The phage T4 *nrdB* intron: a deletion mutant of a version found in the wild

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Bacteriophage T4 possesses three self-splicing group I introns. Two of the three introns are mobile elements; the third, in the gene encoding a subunit of the phage nucleotide reductase (*nrdB*), is not mobile. Because intron mobility offers a reasonable explanation for the paradoxical occurrence of large intervening sequences in a space-efficient eubacterial phage, it is puzzling that the *nrdB* intron is not mobile like its compatriots. We have discovered a larger *nrdB* intron in a closely related phage, and we infer from comparative sequence data that the T4 intron is a deletion mutant derived from this larger intron. This larger *nrdB* intron encodes an open reading frame of 269 codons, which we have cloned and overexpressed. The overexpressed protein shows a dsDNA endonuclease activity specific for the intronless *nrdB* gene, typical of mobile introns. Thus, we believe that all three introns of T4 are or were mobile "infectious introns" and that they have entered into and been maintained in the phage population by virtue of this efficient mobility.

[Key Words: Bacteriophage T4; *nrdB* intron; endonuclease mobility]

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It is generally true that prokaryotic genes do not have introns. Because prokaryotic genomes tend to be smaller and replicate faster than eukaryotic genomes, it is thought that introns and other nonessential DNA sequences have been kept out of prokaryotes by selective pressure to streamline the genome (for particularly cogent reviews of this controversial topic, see Doolittle 1987; Hickey et al. 1989).

An extreme exception is the occurrence of three group I introns in the *Escherichia coli* bacteriophage T4 (for review, see Belfort 1990). Phage T4 has a 166-kb DNA genome, which otherwise appears to be highly streamlined; for example, promoters and even translational initiation regions are often found to lie nested within the coding sequence of upstream genes. That these three introns remain in the space-efficient T4 genome may suggest that there is some selective pressure keeping them there.

The introns occur in the genes encoding the phage dTMP synthase (*td*), the small subunit of the phage nucleotide reductase (*nrdB*), and a protein of unknown function called *sunY*. It is curious that the two proteins of known function catalyze steps in the same pathway—de novo dNTP synthesis from rNTP pools—and, furthermore, that they lie at the only two steps that consume reducing equivalents (ultimately NADPH) from the reserves of the host cell. (Although *sunY* encodes an unknown function, it lies very close to *nrdC*, the phage thioredoxin.) Group I splicing itself relies on an exogenous ribonucleotide and may be inhibited by guanosine analogs; most cofactors involved in redox transactions are analogs of nucleotides and are often derived directly

from adenosine or guanosine (White 1983). These facts have led to speculation that these introns are involved in a feedback regulatory loop that controls the level of dNTP synthesis in the infected cell and, therefore, confer a selective advantage upon intron-containing phage (Gott et al. 1986; Goodrich-Blair et al. 1990).

A different (but not incompatible) hypothesis proposes that the introns are mobile genetic elements. This was suggested by the inconsistent occurrence of the introns in the closely related phages T2 and T6 (T2 has no introns, and T6 has only the *td* intron) (Pedersen-Lane and Belfort 1987; Quirk et al. 1989a). This hypothesis has been substantiated by recent direct experimental evidence demonstrating that the *td* and *sunY* introns are indeed actively mobile (Quirk et al. 1989b); both introns encode DNA endonucleases highly specific for intronless *td* (Bell-Pedersen et al. 1989, 1990; Chu et al. 1990) or *sunY* (Bell-Pedersen et al. 1990) genes. The resulting double-stranded DNA (dsDNA) breaks appear to initiate an efficient unidirectional gene conversion event, resulting in the conversion of intronless to intron-plus genes in a mixed infection. This high-frequency conversion to intron-plus genes might be expected to easily maintain the introns in the phage population, even in the face of other selective pressures to remove them.

Group I intron mobility appears to be a general phenomenon (Dujon 1989a; Perlman and Butow 1989; Scazzocchio 1989). Examples of mobile group I introns now include the yeast mitochondrial ω (Jacquier and Dujon 1985) and *al4* (Delahodde et al. 1989; Wenzlau et al. 1989) introns, a nuclear rRNA intron in *Physarum* (Muscarella and Vogt 1989), and a chloroplast rRNA intron in

Chlamydomonas (Lemieux et al. 1988). Intron mobility may explain apparent horizontal transfer events, in which very similar introns have been found in homologous sites of genes in highly divergent organisms (e.g., Lang 1984; Trinkl and Wolf 1986).

Although high-frequency intron mobility may account for the presence of the *td* and *sunY* introns in T4, it fails to explain the presence of the immobile *nrdB* intron. As ribonucleotide reductase catalyzes the first committed step to phage dNTP synthesis, the *nrdB* intron should be central to any regulation of this process at the level of mRNA splicing. If anything more than intron mobility were maintaining introns in T-even phage populations, we would expect to learn about it from *nrdB*.

We describe here our fortuitous discovery that a wild phage isolate called RB3 possesses a similar but longer intron in its *nrdB* gene. Unlike the 598-bp T4 intron, this 1091-bp RB3 intron encodes a long open reading frame (ORF) of similar length and composition to those encoded by other mobile introns. The RB3 intron ORF produces a DNA endonuclease that targets an intronless *nrdB* gene, a hallmark of a mobile group I intron. We think that the T4 *nrdB* intron is not mobile because it is a deletion mutant of this intron. We propose that all three T4 introns were "infectious introns" (Lambowitz 1989) inserted in the phage genome and maintained there (at least in part) by virtue of an efficient intron mobility mechanism.

Results

PCR fingerprinting of phage isolates

We were interested in surveying the distribution of introns in wild phage populations. We devised a simple polymerase chain reaction (PCR)-based fingerprinting technique to screen single plaques from the wild and find new T-even phages. We used an existing collection of T-even phages—the RB strains, collected from Long Island, New York, in 1964—as positive controls in the development of this PCR fingerprinting screen.

The PCR fingerprints used 10 amplification primers directed at regions of five strategically chosen T4 genes. Two were chosen as genes expected to be highly conserved among T-evens: genes 32 and 43, which encode the single-stranded DNA-binding protein and the DNA polymerase of the phage and which are both required for phage replication. We expected nearly every T-even isolate to possess enough sequence similarity to genes 32 and 43 of T4 that we could amplify one or both of these diagnostic fragments. The other three target genes were the three known intron-containing genes *td*, *nrdB*, and *sunY*. These amplification primers annealed to exon sequence on either side of the intron insertion point, so the fingerprint of an intron-plus phage would show a large PCR product and the fingerprint of an intronless phage would show a small PCR product. We used this mixed 10-primer set to simultaneously amplify fragments from the DNA in resuspended phage plaques.

Figure 1 shows the fingerprints of six of the seven clas-

sic T phages. The technique easily distinguishes a T-even plaque from the plaques of other T phages. However, because some T-even phage may have diverged sufficiently from T4 that some primers fail to anneal, we did not expect to detect all five diagnostic bands from every phage isolate. For example, note that we failed to pick up the intron-plus *td* gene or the intronless *nrdB* gene in T6.

We PCR-fingerprinted 24 phage from the RB collection that were available to us. All 24 phage gave positive fingerprints of from two to five of the expected bands, so we considered the technique adequate for the purpose of screening and rapidly isolating more T-even phage from the wild (S.R. Eddy, L. Zwerdinger, and L. Gold, unpubl.). We were surprised to find that 7 of the 24 phage reproducibly gave a band of unexpectedly large size, and we followed up this observation.

Figure 1 shows the PCR fingerprint of a representative of these seven phage, RB3. Because the fingerprint of RB3 showed the other four products at their expected sizes, we could guess that the larger band was produced from the *nrdB* gene of these phage. Figure 1 also shows that the large product is indeed amplified by the *nrdB*-specific primers alone. Restriction mapping of this PCR fragment indicated that it contained a sequence differing from that of T4 by an insertion of ~500 bp in the amino-terminal part of the *nrdB* intron ORF.

The T4 *nrdB* intron ORF has been enigmatic. It is actually composed of two overlapping ORFs: A short, 22-codon ORF with a translational initiation region blocked by a strong putative secondary structure (a characteristic shared with the other two intron ORFs in T4) overlaps a longer 97-codon ORF with no convincing translational initiation region of its own. The 97-codon ORF possesses a lysine/arginine-rich amino acid composition and two repeats of a zinc-finger-like sequence at its carboxyl terminus (Gott et al. 1986, 1988; Shub et al. 1988; Liu 1990). β -Galactosidase protein fusions into both of these frames had shown activity only from the shorter ORF and none from the longer ORF (Gott et al. 1988; Liu 1990). Ribosomal toeprinting experiments (D. McPheeters and L. Gold, unpubl.) also indicated that the UUG start of the 22-codon ORF was an efficient translational initiation region but that the AUG of the 97-codon ORF was not. These observations led to the suggestion that certain conditions might lead to a ribosomal frameshift event that fused the products of the two reading frames (Gott et al. 1988; Liu 1990). However, the insertion (relative to T4) in the RB3 intron puts the 22-codon ORF and the 97-codon ORF into the same long reading frame, that is, the T4 intron contains a deleted copy of the RB3 intron ORF.

Sequence of the new intron

Using template produced by asymmetric PCR amplification of genomic RB3 phage DNA, we sequenced the entirety of the PCR product produced by *nrdB*-specific fingerprinting primers. As shown in Figure 2, the sequence is virtually identical to that of the T4 intron, except for

Figure 1. PCR fingerprints of phage plaques. Single plaques of the classical phages T2, T3, T4, T5, T6, and T7 were picked from plates and fingerprinted as described in Materials and methods. As expected, only the T-even phages generate fingerprints. Shown next is the fingerprint of the wild isolate RB3 and then a conventional PCR using just the two *nrdB*-specific PCR primers, illustrating that the *nrdB* intron-containing fragment amplified from RB3 is significantly larger than was expected. (Right) A representation of the expected band arrangements from the intron-containing phage T4 and the intronless phage T2, as well as the fingerprint of RB3.

491 bp of new sequence in the intron ORF. As expected, this new sequence puts the two short T4 *nrdB* ORFs in the same long reading frame. The RB3 *nrdB* intron is predicted to encode a 269-amino-acid protein with a molecular mass of ~31 kD.

This predicted protein has features characteristic of some DNA-binding proteins. First, it is highly basic, possessing a large number of lysine and arginine residues. Second, as was noticed in the longer of the T4 ORFs, it has two carboxy-terminal zinc-finger-like motifs of the form CxxC-[12-13]-HxxxC. A search of the NBRF protein data base (release 25) using the FASTA algorithm of Pearson and Lipman (1988), and a TFASTA search of the GenBank nucleic acid data base (release 64), revealed no convincing sequence similarity to known protein sequences. The sequence does not show the LAGLIDAG motif characteristic of most intron ORFs (Hensgens et al. 1983; Waring et al. 1984) nor does it show the GIY-YIG motif shared by the *td* intron ORF and some fungal mitochondrial intron ORFs (Burger and Werner 1985; Michel and Dujon 1986; Cummings et al. 1989). Furthermore, although retroviral endonucleases (e.g., integrases), as well as some group II intron ORFs, often possess carboxy-terminal zinc-finger-like sequences (Doolittle et al. 1989), the RB3 *nrdB* intron ORF sequence showed little similarity to these protein sequences.

The nucleotide sequence suggests how the T4 intron could have arisen. The extra 491 bp in the RB3 intron is

flanked by direct repeats of 12/15-bp identity. Direct repeats have been shown to be hot spots for deletion formation in T4 as well as in other organisms (Singer and Westlye 1988). We propose that the T4 copy of the *nrdB* intron has suffered a simple deletion event, as shown in Figure 3.

Proposed structure of the intron

Figure 4 shows the proposed secondary structure of the RB3 intron, which, like the T4 intron, is a typical group IA intron, drawn according to conventions laid down by Burke et al. (1987). The stem-loop structures drawn here are considered well-proven for the group IA introns on phylogenetic and experimental grounds (Burke 1988; Cech 1988). The T4 and RB3 intron structures differ by two single-base deletions and six base substitutions.

All four of the base changes in canonical group IA stem structures maintain the proposed base-pairing. These compensatory changes add to the already considerable support for the existence of the P6 and P9 pairings (Burke 1988). The P9 changes are particularly noteworthy; the primary sequence of the P9 stem is identical in all three T4 introns. It may be that the homogeneous P9 sequence seen in the T4 introns (or even the overall high level of similarity between the phage introns) is the result of interintron sequence conversions of the type observed by Bryk and Belfort (1990), in which several suppressors of

Figure 2. DNA sequence of the RB3 *nrdB* intron and parts of the flanking exons. Uppercase letters represent the intron itself; lowercase letters denote the exons. The predicted amino acid sequence of the intron ORF is indicated beneath the nucleotide sequence, beginning with a TTG initiation codon; the presumed Shine–Dalgarno sequence for this initiation codon is denoted by stars. The box superimposed on the intron ORF sequence encloses the 491 bases that T4 lacks. Two single-base deletions and 14 base substitutions that RB3 and T4 differ by in this region are noted by the T4 sequence above the RB3 sequence at the appropriate position.

defects in the *td* P7.1 pairing were found to reflect conversion events that replaced the *td* P7.1 with its homologous counterpart in *sunY*. Sequence conversions between similar but ectopic sequences seem to have gen-

erated some of the classic *rII*B mutants as well (Shinedling et al. 1987).

One of the base substitutions alters a newly proposed secondary structure element called P9.0, which is in-

Figure 3. Proposed formation of the T4 intron from an RB3-like ancestor. The 269-codon ORF in RB3 contains two near-perfect direct repeats, of 12/15-bp identity, separated by 476 nucleotides of sequence. We propose that either unequal crossing over during recombination or slipped mispairing during replication deleted these 476 bp and one of the 15-bp repeats, to introduce the 491-bp deletion seen in T4. This deletion frameshifts the remainder of the intron ORF, leading to the overlapping ORF structures in T4 that have been noted previously (Gott et al. 1986, 1988; Shub et al. 1988; Liu 1990).

Figure 4. Proposed structure of the RB3 *nrdB* intron, drawn according to the previously proposed structure for the T4 intron (Shub et al. 1988) and the structural conventions for group IA introns (Burke et al. 1987). The base substitutions by which T4 and RB3 differ in this structure are indicated by arrows, with the T4 sequence in parentheses. The long-range pairing P9.0 is also indicated.

involved in the selection of the 3' splice site. Michel et al. (1990) and Burke et al. (1990) have suggested that the penultimate bases of the intron (here, AC) pair to the short linker between stems P7 and P9 (here, gGU). This interaction generally consists of 2 bp (Michel et al. 1990) in the known group I introns. The RB3 intron has a typical 2-bp P9.0, but a U → A base substitution in the J7/9 linker of the T4 intron creates an A/A mispair. Michel et al. (1990) have noted that the few group I introns that entirely lack recognizable P9.0 pairings tend to have As on both sides, and they suggested that non-Watson-Crick A-A pairs might be accepted in the P9.0 pairing; the U → A base substitution in the T4 *nrdB* P9.0 may support that suggestion.

Cloning and overexpression of the RB3 intron ORF

We predicted that the RB3 *nrdB* intron ORF would produce a DNA endonuclease specific for an intronless *nrdB* gene. The primary sequence of the ORF was suggestive of nucleic acid-binding activity in its overall basic charge and its pair of potential zinc fingers, and the ORF product would be about the same size as other intron ORF-encoded endonucleases. We were prevented from performing RB3 by T4 *nrdB*In⁻ crosses to look for intron mobility, because T4 strongly excludes most regions of T-even genomes from recombinant progeny by an unknown mechanism (Russell and Huskey 1974), and this includes the *nrdB* region of the RB3 genome (data not shown). We decided to overexpress the ORF-encoded product, look for endonuclease activity, and demonstrate intron mobility by using plasmid-encoded ORF endonuclease activity.

We chose to overexpress the protein, using a T7 promoter-driven system (Singer and Gold 1991). We

changed the translational initiation region to eliminate the occluding secondary structure (Gott et al. 1988), using instead a version of a strong ribosome-binding site, called SD8, studied previously in our laboratory (Gold and Stormo 1990). We also changed the initiation codon itself from a UUG to an AUG. These changes were introduced by means of PCR, by designing a 5' primer (5'NOX; for the exact sequence, see Table 1) tagged with the desired changes and a convenient restriction site (Innis et al. 1990).

All nine constructs were transformed into the T7 RNA polymerase-producing host BL21/DE3 (Studier and Moffatt 1986). Upon IPTG induction of the T7 RNA polymerase, these constructs vastly overproduced a protein of ~31 kD not produced by cells that carried the starting vector pDIP19C alone. Small mobility shifts were seen in some of the substitution mutants, which provided further assurance that the protein being overexpressed was encoded by the gene that we had cloned.

In vivo endonuclease activity

We predicted that the intron ORF product should cleave an intronless *nrdB* gene at or near the site of intron insertion. We infected briefly induced pNOX17(BL21/DE3) cells with a high multiplicity of T4 *nrdB*In⁻ phage, allowed 5 min for adsorption and injection of the phage chromosomes into the ORF-expressing cells, and stopped the infection on ice and extracted cellular and phage DNA. Extensions from a primer annealing just downstream of the *nrdB* intron insertion point revealed a double-strand (dsDNA) break that was induced in ORF-expressing cells but not in vector-only negative controls (Fig. 5A). We assume that the ORF encodes this endonuclease activity, although it is possible that the

Table 1. Oligonucleotide sequences

Primer name	Sequence
<i>PCR fingerprinting</i>	
TD125	CCCGGATCCGTGATTTTGGTGGTGTAGAC
TD264	CCCGGATCCAAAGCTCTTTAGGTTACGCC
N114	CCCGGATCCTCTTTACCTCTGTTTGCACG
N215	CCCGGATCCGGAGCATCGGTAATTGGGC
SY259	CCCGGATCCGCTTCTCACC AATACGGCGGAACG
SY389	CCCGGATCCAGTCCAGTCAGTTCCCGTACC
g32-511	CTGAAGATAAAGGCGAGTGGAAAC
g32-564	GTTTGG AATCGCAGATTGATTTCAGG
g43-2048	CGGCCAAGTCGGTATTTCAGTGG
g43-2079	GCGGAGGGCAAGAAATAGCTTCACGG
<i>Cloning</i>	
5'NOX	CCCGGATCCTAAGGAGGAATATAAAATGAAC TATAGAAAAATTTGGATAG
N195	GGGGCATGCTTCTTGTTTCACACTCTTG
<i>Primer extensions</i>	
N101	CCCGGATCCATTGGGCGATATTACGATG
N180	CCATGTTCTTATGGAAGTTAAAGGT

Sequences of oligonucleotides used in this study. Sequences are written 5' to 3'. The first six PCR fingerprinting primers listed include *Bam*HI sites at their 5' ends to facilitate subcloning. The 5'NOX cloning primer replaces the translational initiation region of the *nrdB* intron ORF with the strong Shine–Dalgarno sequence TAAGGAGG and a spacing of 8 nucleotides to an ATG initiation codon. All of the sequences that these primers anneal to are available on the GenBank sequence data base.

ORF product must interact with or modify other cellular gene products.

Alignment of primer extension products with sequencing ladders generated from the primers on both strands permitted us to see the precise location of the dsDNA break (Fig. 5B). In other repetitions of this experiment (not shown here) there is some apparent degradation of the cut ends that varies in extent from experiment to experiment, resulting in a doublet rather than a single band at the cut site.

The unidirectional nature of the gene conversion event that gives rise to intron mobility requires that the dsDNA break occur only in the intronless phage DNA and not in the intron-plus donor. Figure 5C shows the results of an experiment in which we learned that the intron-plus T4 genome is indeed immune to the activity of the ORF product at this site. The ORF-directed cut is specific for an intronless genome.

Figure 5D summarizes these results and shows the location of the dsDNA break on the sequence of an intronless *nrdB* gene. The cut site is located 14–16 bases upstream of the intron insertion site. It has a 2-base 5' overhang. All other intron-encoded endonucleases studied so far leave 2- to 4-base 3' overhangs. This difference may be relevant; to date, our preliminary experiments have not demonstrated that this endonuclease promotes *nrdB* intron mobility (experiments not shown) in plasmid to *In*[−] phage mobility experiments similar to those done for *sunY* and *td* (Quirk et al. 1989b).

Discussion

An *nrdB*-specific intron-encoded endonuclease

Although the T4 *nrdB* intron is clearly not a mobile in-

tron, a related phage, RB3, contains a longer form of the *nrdB* intron that may prove to be mobile. The RB3 *nrdB* intron encodes an endonuclease specific for an intronless *nrdB* gene, a hallmark of the known mobile group I introns. The T4 intron appears to be a simple deletion mutant of an RB3-like ancestor. In accordance with existing nomenclature for intron-encoded endonucleases (Dujon et al. 1989b), we now refer to the RB3 *nrdB* intron ORF gene as the gene *I-TevIII* (intron-encoded T-even endonuclease III, where I and II are encoded by the introns in *td* and *sunY*).

The known examples of eukaryotic intron-encoded endonucleases all cut at or very close to the point of intron insertion. *I-TevIII* shares with the other two T4 intron-encoded endonucleases the peculiar characteristic of cutting at a distance from the point of intron insertion. The *td* endonuclease cuts 23–26 bp upstream of the intron insertion site; the *sunY* endonuclease cuts 13–15 bp downstream of the intron insertion site (Bell-Pedersen et al. 1990). *I-TevIII* cuts 14–16 bp upstream of the *nrdB* intron insertion site. Because intron insertion disrupts the ability of these endonucleases to recognize and cut the gene, we presume that their recognition sites must span the exon 1–exon 2 junction; therefore, the cuts are likely to occur outside the actual recognition site itself. This ability to cut at a precise distance away from a recognition site is reminiscent of the so-called type IIS subclass of restriction endonucleases (Szybalski 1985). The significance, if any, of this difference between the phage intron endonucleases and their eukaryotic counterparts is not known.

We note again that so far we have been unable to demonstrate that *I-TevIII* catalyzes mobility of the RB3 or the T4 *nrdB* intron (work in progress). Keeping this ca-

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Figure 5. (See facing page for legend.)

veat in mind, it nevertheless appears likely that not just two but all three T-even phage introns are (or were) mobile elements. We think it likely that the T4 introns are parasites (or, more properly, commensals, as they do not seem to confer a measurable selective disadvantage or advantage on their host viral genome) (S.R. Eddy and L. Gold, unpubl.); they have been established and maintained in phage genomes by virtue of high-frequency conversion of intronless genomes in the population (Belfort 1989). The peculiar occurrence of the introns in genes of related functions is still mysterious and worthy of further consideration (Goodrich-Blair et al. 1990).

The evolutionary volatility of group I intron ORFs is becoming more and more apparent (Michel and Cummings 1985; Mota and Collins 1988). Our interpretation that phage T4 carries an immobile, deleted version of a mobile intron suggests to us that previous intron mobility may explain the presence and phylogenetic distribution of the other currently immobile group I introns, possibly including stripped-down ORF-less introns such as the canonical 26S rRNA intron in *Tetrahymena*. These introns may well have been mobile introns that lost their endonucleases at some point in their evolutionary history.

Materials and methods

Media

Hershey broth (HB), EHA plates, and EHA top agar were all prepared as described by Steinberg and Edgar (1962).

Phage and bacterial strains

The RB phage are a collection of 31 phage isolated from seven different sewage treatment plants on Long Island, New York, as part of a Cold Spring Harbor summer course in 1964 (Russell and Huskey 1974). They were defined as T-even-like primarily by the criterion that their infectivity could be neutralized by anti-T4 sera. We obtained 24 of these phage from W. Wood's collection in Boulder, Colorado. RB3 is one of eight isolates from Huntington, New York. The T phages T2L, T4D, T6, and T7 were from our laboratory's phage collection. T3 was provided by William Studier, and T5 was provided by Jim McCorquodale. The triple intron deletion strain of T4 was provided by David Shub. Phage stocks were grown according to Steinberg and Edgar (1962) using an *E. coli* B strain NAP IV (Nelson et al.

1982) as the host. The T7 polymerase expressing *E. coli* strain BL21/DE3 was provided by William Studier.

PCR fingerprinting

A single phage plaque ($\sim 10^7$ phage) was picked with a sterile Pasteur pipette and suspended in 100 μ l of distilled water. Of this, 40 μ l was used as template DNA in a 100- μ l PCR reaction that was composed of 50 mM KCl, 10 mM Tris-Cl at pH 8.6, 2.5 mM $MgCl_2$, 170 μ g/ml BSA, 400 μ M of each of the four dNTPs, 50 pmoles of each of 10 oligonucleotide primers (see below), and 3 units of *Taq* DNA polymerase (Promega). These were covered with mineral oil and incubated for 50 cycles of 95°C for 1 min, 50°C for 1 min, and 70°C for 4 min. One-tenth of this reaction was run out on a 1.4% agarose gel, which was stained in ethidium bromide and photographed under short-wave UV illumination. The oligonucleotide primers used to generate a T-even fingerprint were designed to amplify portions of five genes. Two of the products are from genes 32 and 43, essential genes that we presumed would be conserved among all T-even-like phage. The other three products are from the three intron-containing genes *td*, *nrdB*, and *sunY* of phage T4. The primers for these amplification products anneal to exon sequences flanking the intron insertion site; therefore, phage-containing introns amplify large bands and phage without introns give small bands in the resulting fingerprint. The sequences of the primers used are shown in Table 1.

DNA sequencing

Single-stranded (ssDNA) sequencing templates were generated by asymmetric PCR of RB3 phage (Innis et al. 1990). The PCR reaction was carried out exactly as above, except only the two *nrdB*-specific primers N114 and N215 were present in the reaction: 25 pmoles of one, and 0.25 pmole of the other. The products of this reaction (primarily ssDNA) were partially purified by phenol extraction, chloroform extraction, passage over a Sephadex G-50 spin column, and ethanol precipitation. (The G-50 step is crucial; otherwise, significant amounts of dNTPs are carried over from the PCR reaction and the dNTP/ddNTP ratio in sequencing reactions is badly skewed.) Enough template is generated from one 100- μ l PCR reaction to do two or three sequencing reactions. Better results were obtained when the length of the amplified product was short; therefore, templates for many sequencing reactions were generated using PCR primers as close as possible to the annealing site of the sequencing primer. Sequencing was performed by the dideoxy chain-termination method, using a modified T7 DNA polymerase (Sequenase; U.S. Biochemical) essentially as recommended by the

Figure 5. The cloned intron ORF produces a DNA endonuclease specific for an intronless *nrdB* gene. (A) Lanes A, C, G, and T are a sequencing ladder on *nrdBIn*⁻ plasmid template. Shown in the other lanes are results of primer extensions on *nrdBIn*⁻ T4 DNA isolated shortly after infection of expression plasmid-containing cells, where the plasmid is either the starting vector pDIP19C alone or pNOX17, which transcribes a wild-type copy of the ORF-coding sequence. In this particular experiment, two independent preparations of phage DNA were assayed for each of the two plasmids. The primer used is N180, which anneals just downstream of the intron insertion point. The region of the sequence examined here is immediately upstream of the intron insertion point. (B) More precise alignment of the cut site, with sequencing ladders on both strands. Extensions were performed with a primer N101, which anneals ~ 200 bases upstream, and the primer N180, which anneals immediately downstream of the cut site, and aligned with sequencing ladders made with the same primers. The cut is present on both strands. (C) Essentially the same as the experiment in A, except that intron-plus T4 DNA is also tested as a substrate. The upstream primer N101 is used here for primer extensions rather than N180, as N180 anneals to exon 2 and thus gives different sequence ladders on intron-plus and intronless DNA. Only the intronless DNA is recognized and cut by the ORF product. (D) Interpretation of the above results, showing the location of the cuts on both strands of the intronless *nrdB* sequence. The cut is 14–16 nucleotides upstream of the intron insertion site and leaves an end with a 2-base 5' overhang.

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manufacturer, with 5'-³²P-end-labeled synthetic oligonucleotides as primers. Sequence was obtained from both strands of the DNA, using a total of 15 synthetic primers. On average, ~300 nucleotides could be read from each primer, and the primers were spaced 200–300 nucleotides apart on the DNA. There was a single place where a compression artifact appeared (positions 1067–1075, in the P8 stem of the catalytic structure of the intron), and the sequence presented here is that read from the uncompressed strand.

Cloning

DNA manipulations were performed according to standard technique (Maniatis et al. 1982). The vector pDIP19C is a T7 expression vector with certain other features making it useful for T4 work that are not relevant to this paper (Singer and Gold 1991). Briefly, pDIP19C is a pACYC184 derivative (Cam^R, p15A origin) carrying the T7 ϕ 10 promoter driving through the same multiple cloning site as that in pUC19. Primers 5'NOX and N214 were used to amplify a modified RB3 *nrdB* ORF from RB3 phage by PCR; this amplified fragment was phenol- and chloroform-extracted, precipitated, and cut with *Bam*HI and *Kpn*I, and the resulting 971-bp fragment from the synthetic translational start of the gene to the 3' end of the intron was gel-purified from a 1% agarose gel by electroelution onto DEAE paper. (We have found that gel purification of PCR-generated DNA for cloning is often necessary, to avoid cloning low-molecular-weight PCR artifacts—"primer dimers.") This insert was ligated into *Bam*HI/*Kpn*I-cut pDIP19C. Fifty transformants were picked, and nine were found to contain the insert. The insert of each of these constructs was completely sequenced on one strand, using ssDNA template generated by asymmetric PCR from 0.1 ng of plasmid DNA (see above). Three of the constructs were found to be wild type in sequence (pNOX17, pNOX24, and pNOX48), three contained single-base substitutions, two had 2-base substitutions, and one (pNOX47) had three substitutions resulting in two predicted amino acid substitutions. All nine constructs were transformed into the host BL21/DE3 (an *E. coli* B strain carrying a λ lysogen that produces T7 polymerase under IPTG-inducible *lac* promoter control; Studier and Moffatt 1986) for further work on overexpressing the protein. All nine overproduced a protein of ~31 kD upon IPTG induction that was not seen in uninduced cells or in induced cells carrying the parent pDIP19C vector.

In vivo endonuclease activity

pDIP19C or pNOX17 transformants of BL21/DE3 were grown to an OD₆₀₀ of ~0.6 in a volume of 80 ml of HB at 37°C. These cultures were induced by the addition of IPTG to a 1 mM final concentration and incubated for an additional 5 min. T4nrdBIn⁻ or T4D phage at 2.4×10^{10} phage/ml was added in a 20-ml volume, and these infections were allowed to proceed for 5 min at 37°C. Infected cells were poured into centrifuge bottles on ice, and DNA was harvested from them by a standard plasmid isolation protocol, which is essentially the lysozyme/NaOH/SDS lysis protocol described by Silhavy et al. (1984).

One-fifth of the DNA harvested from this procedure was used as input template into primer extensions with Sequenase, using the same conditions as our primer extension sequencing but leaving out the dideoxynucleotides. (The cytosines in T4 DNA are hydroxymethylated and glucosylated. In our hands, AMV reverse transcriptase has failed to effectively extend primers annealed to T4 DNA. Sequenase—modified T7 DNA polymerase—extends on T4 DNA templates well enough for seeing signal from a simple primer extension, although not well enough

to generate sequencing ladders. The sequencing ladders used to calibrate the experiments shown in Figure 5 were generated on double-stranded plasmid clones of the *nrdB* precise intron deletion.) Reaction products were separated by gel electrophoresis on 8% polyacrylamide–8 M urea sequencing gels.

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