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Report

A Free-Standing Homing Endonuclease Targets an Intron Insertion Site in the *psbA* Gene of Cyanophages

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Summary

Homing endonuclease genes are mobile elements that promote their duplication into cognate sites that lack the endonuclease gene [1, 2]. The homing endonuclease initiates this event through site-specific DNA cleavage. Copying of the endonuclease gene follows as a consequence of DNA repair. A genome containing a homing endonuclease gene is subject to self-cleavage. Protection is accomplished through DNA sequence polymorphisms, as is the case in intronless homing of free-standing endonuclease genes [3, 4], or by disruption of the recognition site by a group I intron (or intein) into which the endonuclease ORF is embedded. We describe here a novel free-standing homing endonuclease from cyanobacteriophage S-PM2, which is similar to the DNA resolvase of bacteriophage T4 and is encoded adjacent to an intron-containing *psbA* gene [5, 6]. The endonuclease makes a specific double-strand cut near the intron insertion site (IIS), its DNA recognition site spans the IIS, and it is unable to cleave intron-containing *psbA* genes. This interdependence of a free-standing endonuclease gene and a group I intron, which we denote "collaborative homing," has not been reported previously and gives support to a hypothesis of formation of composite mobile introns by independent convergence of an intron and an endonuclease gene on the same target sequence.

Results

Photosynthesis Genes in Phages of Cyanobacteria

Complete genomic sequences have been determined for five bacteriophages that infect the marine cyanobacteria *Prochlorococcus* and/or *Synechococcus* [6–8]. One of these, P-SSP7, is a podophage with genome size and capsid morphology very similar to the T7 family of phages of enterobacteria. The others, S-PM2, P-SSM2, P-SSM4, and Syn9, are much larger myophages with core replication genes and capsid components very closely related to the T4 family of enterobacteriophages (T-even phages). Remarkably, each of these phages contains photosynthesis genes. In particular, each phage contains a *psbA* gene and three of the myophages also have a *psbD* gene, encoding the D1 and D2 proteins, respectively, that constitute the core of photosystem II. Millard et al. [5] probed a large collection of similar myophages for the presence of *psbA* and determined that most of them had *psbA* genes and many of these also had *psbD*. Partial sequences of several phages that contained both genes showed that they are closely linked, separated by no more than 177bp. Phage S-PM2 presented an interesting exception (Figure 1).

The *psbA* and *psbD* genes of S-PM2 (and the independently isolated phage S-RSM88) are separated by two long ORFs (177 and 178), and the *psbA* gene is interrupted by a 212bp group I intron [5, 6] (Figure 1). (References [5] and [6] refer to these as ORFs 178 and 179, respectively. However, the genomic database entry [accession number NC 006820] now refers to them as ORFs 177 and 178.)

Moreover, although the genomic structure outside the *psbAD* region is different between the two phages, a 3555bp sequence from S-RSM88, containing *psbA* (with its group I intron), *orfs 177* and *178*, and most of *psbD*, is identical to S-PM2, with the exception of a single third position change in *orf 177* that does not change the encoded amino acid. In contrast, the intronless *psbA* gene of another myophage, S-BM4, examined by the same authors, is only 79% identical with that of S-PM2. Considering that S-PM2 and S-RSM88 were isolated at different times from geographically distinct locations (the English Channel and the Gulf of Aqaba, respectively), it was proposed that the *psbAD* region in these two phages is a conserved mobile module [5].

The intron in *psbA* lacks an ORF, so the source of the proposed mobility was not apparent. Interestingly, *orf177* (accession number YP 195212) was annotated as similar to gene *49* of phage T4, which encodes endonuclease VII, a Holliday junction DNA resolvase [5, 6]. Indeed, the two proteins are 40% identical over a 64 amino acid region. We wondered whether ORF177 might be a novel site-specific endonuclease that cleaves intronless *psbA* DNA close to the site of intron insertion. However, such a relationship, of a free-standing endonuclease gene conferring mobility to an intron and its flanking sequences, with the intron providing protection from self-cleavage by the nuclease, had never been described.

orf177 Encodes a Double-Strand DNA Endonuclease, F-CphI

orf177 was transcribed and translated in vitro, yielding a ^{35}S-labeled protein product consistent with its predicted size (16.7 kDa) as determined by mobility on SDS-PAGE (data not shown). Incubation of nonradioactive ORF177 translation products with a 1.7 kb DNA fragment encompassing the phage S-BM4 *psbA* gene (5′ end labeled on either strand) resulted in digestion products of 0.4 kb for the bottom strand and 1.3 kb for the top strand (Figure 2A). No cleavage was observed with an unprogrammed, mock in vitro transcription/translation product. The intron-containing S-PM2 *psbAD* region was not cleaved under identical conditions. The product of ORF177 has been renamed F-CphI (*F*ree-standing homing endonuclease, *C*yano *ph*age, *I*).

Phage P-SSM2 [7] has a gene closely related to F-CphI (gene 287) just downstream of *psbA*, but does not contain

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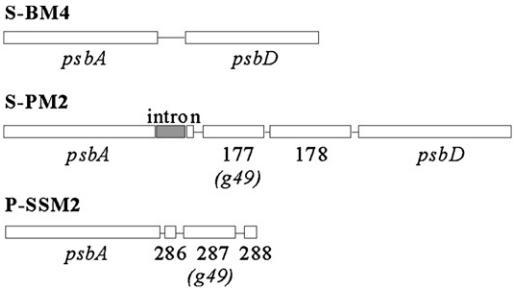

Figure 1. *psbAD* Regions of Cyanobacteriophages

ORFs are shown as boxes approximately to scale and the group I intron in S-PM2 is shaded. ORFs with similarity to phage T4 gene *49* (*g49*) are indicated.

psbD (Figure 1). Attempts to demonstrate endonuclease activity for the translation product of this gene have been uniformly negative, with intronless *psbA* genes from several sources, whereas F-CphI from S-PM2 is able to specifically cleave *psbA* of P-SSM2 (our unpublished results). It is possible that this ORF, which is 21 amino acids shorter than F-CphI with two large gaps in the alignment, may be a pseudogene or may have a substrate specificity different from F-CphI. The sequence surrounding the intron insertion site (IIS) of phage S-PM2 is shown for all three of these phages in Figure 2C.

F-CphI Cleaves the Intronless S-BM4 *psbA* Gene near the IIS

The cleavage site inferred from the sizes of the digestion products in Figure 2A places its location near the IIS. In order to precisely map where F-CphI cuts the S-BM4 *psbA* gene, 200bp surrounding the IIS were PCR amplified, with primers that were individually 5′ end labeled, and digested with in vitro synthesized F-CphI. The cleavage products were denatured and separated next to sequencing ladders generated with the same 5′ end-labeled oligonucleotide primers used in the PCR (Figure 3A). The S-BM4 target is cleaved entirely in exon II. The top- and bottom-strand cleavage products comigrate with the T band corresponding to position +6 (Figure 3A, top) and the G band located at position +2 (Figure 3A, bottom) relative to the IIS, respectively. The resulting cleaved target has 4 nt 3′ single-stranded overhangs (Figure 3B).

The *psbA* Intron Lies within the F-CphI Recognition Site

The lack of purified F-CphI protein prevented direct determination by DNA foot printing of the sequence necessary for its binding. Instead, we used a technique, specific for endonucleases [9], that determines the boundaries of the substrate that allows endonucleolytic cleavage. A population of end-labeled, partially double-stranded DNAs was generated by dideoxy sequencing reactions of the putative target site from S-BM4. The sequencing reactions were digested to completion with F-CphI and the products compared to the undigested ladder (Figure 4A). Molecules containing the complete double-stranded recognition and cleavage sites should be cleaved, but those with an incomplete target site should not. When top-strand-labeled templates were incubated with F-CphI, no band was observed at position +9C but a faint band can be seen at position +8A. Therefore, the downstream boundary is most likely completed by incorporation of +9C. When incubated with F-CphI, the molecule on the bottom strand template ending at −10C remains uncleaved, whereas

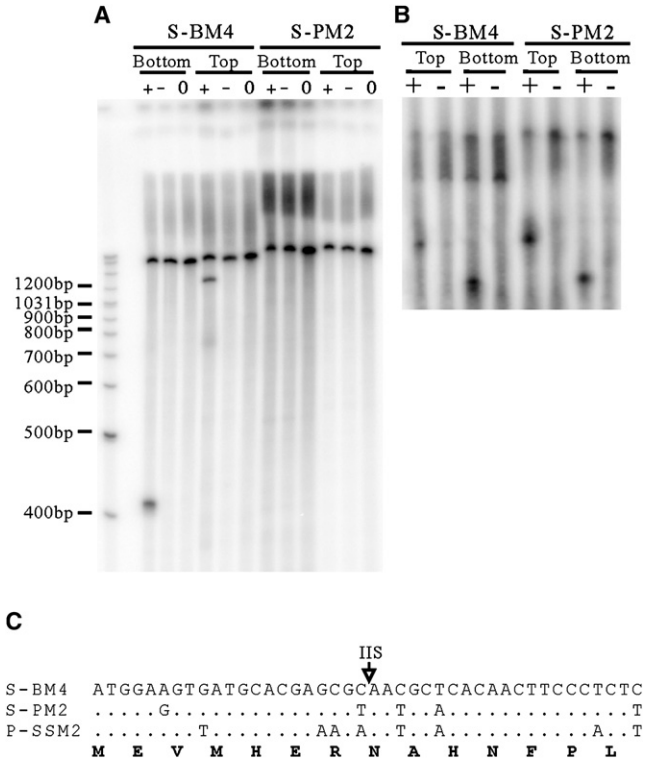

Figure 2. Endonuclease Assay with Phage *psbA* DNA

(A) In vitro synthesized ORF177 was incubated with top- or bottom-strand 5′-end-labeled PCR products from S-BM4 or S-PM2, and the digestion products were separated on a 4% denaturing polyacrylamide gel. Target DNAs were incubated with complete in vitro synthesis reaction (+); mock in vitro synthesis reaction unprogrammed with ORF177 RNA (−); or no in vitro synthesis reaction (0). Labeled DNA ladder is on the left.

(B) 5′-end-labeled 60bp duplexes of S-BM4 DNA and intron-deleted S-PM2 DNA surrounding the IIS were used as substrates in reactions with complete (+) or mock (−) in vitro synthesis products.

(C) 42 nt of the coding strand of S-BM4 *psbA* is shown, with differences in P-SSM2 and the intron-deleted S-PM2 sequences indicated. The IIS is indicated by an open arrowhead. The encoded amino acid sequence is shown at the bottom.

the molecule ending with −11A is cleaved. This indicates that incorporation of −11A is required for completion of the upstream boundary of the recognition site. We conclude that F-CphI cleavage requires a 20bp region spanning sequences in both exons, extending from position −11 to +9 relative to the IIS (Figure 4B).

The S-PM2 *psbA* Intron Protects the Chromosome from F-CphI Cleavage

In addition to the group I intron that interrupts the S-PM2 *psbA* gene, there are several sequence differences between the S-BM4 and S-PM2 sequences around the IIS (Figure 2C). Although it is likely that the intron in S-PM2 is responsible for blocking the activity of F-CphI, these differences might independently prevent endonucleolytic cleavage. To determine whether the intronless S-PM2 *psbA* gene can be cleaved by F-CphI, we used synthetic substrates containing 60 bp of the S-BM4 and S-PM2 (without intron) *psbA* genes centered on the IIS. Incubation with F-CphI resulted in cleavage of both the top and bottom strands of each target (Figure 2B). Because the intronless S-PM2 sequence is sensitive to F-CphI but the

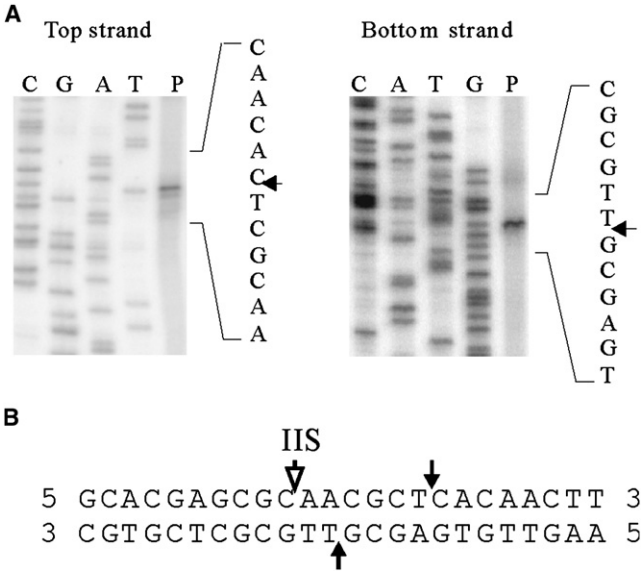

Figure 3. Cleavage Site Mapping

(A) Cleavage products were separated on 4% denaturing polyacrylamide gels along with sequencing ladders generated with the same S-BM4 DNA and labeled primers. The sequence read from the gel is shown on the right and the filled arrowhead indicates the cleavage site.

(B) The cleavage sites (filled arrowhead) and IIS are shown on the S-BM4 sequence.

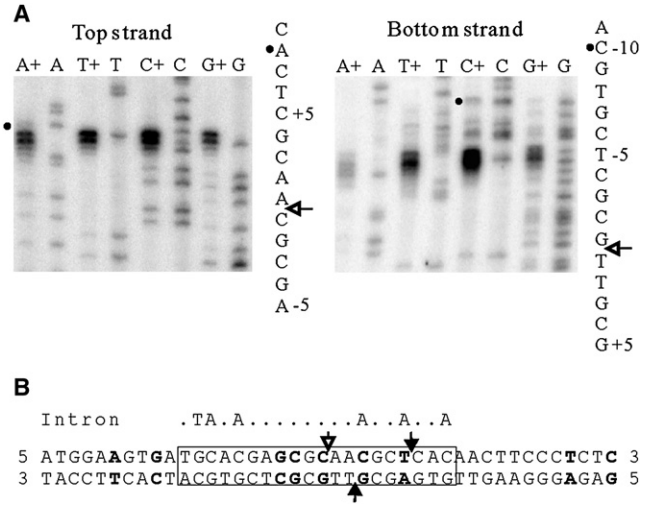

Figure 4. Sequence Needed for Cleavage

(A) Sequencing reactions on S-BM4 DNA were incubated with in vitro synthesized F-CphI (+) and separated next to undigested sequencing ladders on 4% denaturing polyacrylamide gels. The band that represents the last residue that did not complete the F-CphI recognition site and thus remains uncleaved is indicated by a filled circle.

(B) Recognition sequence needed for cleavage is boxed. Bold letters indicate variable nucleotides between S-BM4, S-PM2, and P-SSM2 DNAs. A 20 nt sequence in the S-PM2 *psbA* intron with closest similarity to the boxed recognition sequence is shown above, with differences indicated. Cleavage sites and IIS are indicated as before.

intron-containing sequence is resistant, we conclude that protection of the *psbA* gene is due solely to the presence of the intron.

Discussion

An Intron and a Free-Standing Endonuclease Collaborate to Target the Same DNA Sequence

The usual physical relationship between a group I intron and its homing endonuclease is insertion of the endonuclease gene into the DNA corresponding to a terminal loop of one of the base paired stems of the intron RNA secondary structure [2]. This is a mutually beneficial arrangement, providing both partners with the opportunity to spread to unoccupied chromosomal sites. Cleavage by the enzyme at an unoccupied IIS initiates the sequence of events that leads to spread of the intron [1]. On the other hand, the intron provides the endonuclease gene with a DNA insertion site that is not deleterious for gene expression of the host chromosome. Furthermore, by disrupting the enzyme recognition site, the intron prevents self-cleavage by the enzyme after a successful homing event.

Homing endonuclease genes are not always associated with introns. They are sometimes encountered as optional free-standing genes that are inserted between two conserved genes. Some of these homing endonucleases have been shown to cleave within a conserved sequence of an adjacent gene in genomes that lack the homing endonuclease. By a mechanism analogous to that involved in intron homing, repair of the DNA results in copying the homing endonuclease gene into the recipient, a process that has been called intronless homing [3].

One major difference between intron and intronless homing is how self-cleavage of the donor (and the newly converted recipient) is prevented. Except for one interesting exception, the mobile intron in *Bacillus subtilis* phage SP82 [10], the intron itself confers resistance by disrupting the enzyme recognition site. However, in all documented examples of intronless homing, sequence differences in the recognition sites of the donor and recipient are responsible for prevention of self-cleavage. Therefore, homing of a free-standing endonuclease gene must transfer both the gene and its resistant cleavage site to the recipient.

As far as we are aware, the situation described here, where an intron and a nearby free-standing endonuclease gene collaborate as independent units, has not been described previously. However, we have proposed that precisely this organization should exist, and may have been an intermediate in the formation of the more usual chimeric mobile introns [11].

Implications for the Formation of Mobile Group I Introns

The IISs of group I introns tend to be in highly conserved, functionally important regions of essential genes. Because the sequence at the intron ends is not repeated, loss of an intron by exact deletion will be rare, and inexact deletion at such a site will create a lethal mutation. So introns are found where they have the highest likelihood of survival. Interestingly, cleavage sites of free-standing homing endonucleases have the same properties as intron IISs. Because homing endonucleases are not essential, they are easily lost by mutation. Their survival depends on the ability to spread to unoccupied sites during mating (or mixed infection as in the case of phages) by the genomes in which they reside. Therefore, they tend to target the sequences that are most frequently encountered in the gene pool that they inhabit.

The IIS and endonuclease cleavage site in the *psbA* gene of phage S-PM2 fits these criteria perfectly. The protein product of *psbA*, the D1 protein, is at the core of the reactive center of photosystem II and has remained remarkably conserved in chloroplasts and cyanobacteria in spite of $>10^9$ years since the divergence of these genomes. Furthermore, the IIS is one of the most highly conserved regions of this gene, encoding a region of the D1 protein that binds the Mn-Ca cluster of the oxygen-evolving center, which is at the catalytic heart of the protein [12]. We have proposed elsewhere that such sites will be targeted separately by group I introns and homing endonuclease genes, which can then be united into the same genome by recombination in the sequence between the intron and endonuclease [11]. Collaborative homing would allow both entities to spread through the population. However, because they can also be separated occasionally by recombination [4, 11], the most stable combination will occur when the homing endonuclease gene invades a nonessential region of the intron, the situation typically encountered in mobile group I introns. This may explain why the situation reported here, where endonuclease and intron are separate but collaborate in homing as independent entities, is so rare.

It has been noted that sequences flanking the endonuclease genes of some mobile group I introns resemble the IIS and could have provided the entry point for insertion of the endonuclease gene [13]. Interestingly, a sequence of 20 nt that differs at six positions from the minimal sequence required for cleavage (Figure 4B) is located near the beginning of the intron, in the highly variable loop (L1) of pairing element P1, which is the location of ORFs in some group I introns [14, 15]. Two of the six differences occur at positions that are variable in natural substrates of F-CphI (Figure 2C). Although there may not yet be a sequence in a dispensable portion of the *psbA* intron that provides an entry point for invasion by F-CphI, it is reasonable to suppose that eventually one will be generated, perhaps by further variation of the sequence in L1 that already bears some similarity to the IIS.

A New Family of Homing Endonucleases Related to a Holliday Junction Resolvase

ORF177 was annotated as resembling gp49, the Holliday junction resolvase of phage T4 and its relatives. However, we think it unlikely that it has this function, because the gene is present in some cyanophage genomes and absent in others. We have shown here that the protein product of *orf 177* is an endonuclease, F-CphI, whose activity requires a particular DNA sequence surrounding the *psbA* IIS rather than a specific DNA conformation. BLAST analysis [16] against the entire protein database reveals a large number of hits with much lower E values than gp49, all of which are from bacteria or bacteriophages. Many of these are also annotated as gp49-like, but none of them have been assayed for enzymatic activity. We suggest that this family of proteins likely represents a new category of homing endonucleases that utilize the same protein fold as the catalytic part of gp49.

We do not assume, however, that the fold originated in a resolvase gene and was appropriated by homing endonucleases. There are four unrelated families of DNA resolvases, and endonuclease VII appears only in a very restricted group of organisms: the closely related T-even phages [17]. In contrast, the large number of proteins that are most similar to F-CphI are distributed over a very wide range of bacteria and their viruses, suggesting that endonuclease VII may have been created by appropriating its catalytic fold from a widely distributed family of homing endonucleases related to F-CphI.

Implications for Evolution of Photosystem II Genes in Cyanobacteria and Their Viruses

Surveys of myoviruses of *Prochlorococcus* and *Synechococcus* indicate that virtually all of them have a *psbA* gene and about 2/3 also have *psbD* [18]. Evidence is accumulating for exchange of these photosystem II genes among phages, from host to phage, from phage to host, and for intragenic recombination in *psbA* [5, 7, 18, 19]. The existence of F-CphI in cyanophages may have created a hot spot for recombination within *psbA*, which may have played a major role in distribution and diversification of photosystem II genes in the marine environment.

Conclusions

We describe F-CphI, a free-standing homing endonuclease encoded immediately downstream of gene *psbA* in cyanophage S-PM2. F-CphI is the first example of a homing endonuclease that is closely related to gp49, the DNA resolvase of *E. coli* phage T4. Unlike every other free-standing homing endonuclease described, F-CphI is prevented from cutting its own genome by the presence of a group I intron interrupting its DNA recognition/cleavage site in *psbA*. This association of an endonuclease-lacking group I intron and a free-standing endonuclease that targets the IIS supports a proposed pathway for evolution of mobile group I introns whereby introns and homing endonucleases independently target the same conserved sequences. This site-specific double-strand DNA endonuclease may be an important stimulus for recombination between *psbA* genes of cyanophages, and may be responsible for frequent genetic exchange between host and phage versions of this gene.

Experimental Procedures

Bacteriophages and Recombinant Plasmids

Plasmid pCR 2.1 (Invitrogen) was used to carry the desired gene fragments. Recombinant plasmid pSBM4-TA contains *psbA* and part of *psbD* of phage S-BM4. These two recombinant plasmids and cyanophage S-PM2 were provided by Millard et al. (University of Warwick, Coventry, United Kingdom). *E. coli* strain XL-1 Blue (Stratagene) was used as host for these plasmids.

In Vitro Expression of F-CphI Protein

Templates for in vitro protein synthesis were obtained by amplification of S-PM2 *orf177* with primers S-PM2-1 (which contains a phage T7 RNA polymerase promoter sequence fused to the upstream sequence of *orf 177*) and S-PM2-2. PCR was carried out with 0.01 U/μl Taq DNA polymerase (Fermentas) in 10 mM Tris-HCl (pH 8.8), 5 mM KCl, 0.008% Nonidet P40, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 40 pM of each primer. PCR cycling conditions consisted of a hot start at 94°C for 5 min, followed by 35 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 2 min), followed by incubation at 72°C for 5 min. The amplified product was purified with QIAquick PCR Purification Kit (QIAGEN) transcribed with T7 RNA polymerase. *orf 177* RNA was translated in vitro with TNT Quick Coupled Transcription/Translation Systems (Promega) according to the manufacturer's specification. ORF177 protein synthesis was monitored by ^{35}S-methionine incorporation on SDS-PAGE. Nonlabeled ORF177 protein was made separately for endonuclease assays.

Generation of DNA Substrates

Naturally occurring *psbA* genes used as targets in endonuclease assays were amplified as described above except that the number of cycles was reduced to 25. M13 Reverse and M13 Forward primers were used to amplify the bottom and top strands, respectively, from plasmids pSBM4-TA and pAM3. Synthetic *psbA* targets were generated by annealing complementary oligonucleotides SBM4-60Top and SBM4-60Top-r, or oligonucleotides

SPM2-60Top and SPM2-60Top-r corresponding to the S-BM4 and intronless S-PM2 targets, respectively. Individually 5′ end-labeled targets were generated by labeling one of the oligonucleotides on its 5′ end (with [γ-^{32}P]ATP and T4 polynucleotide kinase) before use in either PCR or annealing reactions with an unlabeled primer partner. Oligonucleotides were annealed by incubating labeled oligonucleotide and its complement at 90°C for 5 min and cooling to 55°C in 25 mM Tris/HCl (pH 8.0) and 50 mM NaCl. Labeled PCR products and duplexes were purified with QIAquick PCR Purification Kit (QIAGEN).

Endonuclease Assays

Endonuclease assay reactions (10 μl) containing 2 μl of in vitro synthesis reaction and 4 μl DNA substrate were incubated at 30°C for 30 min in 0.05 M NaCl, 0.05 M Tris (pH 7.5), and 0.5 μg Poly dI-dC. Reaction products were extracted with an equal volume of phenol before separation on a 4% denaturing polyacrylamide gel.

Cleavage Site Mapping

The location of the F-CphI cleavage site was mapped on the top and bottom strands of the S-BM4 *psbA* gene with substrates amplified from pS-BM4-TA with primers SBM4In3 and SBM4In5, respectively. PCR amplification, 5′ end labeling, and endonuclease assay conditions used were the same as described above. Cleavage products were separated by electrophoresis on 4% denaturing polyacrylamide gel next to sequencing ladders generated with the same template DNA and labeled primers.

Determination of the Boundaries Allowing Endonucleolytic Cleavage

The minimum size of the substrate was determined with the method of Wenzlau et al. [9]. Sequencing ladders were generated with 5′ ^{32}P-end labeled primers and pSBM4-TA. 1 μl of each sequencing ladder was incubated with 8 μl in vitro protein synthesis reaction in a total volume of 10 μl at 30°C for 30 min in 0.05 M NaCl, 0.05 M Tris (pH 7.5), and 0.5 μg Poly dI-dC. Reaction products were extracted with equal volume of phenol and separated next to undigested sequencing ladders on a 4% denaturing polyacrylamide gel.

Oligonucleotides

Numbers in parentheses indicate location in the database entry. S-PM2-1, AAATTAATACGACTCACTATAGGGAGACAAGCCAGGAGCCACCATGACTAAACTATACTCTGATTTGTAT (1507–1533, AY329638) (noncoding sequence containing a T7 promoter is underlined); S-PM2-2, TCCATATTCCTCATAAGAATAAT (complement 1970–1948, AY329638); SBM4In5, TGGGTAAAAAAATTGGCGGGAAATT (1036–1060, AJ628858); SBM4In3, GGCATTCAACCTGAACGGTTTCA (complement 1299–1277, AJ628858); M13 reverse primer, CAGGAAACAGCTATGAC; M13 forward primer, GACCGGCAGCAAAATG; SBM4-60Top, TGGTCTGGGTATGGAAGTGATGCACGAGCGCAACGCTCACAACTTCCCTCTCGACCTTGC; SBM4-60Top-r, complement of SBM4-60Top; SPM2-60Top, GGTCTGGGTATGGAGGTGATGCACGAGCGTAATGCACACAACTTCCCTCTTGACCTTGCA; SPM2-60TopC-r, complement of SPM2-60Top.

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