genomes, coding regions are sometimes interrupted by self-splicing introns or inteins, which can encode mobility-promoting homing endonucleases. Homing endonuclease genes are also found free-standing (not intron- or intein-encoded) in phage genomes where they are inserted in intergenic regions. One example is the HNH family endonuclease, *mobE*, inserted between the large (*nrdA*) and small (*nrdB*) subunit genes of aerobic ribonucleotide reductase (RNR) of T-even phages T4, RB2, RB3, RB15, and LZ7. Here, we describe an insertion of *mobE* into the *nrdA* gene of *Aeromonas hydrophila* phage Aeh1. The insertion creates a unique genes-in-pieces arrangement, where *nrdA* is split into two independent genes, *nrdA-a* and *nrdA-b*, each encoding cysteine residues that correspond to the active-site residues of uninterrupted NrdA proteins. Remarkably, the *mobE* insertion does not inactivate NrdA function, although the insertion is not a self-splicing intron or intein. We copurified the NrdA-a, NrdA-b, and NrdB proteins as complex from Aeh1-infected cells and also showed that a reconstituted complex has RNR activity. Class I RNR activity in phage Aeh1 is thus assembled from separate proteins that interact to form a composite active site, demonstrating that the *mobE* insertion is phenotypically neutral in that its presence as an intervening sequence does not disrupt the function of the surrounding gene.

bacteriophage Aeh1 | gene structure | intervening sequence

Homing endonucleases are a distinctive class of site-specific DNA endonucleases that promote the lateral transfer of their own coding region and flanking DNA between genomes by a recombination-dependent process termed homing (reviewed in ref. 1). Homing endonucleases are often encoded within self-splicing introns and inteins (2–4), but many bacterial and phage genomes possess a significant number of so-called free-standing homing endonucleases that are not encoded within introns or inteins (5, 6). Free-standing endonucleases do not have the benefit of a self-splicing element to minimize their impact on host gene structure and function and, thus, are found at genomic insertion sites that are of low impact, such as intergenic regions. In T4-like phages that infect *Escherichia coli* and related bacteria, free-standing endonucleases are more abundant than intron-encoded versions (6–9) and promote their spread to phage genomes lacking the endonuclease by an intronless homing pathway (10–12).

As a consequence of their abundance in T4-like phages, free-standing endonucleases represent a significant source of genetic variation by promoting recombination between genomes. Many characterized homing endonucleases have recognition sites that lie in genes that function in DNA metabolism, including those that encode aerobic and anaerobic ribonucleotide reductases (RNRs) (13). Three classes of RNRs have been described to date, based on required metal cofactors used to generate a radical intermediate and on structural differences (14). Prokaryotic class Ia aerobic enzymes, typified by the *Escherichia coli* *nrdA* and *nrdB* genes, are  $\alpha_2\beta_2$  heterodimers of

the large NrdA ( $\alpha$ ) and small NrdB ( $\beta$ ) proteins, respectively (15). Moreover, the *nrdA* and *nrdB* genes are well conserved, offering a good target for homing endonucleases. Consistent with this prediction, many phage- and bacterial-encoded RNR genes are interrupted by self-splicing introns or inteins, with the insertion sites often near the active site of the enzymes (16–18). In phages T4 and RB3, the *nrdB* gene is interrupted by a group I intron encoding a HNH family homing endonuclease, I-TevIII (19–21). Of relevance to this study is the free-standing HNH endonuclease, *mobE*, that is inserted between the *nrdA* and *nrdB* genes of phages T4, RB2, RB3, RB15, and LZ7 (9, 22) (Fig. 1A). MobE efficiently spreads between T-even phages, and likely possesses a cleavage site within or nearby *nrdA* or *nrdB* (12, 22). The *nrdA-nrdB* region of T-even phages thus appears to be a hotspot of homing endonuclease-mediated recombination.

Here, we examine the impact of free-standing endonucleases on gene structure and function. We describe a gene arrangement created by the insertion of *mobE* into the coding region of the *nrdA* gene of *Aeromonas hydrophila* T-even phage Aeh1 that possesses the characteristics of a transposition event. The *mobE* insertion fragments the *nrdA* gene at the active site, creating two independent genes that each encode key active site residues of NrdA proteins. Remarkably, the *mobE* insertion is not inactivating for RNR function, and we show that the split NrdA-a and NrdA-b proteins assemble into a complex with the NrdB protein that retains RNR activity. Our results demonstrate that the *mobE* insertion is phenotypically neutral with respect to NrdA function and that, despite the absence of splicing, the presence of an intervening sequence does not disrupt the function of the surrounding gene.

## Results

**A Split *nrdA* Gene in Phage Aeh1 Created by the Insertion of an HNH Homing Endonuclease.** Database searches revealed a *mobE* homolog in the sequenced genome of the *A. hydrophila* T4-like phage Aeh1 (6, 23). Intriguingly, the Aeh1 *mobE* gene is inserted in a different genomic position relative to *mobE* endonucleases in phages T4, RB2, RB3, RB15, and LZ7, where it is inserted between *nrdA* and *nrdB* (Fig. 1A). The Aeh1 *mobE* insertion interrupts the *nrdA* gene, splitting it into two smaller coding

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Abbreviation: RNR, ribonucleotide reductase.

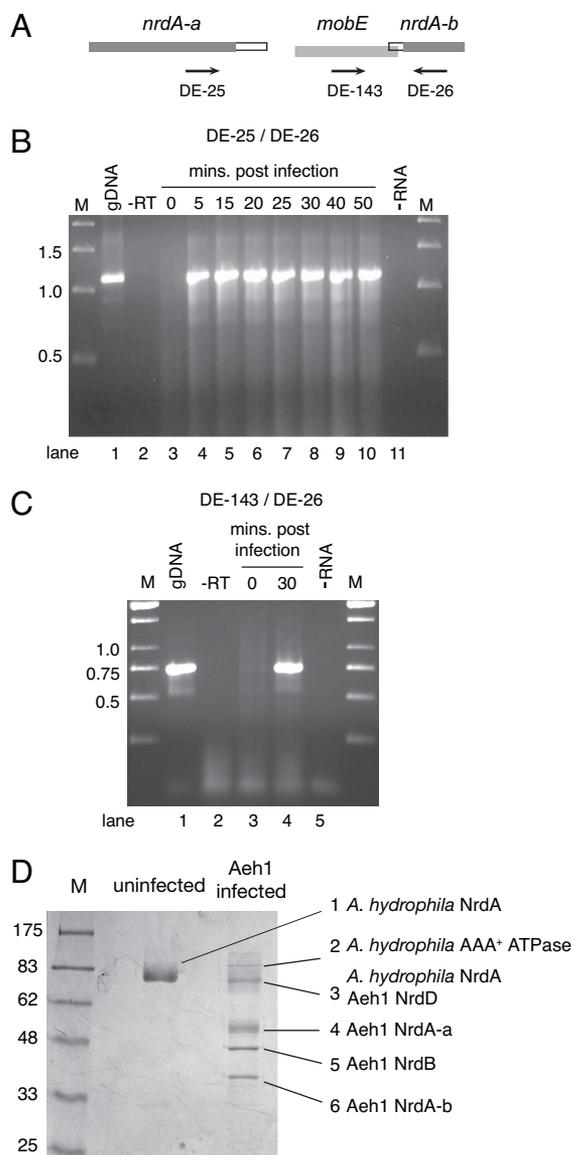
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**Fig. 2.** The Aeh1 *mobE* insertion is not a self-splicing intron or intein. (A) Schematic of the Aeh1 *mobE* insertion indicating the approximate position of primers. (B) RT-PCR with primers DE-25/DE-26. Shown is a 1% agarose gel of aliquots of RT-PCRs using total RNA isolated at the indicated times after Aeh1 infection. gDNA, PCR with Aeh1 genomic DNA; –RT, PCR performed without prior reverse transcriptase step; –RNA, reaction performed without RNA; M, molecular weight markers with sizes indicated in kilobases. (C) RT-PCR with primers DE-143/DE-26 and labeled as in B. (D) Purification of phage Aeh1-encoded RNR proteins from infected *A. hydrophila* cell extracts by dATP-Sepharose chromatography. Shown is a Coomassie-stained 10% SDS/PAGE gel of a dATP-Sepharose purification using uninfected or Aeh1-infected *A. hydrophila* cell extracts. Bands corresponding to proteins identified by mass spectrometry are numbered. M, molecular mass marker with sizes indicated in kilodaltons.

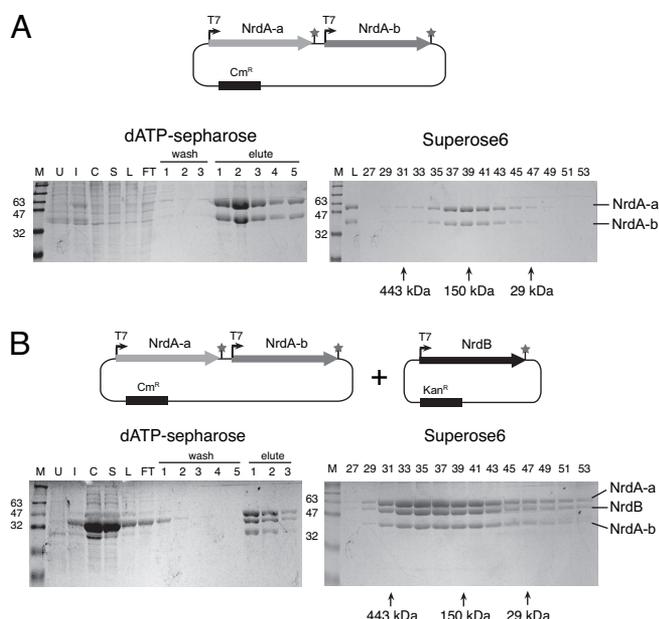
to the genomic sequence. A second RT-PCR was performed such that a product would be amplified only when the *mobE* insertion was not removed by RNA splicing (Fig. 2C). Using RNA isolated 30-min after infection, we amplified an RT-PCR product of 0.73 kb (Fig. 2C, lane 4), the same size as the genomic amplification product (Fig. 2C, lane 1). A similar result was obtained using RNA isolated at earlier time points after Aeh1-infection (not shown). Thus, two independent RT-PCRs demonstrate that the *mobE* insertion is not a self-splicing intron and that the *nrdA-a*, *mobE* and *nrdA-b* genes are cotranscribed.

To determine whether protein splicing created a contiguous NrdA protein, we used dATP-Sepharose chromatography to purify the protein from phage-infected *A. hydrophila* cell extracts (30–32). Class I aerobic RNRs are allosterically regulated by dATP, which binds to a site in the NrdA subunit (24). Based on sequence similarity to the *E. coli* enzyme, the dATP-binding site is located in the Aeh1 NrdA-a protein. As shown in Fig. 2D, five polypeptides eluted from the dATP-Sepharose column when an Aeh1-infected cell extract was applied (Fig. 2D, Aeh1 infected). One of these polypeptides (band 4) was positively identified as the Aeh1 NrdA-a protein by mass spectrometry, consistent with this polypeptide possessing a dATP-binding site. Significantly, two additional polypeptides (bands 5 and 6) were identified by mass spectrometry as the Aeh1-encoded NrdB and NrdA-b proteins, respectively. As expected, the *A. hydrophila* NrdA protein was also purified from both uninfected (Fig. 2D, uninfected, band 1) and Aeh1-infected cells (Fig. 2D, band 3). Mass spectrometry of band 3 revealed a mixture of the *A. hydrophila* NrdA protein and the Aeh1-encoded NrdD protein, the anaerobic RNR.

Purification of proteins that correspond to the predicted sizes of the Aeh1-encoded NrdA-a and NrdA-b proteins is significant because it rules out intein splicing or ribosomal hopping (33, 34) as a mechanism to restore a contiguous large subunit protein. Our data also show that the *nrdA-a* and *nrdA-b* genes have their own ribosome-binding sites (SI Fig. 5) and are independently translated from a common transcript. Moreover, the observation that the Aeh1 NrdA-a, NrdA-b and NrdB proteins coelute from the dATP-Sepharose column implies that they form a tight complex, and that formation of this complex likely occurs by a posttranslational mechanism.

**The NrdA-a and NrdA-b Proteins Assemble Independently of Interactions with NrdB.** To facilitate further characterization of the split Aeh1 RNR gene products, we cloned the Aeh1 *nrdA-a*, *nrdA-b*, and *nrdB* genes into compatible *E. coli*-based overexpression vectors. We found that the NrdA-a or NrdA-b proteins were insoluble when individually expressed, but coexpression of the two proteins increased solubility (not shown). We thus transformed an *E. coli* expression strain with a pNrdA-a/NrdA-b dual-expression plasmid (Fig. 3A) to ask whether the proteins could interact and form a heterodimeric complex independent of interactions with the NrdB small subunit. As shown in Fig. 3A, the NrdA-a and NrdA-b proteins coeluted from the dATP-Sepharose column. To confirm that the NrdA-a and NrdA-b proteins formed a complex, we applied the peak dATP-Sepharose fraction to a gel-filtration column. Both proteins coeluted, and we estimated the molecular mass of the NrdA-a/NrdA-b complex to be  $\approx 186$  kDa with a  $\approx 1:1$  stoichiometry of NrdA-a:NrdA-b (Fig. 3A), in agreement with a predicted 181 kDa  $(\alpha\text{-a}/\alpha\text{-b})_2$  dimer of heterodimers. We repeated the above experiment with the NrdA-a and NrdA-b proteins expressed *in trans* from two compatible *E. coli* plasmids and obtained similar results (not shown). Collectively, these results show that the NrdA-a and NrdA-b proteins form a complex independent of interactions with the NrdB protein.

**Reconstitution of an Active RNR Complex.** We next coexpressed the NrdA-a, NrdA-b, and NrdB proteins in the same *E. coli* strain (Fig. 3B). All three proteins coeluted from the dATP-Sepharose column, consistent with the purification of the three proteins from Aeh1-infected cells (Fig. 2D). We separately purified the NrdB protein by ion-exchange chromatography, and determined that the purified protein did not bind to the dATP-Sepharose column (data not shown), indicating that coelution of NrdB with NrdA-a and NrdA-b from the dATP-Sepharose column was due to its interactions with the large subunit proteins. The dATP-Sepharose fractions were applied to a gel-filtration column, and



**Fig. 3.** Reconstitution of RNR complexes using purified proteins. (A) Coexpression and purification of NrdA-a and NrdA-b by dATP-Sepharose and Superose6 gel-filtration chromatography. A schematic of the pNrdA-a/NrdA-b expression plasmid is shown, with T7 RNA polymerase promoters (T7) indicated by right-facing arrows and transcriptional termination signals indicated by stars. Shown are 12% SDS/PAGE gels with aliquots of each purification step. Fractions from wash or elution steps are indicated above the gel. M, molecular mass standards (in kilodaltons); U, uninduced extract; I, induced extract; C, crude lysate; S, soluble fraction; L, column load; FT, column flowthrough. (B) Coexpression and purification of NrdA-a, NrdB, and NrdA-b, labeled as in A.

we found that all three proteins coeluted in the same fractions, independent of the initial concentration of the sample (Fig. 3B). Estimation of the size of the NrdA-a/NrdA-b/NrdB complex was problematic, however, because the complex eluted over a broad range of fractions, and because the elution profile of the complex depended on the initial sample concentration. At 0.1 mg/ml, the size of the complex was estimated to be  $\approx 256$  kDa, with the NrdB and NrdA-b proteins present in a 0.89:0.95 ratio relative to NrdA-a, suggesting a  $(\alpha\text{-a}/\alpha\text{-b})_2\beta_2$  structure mimicking the  $\alpha_2\beta_2$  bacterial class I RNRs. The separately purified NrdB protein was also applied to the gel-filtration column, and found to elute in a different range of fractions than those of the NrdA-a/NrdA-b complex, and the NrdA-a/NrdA-b/NrdB complex (data not shown).

We assayed the purified proteins for aerobic RNR activity. No RNR activity was detected with the NrdA-a/NrdA-b complex alone, or with the NrdB protein by itself (Table 1). Instead, we observed RNR activity when the separately purified NrdA-a/NrdA-b and NrdB proteins were combined (Table 1). The purified NrdB required reconstitution by iron to acquire the typical diiron/tyrosyl radical cofactor (0.47 radical/dimer) (Fig. 4A). Using the reconstituted NrdB protein and DTT as an artificial electron donor, we observed a specific activity of close to 100 units/mg protein for the complex, and the dissociation constant for NrdA-a/NrdA-b versus NrdB was estimated to be  $0.03 \mu\text{M}$  (Fig. 4B). Enzymatic activity was allosterically stimulated by both ATP ( $K_L = 1.2$  mM) and dATP ( $K_L = 0.12$  mM) (Fig. 4B Inset). The Aeh1 NrdA-a protein appears to have an N-terminal ATP-cone domain, which is responsible for dATP-dependent inhibition of enzyme activity in most RNRs (14, 24). However, two critical histidine residues responsible for this inhibition are replaced by Gln-54 and Ala-83 in the Aeh1 NrdA-a protein (15), which plausibly explains its lack of dATP-

**Table 1.** The Aeh1 NrdA-a/NrdA-b/NrdB complex has high enzyme activity

Added protein	Specific enzyme activity, units/mg	
	Aeh1 NrdA-a/NrdA-b	Aeh1 NrdB
Aeh1 NrdA-a/NrdA-b	0.8*	94 $\pm$ 1
Aeh1 NrdB	94 $\pm$ 1	0.3*
<i>A. hydrophila</i> NrdA	n.a.	2.4 $\pm$ 0.3 <sup>†</sup>
<i>E. coli</i> NrdA	n.a.	1.3 $\pm$ 0.02
<i>E. coli</i> NrdB	0.5 $\pm$ 0.01	n.a.

Amounts of protein used were 4  $\mu\text{g}$  of Aeh1 NrdA-a/NrdA-b, Aeh1 NrdB, *A. hydrophila* NrdA, and *E. coli* NrdB, and 1  $\mu\text{g}$  of *E. coli* NrdA. n.a., not applicable.

\*Background enzyme activity of 4  $\mu\text{g}$  of Aeh1 NrdA-a/NrdA-b, or 4  $\mu\text{g}$  of Aeh1 NrdB.

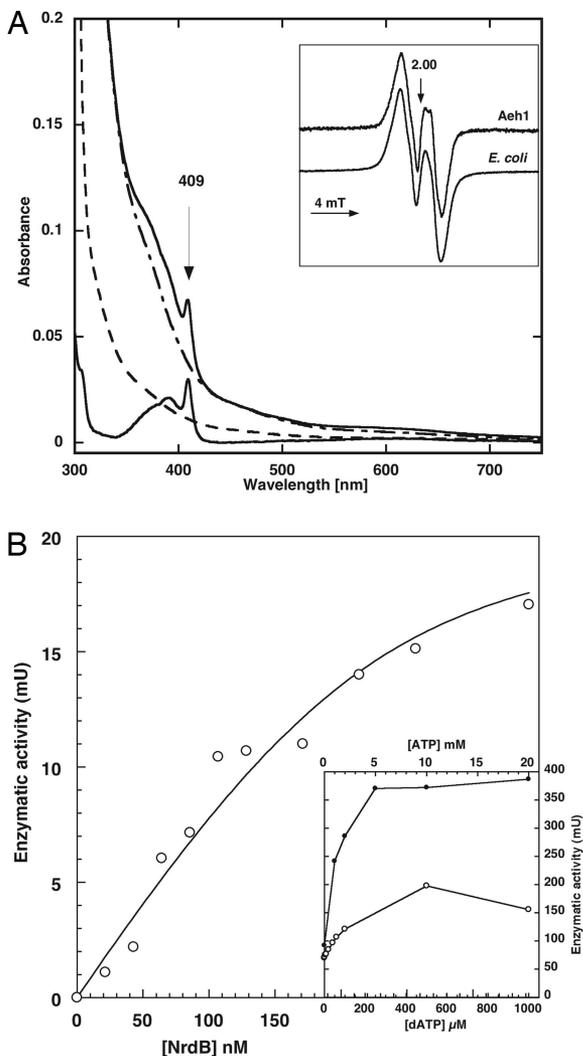
<sup>†</sup>Background activity of 4  $\mu\text{g}$  of *A. hydrophila* NrdA was 1.8 units/mg.

dependent inhibition. Importantly, the Aeh1 NrdA-a/NrdA-b and NrdB components showed no cross reactivity with the *A. hydrophila* NrdA protein, or with the *E. coli* NrdA or NrdB proteins (Table 1). Collectively, these results show that the NrdA-a, NrdA-b, and NrdB proteins copurify through two columns, form a tight complex, and exhibit RNR activity.

## Discussion

Phage genomes are littered with free-standing homing endonuclease genes (6, 7, 9), characterized examples of which function as selfish genetic elements by promoting their spread between phage genomes (10–12). Many free-standing endonucleases are found at genomic insertion sites that are tolerated by the host because they are of low impact, such as intergenic regions. Here, however, we describe an insertion of a homing endonuclease gene, *mobE*, into a conserved and critical gene of DNA metabolism, the large subunit gene (*nrdA*) of aerobic RNR. Significantly, the *nrdA* gene of phage Aeh1 is split such that active site residues in the  $\beta/\alpha$ -barrel domain are encoded in two separate genes, *nrdA-a* and *nrdA-b*, a unique organization for RNR genes. We show that, despite the *mobE* insertion, phage Aeh1 possesses a functional class I RNR enzyme with a composite active site formed by residues contributed by the NrdA-a and NrdA-b proteins in complex with the NrdB protein. Our results have implications for the evolution of structure and function of RNRs, and more generally suggest that novel catalytic sites could be created from polypeptides that have been shuffled between genomes as a consequence of homing endonuclease mobility.

The observation that T4-like phages related to Aeh1 have an uninterrupted *nrdA* coding region strongly suggests that the Aeh1 split gene structure resulted from the insertion of *mobE* into *nrdA*. Furthermore, the intergenic location of *mobE* in phages T4, RB2, RB3, RB15, and LZ7 also supports a scenario whereby *mobE* invaded a contiguous *nrdA*-coding region in a phage ancestral to Aeh1 (6, 22). The alternative hypothesis, that the *nrdA* gene was already split, and *mobE* invaded the *nrdA-a* and *nrdA-b* intergenic region is unlikely, because *nrdA* genes are contiguous in all organisms examined to date. Although the recognition and cleavage sites of *mobE* are uncharacterized, inheritance of genetic markers in phage crosses indicate that the *mobE* cleavage sites are likely located within or nearby the *nrdA* or *nrdB* genes (22), suggesting that a rare transposition event may have resulted in *mobE* invading the Aeh1 *nrdA* gene. Transposition of homing endonucleases to nonallelic sites is thought to result from the sequence-tolerant binding ability of homing endonucleases, facilitating binding and cleavage at sites that share nucleotide similarity with the endonuclease's primary recognition site. Illegitimate DSB-repair of the cleaved non-allelic site by using short stretches of DNA homology surrounding the endonuclease's insertion site may provide a mechanism



**Fig. 4.** Enzyme activity of Aeh1 NrdA-a/NrdA-b and NrdB proteins. (A) Uv-vis spectra of 18  $\mu\text{M}$  Aeh1 NrdB protein: as purified (dashed line), after iron reconstitution (upper solid line), after iron reconstitution and scavenging of tyrosyl radical by 10 mM hydroxyurea treatment (dash-dotted line), subtraction of before and after hydroxyurea treatment (lower solid line). (Inset) Electron paramagnetic resonance spectrum of iron reconstituted Aeh1 NrdB (45  $\mu\text{M}$  protein, 0.47 radical/dimer) and *E. coli* NrdB. (B) Titration of enzyme activity in presence of increasing concentrations of Aeh1 NrdB. Curve fitting was performed as described (42) and gave  $K_D$   $0.034 \pm 0.057$   $\mu\text{M}$  (the enzyme activity level required an Aeh1 NrdA-a/NrdA-b concentration of 0.22  $\mu\text{M}$ , which results in high standard error). (Inset) Titration of enzyme activity in presence of increasing concentrations of ATP (filled circles) and dATP (open circles).

by which the endonuclease could transpose to a new genomic location (35).

Because many characterized homing endonucleases have recognition and cleavage sites within protein-coding genes, transposition of free-standing endonucleases into coding regions by illegitimate DSB repair may occur at an appreciable rate (36–38). A Seg-like homing endonuclease is found associated with a split gene structure in the family B DNA polymerase (gp43) of *Aeromonas salmonicida* phage 25 (23, 39). Related phage possess a split gene 43 separated by up to  $\approx 3$  kb of intervening sequence without a Seg-like endonuclease. The insertions in gene 43 occur such that the DNA polymerases are split between two defined units of protein structure, separating amino acid residues involved in polymerase function; presumably these split gene

products must assemble to form a functional enzyme. A similar situation is observed in the case of the T4-encoded type II topoisomerase, which is split into two smaller genes (39 and 60) by the insertion of an HNH endonuclease, *mobA* (7) (D. Shub, personal communication).

Previously characterized bacterial class I RNR enzymes are a  $\alpha_2\beta_2$  complex composed of NrdA ( $\alpha$ ) and NrdB ( $\beta$ ) homodimers (15), although recent characterization of a *P. aeruginosa* class I enzyme suggested a higher oligomeric structure (40). In phage Aeh1, however, we show that the RNR holocomplex is composed of two split large subunit proteins, NrdA-a and NrdA-b, and the small subunit protein, NrdB. Interestingly, a documented example of a split RNR gene involves a class II RNR enzyme, *nrdJ*, in *P. aeruginosa* and other  $\gamma$ -proteobacteria (41). The two halves of *nrdJ* are translated separately, and class II RNR activity was detected in *P. aeruginosa* cell extracts. The split, however, is not at the active site of the enzyme (as is described here), and is not created by the insertion of a homing endonuclease.

Our data raise the intriguing question as to the nature of the mechanism(s) by which active site residues on the separate NrdA-a and NrdA-b proteins are assembled correctly to form a composite active site domain and a fully functional large subunit. This mechanism must involve protein-protein interactions, because we find no evidence of RNA or protein splicing to remove the *mobE* insertion from the *nrdA-a/mobE/nrdA-b* transcript, and because we can purify a NrdA-a/NrdA-b/NrdB complex from Aeh1-infected cells. In this regard, the dimerization interface of the *E. coli* NrdA subunit has been well characterized (15), and the amino acid residues involved in dimerization are conserved in the phage Aeh1 NrdA-a protein, suggesting a mechanism by which the NrdA-a protein might dimerize. Moreover, the observation that we can purify an NrdA-a/NrdA-b complex in the absence of NrdB shows that heterodimerization does not require interactions with NrdB. In considering a potential mechanism by which the two proteins assemble, we noted that the amino acid composition of the NrdA-a and NrdA-b specific ‘tails’ are enriched in charged residues (Fig. 1C). An attractive hypothesis is that the tails facilitate association of NrdA-a and NrdA-b by mediating protein-protein interactions. Interestingly, our data indicate that the split NrdA-a/NrdA-b proteins form a complex with NrdB (Fig. 4B) that has a dissociation constant almost 10-fold stronger than the corresponding *E. coli* NrdA/NrdB complex (42).

One critical finding was that the split Aeh1 RNR proteins have an activity ( $\approx 100$  units/mg) similar to that reported for class I RNR  $\alpha_2\beta_2$  enzymes that are composed of a contiguous NrdA protein, including the enzyme from the related T-even phage, T4 (SI Table 2). In phage T4, the *nrdA* gene is not essential, but *nrdA* mutants have a reduced burst size and a delayed DNA synthesis phenotype compared with wild-type phage (43, 44). The phenotype of *nrdA* mutant phage suggests a strong selective advantage for T-even phage to possess a functional class I RNR, as wild-type phage would quickly out-compete mutant phage. It is tempting to speculate that the *mobE* insertion into the Aeh1 *nrdA* gene may have been tolerated initially because the gene is not essential. We suggest, however, that natural selection would quickly favor phage variants in which a mechanism arose to assemble a functional large subunit from two split halves, because such phage would be at a replicative advantage over *nrdA* mutant phage. Although the molecular mechanism(s) that promotes assembly of the split RNR proteins remains to be elucidated, it is clear that the *mobE* insertion is phenotypically neutral with respect to *nrdA* function, thus minimizing the impact of the insertion on phage viability.

## Materials and Methods

**Bacteria and Phage.** *E. coli* DH5 $\alpha$  was used for cloning, and *E. coli* BL21(DE3) was used for protein expression. *A. hydrophila* C-1

was used for propagation of phage Aeh1 (obtained from the Félix d'Hérelle Reference Center for Bacterial Viruses at Laval University, Quebec, Canada).

**Phage Infections.** Overnight cultures of *A. hydrophila* C-1 were diluted 1/100 in fresh TSB media (EMD BioScience, San Diego, CA) and grown at 30°C to an  $A_{600}$  of 0.4 ( $\approx 2 \times 10^8$  cells per ml). Aeh1 was infected at a multiplicity of infection of 4–8. For RNA isolation, 3-ml aliquots were removed at various time points. RNA was isolated according to manufacturer's guidelines (Qiagen, Valencia, CA) and treated with TURBO DNase (Ambion, Austin, TX). For protein isolation, 300 ml of *A. hydrophila* was infected as above, and cells were collected after 20 min and frozen at  $-80^\circ\text{C}$ .

**RT-PCR.** RT-PCR was performed by using 5  $\mu\text{g}$  of total RNA, 20 pmol of primer DE-26, and M-MuLV reverse transcriptase (NEB, Pickering, Canada) in the supplied buffer. A 5- $\mu\text{l}$  aliquot of each reverse transcription reaction served as template for the amplification of the cDNA using *Taq* DNA polymerase (NEB) in supplied buffer with primers DE-25/DE-26 or DE-143/DE-26.

**Protein Purification.** Details of cloning the Aeh1 *nrd* genes and purification of RNR proteins are available in *SI Materials and Methods*.

**RNR Assays.** In standard assays, proteins were incubated in 50 mM Tris-HCl (pH 7.5)/5 mM ATP/30 mM magnesium acetate/30 mM DTT/0.7 mM [ $^3\text{H}$ ]CDP ( $\approx 20,000$  cpm/pmol) for 20 min at 25°C in a final volume of 50  $\mu\text{l}$ . The amount of dCDP formed was determined by the standard method as described (45). One unit of enzyme corresponds to 1 nmol of dCDP formed per min, and

specific activity is units/mg of protein.  $K_D$ ,  $V_{\text{max}}$ , and  $K_L$  values were obtained by direct curve fitting as described (15, 42) by using KaleidaGraph software (Synergy Software). Before assaying, the NrdB protein was reconstituted at 25°C by reaction with anaerobic ferrous iron ascorbate solution under aerobic conditions as described (46) with a molar ratio of three irons per NrdB polypeptide. After 5 min, the reaction mixture was desalted on a NAP-10 column (GE Healthcare, Piscataway, NJ) equilibrated with buffer B and concentrated with Centricon 30 (Millipore, Bedford, MA).

**Mass Spectrometry.** Mass spectrometry was performed at the Functional Proteomics Facility and the Biological Mass Spectrometry Laboratory at the University of Western Ontario. The Aeh1-encoded NrdA-a, NrdA-b, and NrdB proteins were identified by MALDI analyses of proteolytic digestions of excised spots. The *A. hydrophila*-encoded NrdA, AAA+ ATPase, and Aeh1-encoded NrdD proteins were identified by Q-TOF MS/MS analyses after separation of peptides by liquid chromatography.

**Oligonucleotides.** Sequences of oligonucleotides used in cloning and RT-PCR experiments are available in *SI Materials and Methods*.

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