Incision at Nucleotide Insertions/Deletions and Base Pair Mismatches by the SP Nuclease of Spinach†

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ABSTRACT: Spinach leaves contain a highly active nuclease called SP. The purified enzyme incises single-stranded DNA, RNA, and double-stranded DNA that has been destabilized by A-T-rich regions and DNA lesions [Strickland et al. (1991) *Biochemistry 30*, 9749−9756]. This broad range of activity has suggested that SP may be similar to a family of nucleases represented by S1, P1, and the mung bean nuclease. However, unlike these single-stranded nucleases that require acidic pH and low ionic strength conditions, SP has a neutral pH optimum and is active over a wide range of salt concentrations. We have extended these findings and showed that an outstanding substrate for SP is a mismatched DNA duplex. For base-substitution mismatches, SP incises at all mismatches except those containing a guanine residue. SP also cuts at insertion/deletions of one or more nucleotides. Where the extrahelical DNA loop contains one nucleotide, the preference of extrahelical nucleotide is A ≫ T ∼ C but undetectable at G. The inability of SP to cut at guanine residues and the favoring of A-T-rich regions distinguish SP from the CEL I family of neutral pH mismatch endonucleases recently discovered in celery and other plants [Oleykowski et al. (1998) *Nucleic Acids Res. 26*, 4597−4602]. SP, like CEL I, does not turn over after incision at a mismatched site in vitro. Similar to CEL I, the presence of a DNA polymerase or a DNA ligase allows SP to turn over and stimulate its activity in vitro by about 20-fold. The possibility that the SP nuclease may be a natural variant of the CEL I family of mismatch endonucleases is discussed.

Nucleases participate in many essential cellular functions (*1*). Some nucleases are highly specialized in DNA recombination and repair while others enable general degradation of dietary nucleic acids. Of the latter, the secreted fungal nucleases, S1 (*2*) and P1 (*3*), and the pancreatic DNase I (*4*) are the best characterized. Often, a nuclease may possess multiple activities within one polypeptide, thus enabling it to perform both general nucleic acid degradation and unique steps in DNA replication, recombination, or repair. For example, Exo III of *E. coli* is a powerful 3′ to 5′ exonuclease as well as being the major apurinic endonuclease in this organism and a 3′ phosphatase (*5*). The recBCD recombination nuclease is a potent 5′ to 3′ and 3′ to 5′ exonuclease and a helicase (*6*, *7*).

Spinach (*Spinacia oleracea*) contains a nuclease called SP (*11*, *12*) that has multiple activities. The purified SP, similar to S1, P1, and mung bean nuclease (*13*−*15*), is able to degrade single-stranded DNA, double-stranded DNA, and RNA. Instead of having an acidic pH optimum like S1, P1, and mung bean nuclease, SP has a neutral pH optimum. Interestingly, SP incises DNA-containing cisplatin adducts, the $TC_{(6-4)}$-type pyrimidine dimers, but not the cyclobutane-type pyrimidine dimers (*12*). Such properties suggest that SP could be a repair enzyme. We report here an unexpected prominent property of SP: the incision at DNA insertion/deletion loops, and at base-substitution mismatches, under physiological conditions. We also show that in vitro SP mismatch incision activity is stimulated by the presence of a DNA polymerase or a DNA ligase.

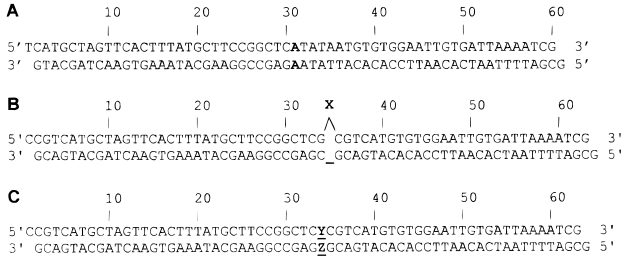

FIGURE 1: Heteroduplex DNA substrates. (A) The oligonucleotide duplex substrate containing an A/A mismatch at position 30, next to an A-T-rich region. (B) The substrates containing an extrahelical DNA loop X with one or more nucleotides. (C) The base-substitution substrates. Y and Z are various nucleotides that can be substituted in to produce the mismatches used in this study.

EXPERIMENTAL PROCEDURES

SP Nuclease. SP nuclease for the initial mismatch endonuclease assays was generously provided by Dr. Doetsch of Emory University. Subsequent experiments used SP prepared in our laboratory according to the published protocol (*11*).

Preparation of Plant Extracts. Various plant tissues were homogenized in a blender at 4 °C and adjusted with a 10× solution to give the composition of buffer A [0.1 M Tris-

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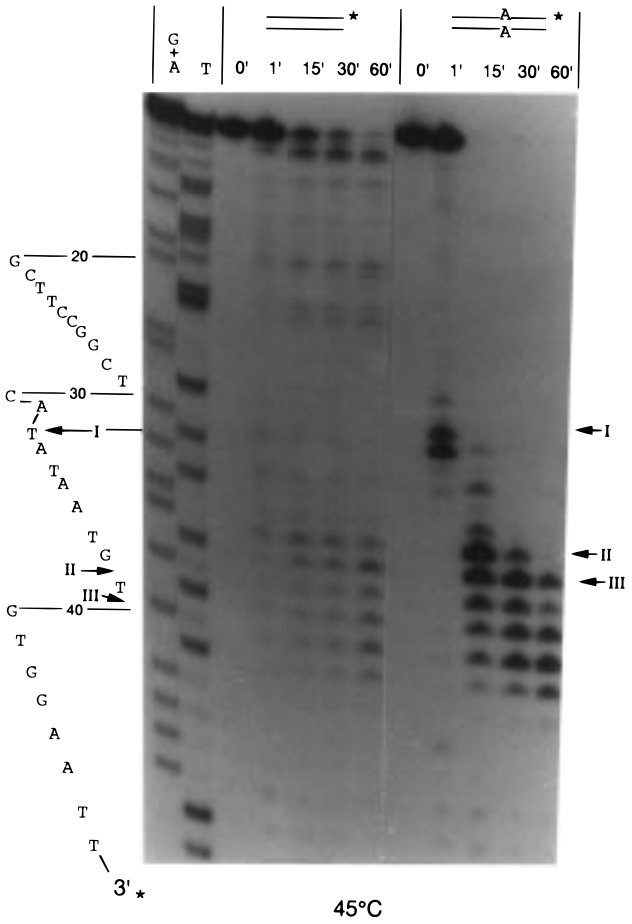

FIGURE 2: Incision of mismatched substrates by the spinach SP nuclease. Fifty femtomoles of no-mismatch substrate, or A/A base-substitution substrate of Figure 1A, 3′-labeled (*) in the top strand was incubated with 1 ng of SP in buffer C for various durations at 45 °C. The 20 μL reaction was terminated by adding 10 μL of 1.5% SDS, 47 mM EDTA, and 75% formamide plus tracking dyes and analyzed on a denaturing polyacrylamide gel in 7 M urea and 50 °C. The autoradiogram is shown. Chemical DNA sequencing ladders are shown for determining the positions of incisions (I, II, and III) in the DNA sequence. For a 3′-labeled substrate, when a nuclease nicks 3′ of a nucleotide and produces a 5′-PO_4 terminus, the labeled truncated band comigrates with the band for that nucleotide in the chemical DNA sequencing reaction lane (*13*).

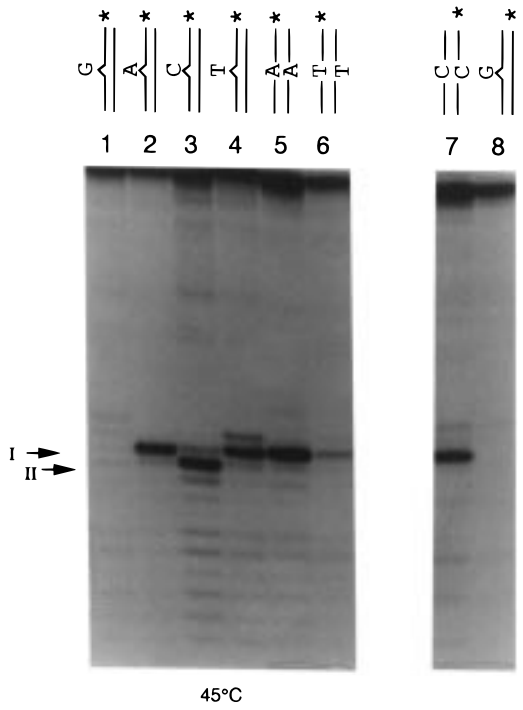

FIGURE 3: SP incision at various mismatches. Substrates were made from the sequences in Figure 1B,C with the position of the radioactivity indicated by an asterisk. Fifty femtomoles of DNA substrate was incubated with 5 ng of SP in buffer B for 30 min at 45 °C. Lanes 1−6 were 3′-labeled in the top strand. Lanes 7 and 8 were 5′-labeled in the bottom strand. SP incision produced bands I and II about 32 nucleotides (nt) long. The substrates with the extrahelical nucleotides are shown in lanes 1−4. Base-substitution heteroduplexes are shown in lanes 5−7. I = incision position at the 3′ side of the extrahelical A nucleotide. II = the incision from an alternate extrahelical C base-pairing permissible in this sequence.

HCl, pH 7.7, plus 10 μM phenylmethanesulfonyl fluoride (PMSF)]. The extracts were stored at −70 °C. Alternatively, the tissues were frozen in liquid nitrogen, ground to a powder with a mortar and pestle, and then extracted with buffer A on ice. Both types of extract provided equivalent data.

Preparation of Extracts of Spinach Seedlings. Seeds of several spinach varieties were purchased from gardening centers in Philadelphia, PA. The seeds were soaked for 3 h in water and planted in soilless potting soil and allowed to grow for 3 weeks before harvest. The plant tissues were frozen in liquid nitrogen and ground to a powder in a liquid nitrogen-cooled mortar and pestle. The powder was extracted with buffer A and stored at −70 °C.

Preparation of Heteroduplexes Containing Various Mismatches. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by electrophoresis in a denaturing polyacrylamide gel in the presence of 7 M urea at 50 °C. The purified single-strand oligonucleotides were hybridized with appropriate opposite strands to construct DNA heteroduplex substrates, 61−65 bp[1] long containing base-substitution mismatches, or insertion/deletion DNA loops (Figure 1). The DNA duplexes were labeled with ^{32}P at one of the four termini so that DNA endonuclease incisions at the mispaired nucleotides could be identified as truncated DNA bands on denaturing DNA sequencing gels (16). The 5′-labeled substrates were labeled as single-strand DNA with T4 polynucleotide kinase and [γ-^{32}P]ATP before annealing to its opposite strand. The 3′-labeled substrates were labeled by the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP and [α-^{32}P]dGTP after annealing. All the labeled duplexes were made blunt-ended by the fill-in reaction of DNA polymerase I Klenow fragment using dCTP and dGTP, and purified by electrophoresis in a nondenaturing polyacrylamide gel as described (16). DNA was electroeluted from the gel slice in a Centricon unit with an AMICON Model 57005 electroeluter. The upper reservoir of this unit has been replaced with one having watertight partitions to prevent cross-contamination.

Mismatch Endonuclease Assay. Ten to fifty femtomoles of ^{32}P-labeled substrates was incubated with 0.3−5 ng of the purified SP preparation in buffer B (20 mM Tris-HCl, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$) for 30 min. The 20 μL reaction was terminated by adding 10 μL of 1.5% SDS, 47 mM EDTA, and 75% formamide plus tracking dyes and analyzed by denaturing polyacrylamide gel electrophoresis

[1] Abbreviations: bp, base pair; nt, nucleotide(s); PCR, polymerase chain reaction.

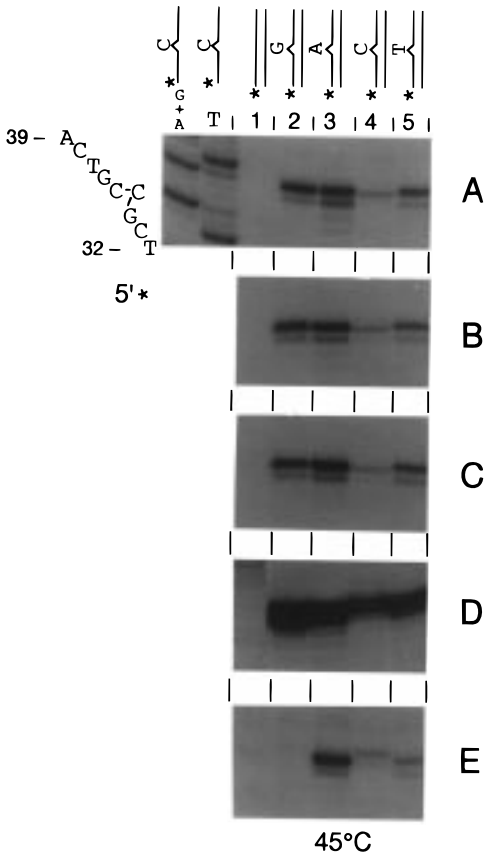

FIGURE 4: Comparison of various plant extracts for the ability to incise at extrahelical nucleotide residues. Fifty femtomoles of each substrate labeled at the 5′ termini of the top strand was incubated with 1 μL of various plant extracts for 30 min at 45 °C. The treated DNA was analyzed as described the experiment in Figure 2. Panels A, B, C, D, and E represent extracts from broccoli, cabbage, cauliflower, celery, and spinach, respectively. Chemical DNA sequencing ladders are used for deducing the positions of the incisions in the substrates. For a 5′-labeled substrate, when a nuclease nicks 5′ of a nucleotide and produces a 3′-OH terminus, the truncated band migrates half nucleotide spacing slower than the band for that nucleotide in the lane containing the products of the chemical DNA sequencing reaction (*13*).

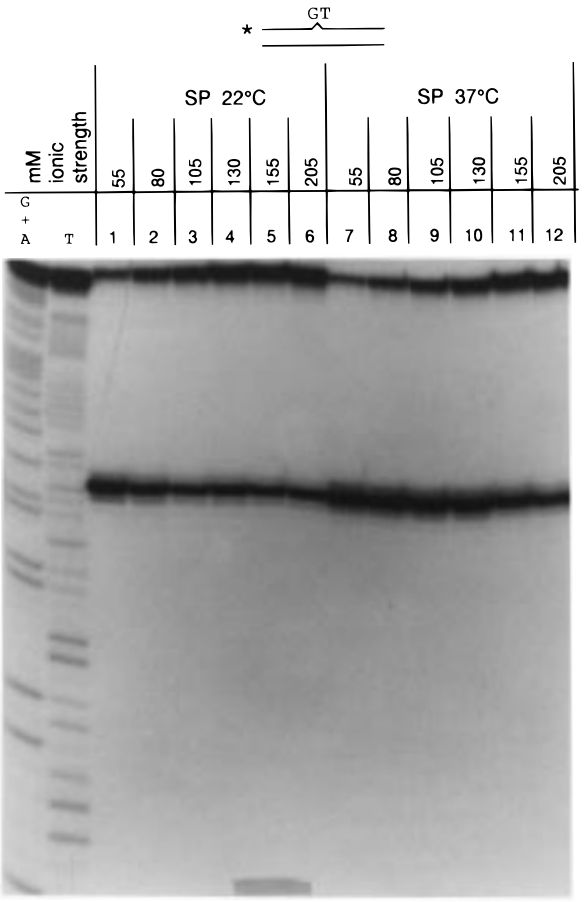

FIGURE 5: Ability of SP to incise at a GT loop mismatch under physiological conditions. Fifty femtomoles of 5′ top-strand-labeled extrahelical GT loop substrate was incubated with 5 ng of SP for 30 min at 22 or 37 °C. NaCl was added to buffer B to reach various ionic strengths. Procedures were as described in Figure 2.

as described (*13*). When Ampligase (a thermostable DNA ligase from Epicentre Technologies) was present in the assay, the reaction was carried out in buffer C (buffer B plus 0.001% Triton X-100 and 6 mM NAD).

RESULTS AND DISCUSSION

Incision of SP at an A/A Mismatch Located Next to an A-T-Rich Region. Incubation of SP with an A/A mismatch-containing substrate results in incision near the mismatch (Figure 2). SP is known to cleave DNA to produce 3′-hydroxyl and 5′-phosphoryl termini (*12*). The incisions at 1 min in the 3′-labeled top stand were traced to the first and second phosphodiester bonds 3′ of the A/A mismatch site. The shorter bands at later time points were probably produced by further SP digestion of the A-T-rich region destabilized by the DNA nicking at the mismatch.

Low-level nonspecific DNA nicking of the nonmismatched substrate by SP at the A-T-rich region destabilized by 45 °C (Figure 2) shows that SP exhibits properties similar to those of S1 type of single-strand-specific nucleases, except it does so at neutral pH.

Because A-T-rich regions enhance the ability of incision at a mismatch, we redesigned the substrates to include the challenge of G-C-rich flanking sequences (Figure 1B,C). These G-C-rich substrates are used in all experiments in Figures 3−7. DNA nibbling from the mismatch cut site does not occur for these substrates in which the mismatch is not in an A-T-rich region. Figure 3 illustrates the result of SP incision at substrates containing an extrahelical nucleotide of A, C, or T residue. Incision was not observed in the substrate with an extrahelical G residue. The absence of cutting in the top strand of this substrate is not due to the possibility of cutting being directed to the bottom strand. In fact, incision was not observed in the bottom strand of this substrate either (lane 8). In another control experiment to evaluate the possibility that flanking G-C-rich sequences may have inhibited SP cutting at guanine residues, we found that SP did not incise at either a single guanine residue or a loop of five guanine residues, inserted in an A-T-rich region (data not shown). Thus, the reactivity of SP with insertion/deletion mismatches is consistent with the known intrinsic preference of SP for A and T residues (*12*).

In lane 3 of Figure 3, the extrahelical C substrate produced a band one nucleotide shorter than the extrahelical A and T substrates in lanes 2 and 4, respectively. The likely reason is that the extrahelical C in this substrate was located 5′ to another C residue, therefore allowing the two C residues to alternate in base-pairing with the G residue in the opposite

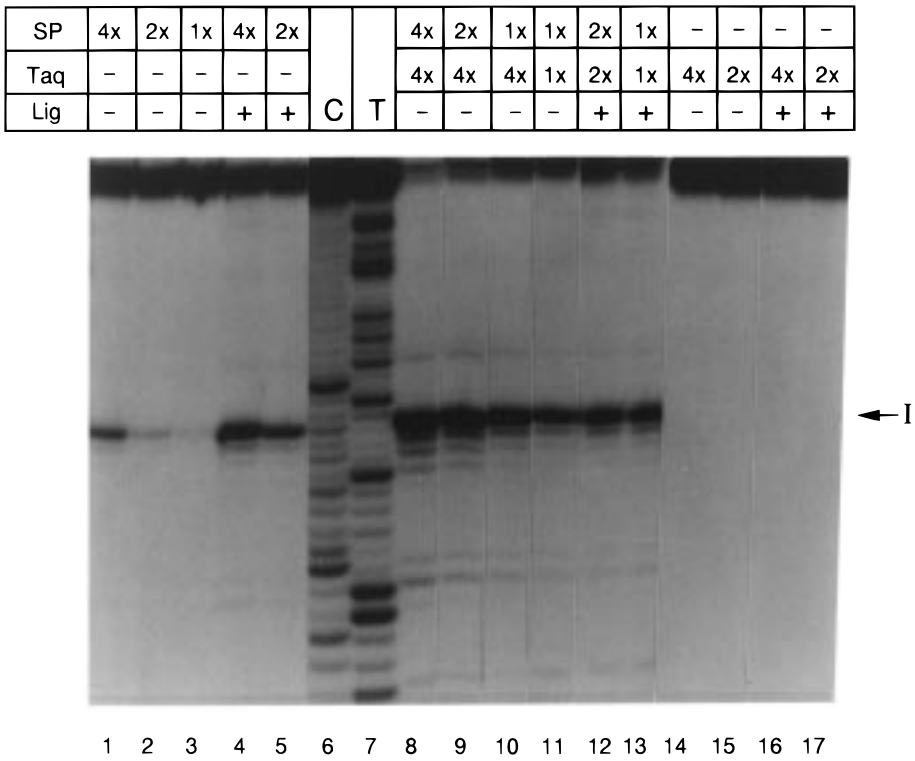

FIGURE 6: Stimulation of SP by DNA polymerase and DNA ligase. Lanes 1−3 are a dilution series of SP, 1.2, 0.6, and 0.3 ng, respectively, incubated with 50 fmol of top strand 5′-labeled A/A-mismatched heteroduplex for 30 min at 45 °C. SP nicking of the mismatch was barely visible at 0.3 ng. However, if 12.5 units of Ampligase were present, and/or 0.125 unit (1×) of Taq DNA polymerase was present, the SP cutting at the mismatch was greatly stimulated. I = incision site at the first phosphodiester bond 3′ of a mismatch. C and T chemical DNA sequencing ladders are shown for position reference.

strand. One of these C mismatch conformations is favored in the reaction.

Lanes 5, 6, and 7 of Figure 3 showed that A/A, T/T, and C/C mismatches are also incised, but T/T is cut less well by SP. Other base-substitution mismatches were tested under these conditions or under more favorable conditions and will be described after we show how the favorable conditions were established.

To ascertain whether the lack of incision at guanine nucleotides by the purified SP was due to the loss of some activity or protein factors during enzyme purification, we performed an assay of the crude cell extract (Figure 4). Extracts of broccoli, cabbage, cauliflower, and celery were able to incise at all four substrates containing an extrahelical nucleotide (panels A−D, lanes 2−5) without significant background cutting in the no-mismatch substrate (lane 1), as expected due to the presence of a CEL I-like activity (*16*). In contrast, spinach extract (panel E) failed to incise at the substrate containing the extrahelical G residue, but it was able to cut the three substrates with A, C, or T extrahelical nucleotides. As a further survey, extracts of 3-week-old seedlings of six varieties of spinach were tested with this extrahelical G substrate, and all were found to be unable to incise at this mismatch (data not shown). The spinach varieties tested were all of 1998 lots: Avon Hybrid, Melody Hybrid, TYEE, Indian Summer Hybrid, and two varieties of Bloomsdale Long-Standing spinach. This finding suggests that the inability of SP to cut at a G mismatch is not unique to one variety of spinach.

Incision of Mismatch by SP under Physiological Conditions. Figure 5 shows that SP is efficient at mismatch recognition at 22 and 37 °C under a variety of ionic strength conditions and neutral pH. These conditions are not known to favor the mechanisms of S1, P1, and mung bean nuclease type nucleases. For example, S1 nuclease does not cleave DNA at single-base mismatches at pH 4.6 (17) or pH 7.5 (data not shown). The efficiency of mung bean nuclease to nick supercoiled DNA is 5 orders of magnitude higher at pH 5 than at pH 8 (18). In this substrate containing two extrahelical GT nucleotides, the incision by SP occurred between the GT dinucleotides, at the 3′ side of the G residue, as determined by comparison with the chemical DNA sequencing ladder on the side (19). Whether the apparent incision position 3′ of a G residue is the result of incision at the 3′ side of the T residue, followed by exonuclease removal of the T residue, was not tested. It will be interesting to elucidate the parameters that govern the nucleotide specificity of these nucleases.

Mechanism of Turnover of the SP Nuclease. Figure 6 illustrates the ability of Taq DNA polymerase and Ampligase to stimulate SP activity. In Figure 6, lanes 1−3, decreasing amounts of SP were incubated with 50 fmol of A/A-mismatched substrate for 45 min. The SP mismatch-specific incision band was barely visible in lane 3. In lanes 4 and 5, the presence of the Ampligase during the SP incubation greatly enhanced the SP activity. In lanes 8−13, various combinations of SP and Taq DNA polymerase, with or without the DNA ligase present, stimulated the SP activity. All incubations were performed in buffer C, and dNTP was absent in these incubations. Comparing lanes 13 and 3, one can see that the SP stimulation by DNA polymerase and DNA ligase is over 20-fold. Exo$^-$ Klenow DNA polymerase

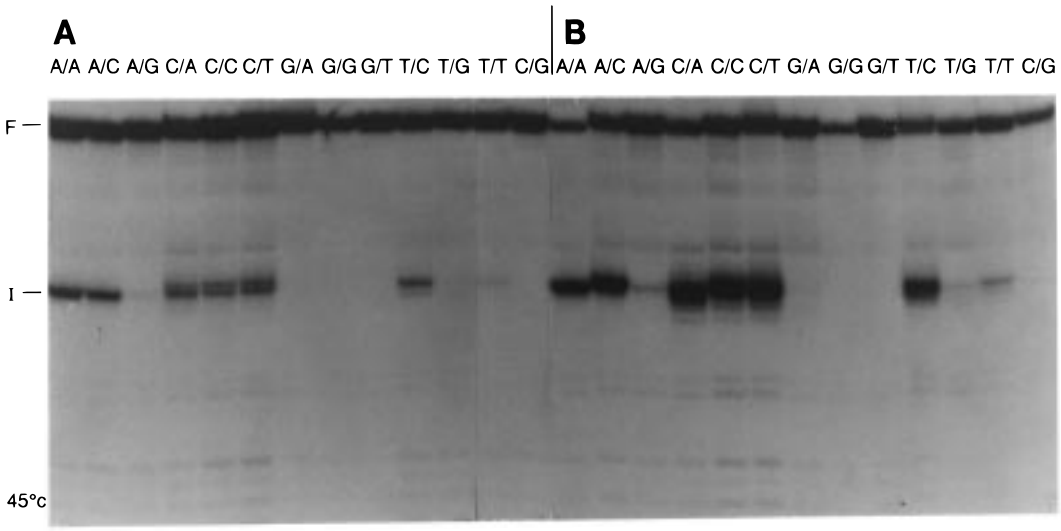

FIGURE 7: Recognition of base-substitution mismatches by SP. 5′-Labeled substrates described in Figure 1C were incubated with 5 ng of SP for 30 min at 45 °C. Samples were analyzed as described in Figure 2. Panel B is the same as panel A, except for the presence of 0.5 unit of Taq DNA polymerase, but no dNTP. F = position of full-length single-strand DNA. I = incision site at the first phosphodiester bond 3′ of a mismatch.

I fragment missing the 3′ to 5′ exonuclease activity can substitute for the Taq DNA polymerase to stimulate SP activity (data not shown), although a thermostable DNA polymerase is more appropriate at 45 °C. The DNA polymerase and DNA ligase, by themselves or together, do not lead to mismatch nicking (lanes 14−17). This lack of incision by a DNA polymerase on mismatch substrates is in contrast to a Y-type junction that can be nicked by eubacterial DNA polymerases (*20*).

The incision of SP at various base-substitutions, in the absence or presence of stimulation by Taq DNA polymerase, is shown in Figure 7. Some base-substitutions are better substrates for SP than others. To the best of our knowledge, no single-strand-specific nuclease other than the CEL I family (*16*) has been able to make such dramatic mismatch-specific incisions. Guanine residues in base-substitutions (G/A, G/G, G/T, A/G, and T/G) and T/T in our model substrate sequence are not incised by SP appreciably in the absence or presence of stimulation by Taq DNA polymerase.

An interesting possibility, but not the only one, is that the spinach SP nuclease may be a natural variant among the CEL I family of mismatch endonucleases. If this were true, sequence comparison will facilitate the identification of the active site and the elucidation of the parameters that control nucleotide specificity. Furthermore, the absence of the guanine cutting ability in SP is coincident with the presence of cutting at A-T-rich sequences. The latter property is not observed for the CEL I family of nucleases, but is a feature of the S1 family of nucleases and the mung bean nuclease (*21*). Therefore, the properties of SP seem to be intermediate between those of S1-type nucleases and CEL I-type nucleases. The availability of the sequences of these nucleases in the future may shed light on their evolutionary relationships and should clarify why SP cannot cut at most guanine nucleotides.

While the mismatch-removal function of SP, coupled with the proofreading and nick-translation ability of a DNA polymerase, forms an efficient mismatch-removal system in vitro, it is unclear whether mismatch repair is a role for SP in vivo. For example, SP is unable to determine which strand should be preserved as template in the mismatch correction process in vitro. However, its activity is consistent with the characteristics of gene conversion where different species, and different gene regions are known to exhibit unequal amounts of sequence conversion. In such a role, the inability to incise a mismatch at a guanine residue may lead to less gene conversion at some sites.

It was previously shown that SP can incise at pyrimidine $TC_{(6-4)}$ dimers and cisplatin adducts (*12*), suggesting that SP may have a role in DNA repair of these lesions. Those studies were done without using a DNA polymerase or a DNA ligase to stimulate SP. It will be interesting to determine whether the SP incision at these adducts will be more efficient under the conditions established in this paper for mismatch incision.

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