Self-catalyzed Site-specific Depurination of G Residues Mediated by Cruciform Extrusion in Closed Circular DNA Plasmids

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Background: Self-catalyzed depurination of G residues occurs readily in single-stranded stem-loops of appropriate sequence.
Results: The catalysis occurs in duplex DNA of comparable sequence upon cruciform extrusion, but not in relaxed circular plasmids.
Conclusion: The catalytic reaction for self-depurination can occur under essentially in vivo conditions.
Significance: Mutagenic consequences of the error-prone repair of resultant apurinic sites should be manifest in vivo.

A major variety of “spontaneous” genomic damage is endogenous generation of apurinic sites. Depurination rates vary widely across genomes, occurring with higher frequency at “depurination hot spots.” Recently, we discovered a site-specific self-catalyzed depurinating activity in short (14–18 nucleotides) DNA stem-loop-forming sequences with a 5’-G(T/A)GG-3’ loop and T-A or G-C as the first base pair at the base of the loop; the 5’-G residue of the loop self-depurinates at least 103-fold faster than random “spontaneous” depurination at pH 5. Formation of the catalytic intermediate for self-depurination in double-stranded DNA requires a stem-loop to extrude as part of a cruciform. In this study, evidence is presented for self-catalyzed depurination mediated by cruciform formation in plasmid DNA in vitro. Cruciform extrusion was confirmed, and its extent was quantitated by digestion of the plasmid with single strand-specific mung bean endonuclease, followed by restriction digestion and sequencing of resulting mung bean-generated fragments. Appearance of the apurinic site in the self-depurinating stem-loop was confirmed by digestion of plasmid DNA with apurinic endonuclease IV, followed by primer extension and/or PCR amplification to detect the endonuclease-generated strand break and identify its location. Self-catalyzed depurination was contingent on the plasmid being supercoiled and was not observed in linearized plasmids, consistent with the presence of the extruded cruciform in the supercoiled plasmid and not in the linear one. These results indicate that self-catalyzed depurination is not unique to single-stranded DNA; rather, it can occur in stem-loop structures extruding from double-stranded DNA and therefore could, in principle, occur in vivo.

Spontaneous generation of apurinic sites, i.e. depurination, is a major type of endogenous DNA damage (1). It has been estimated to occur in vivo at a rate of ~104 AP sites/cell/day, or 3 x 10^{-9} min^{-1} (2). AP sites are mutagenic because of their error-prone repair (3, 4), and potentially lethal because they can block replication and transcription (5–8). Although such sites can arise during base excision repair (6), other sources of their endogenous generation are less well understood. Recently, we described a novel mechanism for endogenous AP site generation caused by a DNA self-catalyzed mechanism for site-specific depurination of singular G residues present in a frequently occurring consensus sequence in the human genome (9, 10). This self-depurinating consensus sequence has a potential for forming a stem-loop structure, with a specific 6-nucleotide sequence, 5’-(T/G)G(T/A)GG(A/C)-3’ consisting of a 4-residue loop and the first base pair of the stem, in which the 5’-G residue of the loop is uniquely depurinated. The self-catalyzed depurination rate measured in vitro for stem-loops formed by single strands under “physiological conditions” mimicking intracellular macromolecular crowding (11, 12), i.e. a high concentration of polyethylene glycol in a pH 7 buffer with 0.1 M NaCl and 0.005 M MgCl2, is between 8 x 10^{-6} and 4 x 10^{-5} min^{-1}, depending primarily on the stability of the stem but also on the somewhat variable loop sequence (Fig. 5 in Ref. 10). In this rate range, the self-depurination mechanism might generate at least several thousand apurinic sites/cell/day if the stem-loop catalytic intermediate were to extrude as part of a cruciform for a length of time sufficient for catalysis to occur at each site.

Stem-loops in the form of cruciforms were shown previously to extrude readily under superhelical stress, as does hinged DNA (which contains a three-stranded element formed by a segment of homopyrimidine strand folding back on the duplex at its point of symmetry in an inverted repeat sequence, whereas the homopurine complement serves as a non-hydrogen-bonded linker) (13–17). Their formation has been directly demonstrated in bacteria (13, 15, 16), and there is much evidence suggesting formation of such structures in eukaryotic cells as well (15, 17–21).

DNA cruciform-binding proteins have been characterized in mammalian cells and shown to bind to chromatin sections.
that contain inverted repeats (15, 18, 19, 21); such stem-loop and cruciform structures are also strongly implicated in mutational events and genome instability (15, 22, 23). A source of DNA supercoiling necessary for cruciform extrusion can be provided, for example, by a wave of supercoiling traveling through chromatin at times of transcription and replication (24) or by action of certain DNA-binding proteins, e.g. DNA gyrase.

As a step toward demonstrating the potential of this self-depurinating mechanism in vivo, the present study focuses on its occurrence in supercoiled plasmid DNA in vitro. Sequences containing either the self-depurinating loop 5′-GTGG-3′ or a nondepurinating 5′-TTTT-3′ loop, surrounded by an (A-T)$_{16}$ stretch with a potential to form a stem-loop structure (see Table 1) were cloned into a pUC19 vector, which in turn was transfected into Escherichia coli. Cruciform extrusion in those supercoiled plasmids capable of self-depurination enabled detection and identification of apurinic sites and demonstration of their absence when self-depurination was not possible, either because of loop sequence or because the plasmid was relaxed or linearized. These results indicate that self-catalytic depurination is not unique to single-stranded DNA and is relevant to the principal genomic form in vivo, that of the supercoiled duplex.

**EXPERIMENTAL PROCEDURES**

*Deoxyoligonucleotides*—These were commercially synthesized (IDT), purified on denaturing 12% PAGE, eluted from the gel using the “freeze and squeeze” method (25), and desalted using Sep-Pak C18 columns (Waters). They were eluted with 50% acetonitrile, dried in a vacuum concentrator, and dissolved in Tris/EDTA buffer, pH 7, or Aldrich ACS reagent grade water.

*Kinetics of Deoxyoligonomer Depurination*—Kinetics of deoxyoligonomer depurination were determined as in Ref. 10.

*Cloning of Stem-Loop-forming Inserts*—The strands used for the self-depurinating and control stem-loops were designed so as to form a complementary duplex with sticky ends compatible with BamHI or HindIII restriction fragments. Duplexes were formed by slow annealing of equimolar amounts of the complementary strands. The duplexes were ligated with a pUC19 vector (2686 bp) pretreated with BamHI and HindIII, then ligated with alkaline phosphatase, and gel-purified before ligation. Asymmetric noncompatible ends of the vector and the alkaline phosphatase pretreatment efficiently prevented self-ligation, whereas the BamHI- and HindIII-compatible ends of the duplex inserts ensured unidirectional cloning. *E. coli* SURE strain (Stratagene) was transformed with ligation mixtures. Clones were analyzed by sequencing (Genewiz), using the pUC primers at positions 24 and 501 (the insertion point was at position 426).

*Enzymes*—Restriction endonucleases BamHI, HinfIII, NdeI, and BssSI were from New England Biolabs (NEB), and digests were conducted according to the supplier’s instructions. Single strand-specific mung bean endonuclease (NEB) was used to detect cruciform extrusion; endonuclease IV (NEB) to detect AP sites.

*Preparation of Supercoiled Plasmids Free of AP Sites*—To create a population of plasmids free of apurinic sites that inevitably accumulate during plasmid growth and purification, mainly supercoiled plasmids were treated with piperidine that converted existing AP$^+$ sites into strand breaks and, as a result, converted supercoiled plasmids into the open circular form. Lacking an AP site, the remaining supercoiled fraction was separated from the open circular fraction by agarose gel electrophoresis and purified by gel purification kits from Qiagen.

*Self-depurination Kinetics*—Supercoiled plasmid, prepared as described above, was incubated at 22 °C, pH 5, and aliquots were taken at intervals and frozen on dry ice. The self-depurination products in the aliquots were then simultaneously treated with AP endonuclease to convert the accumulated AP sites into strand breaks, and the resulting fragments were digested with BssI, purified on Qiagen PCR purification columns, and used as templates for asymmetric PCR amplification.

**RESULTS**

*Self-depurination in Deoxyoligonomer Stem-Loops*—The duplex sequences used for cloning the stem-loop-forming inserts into the pUC19 vector are shown in Table 1, and Fig. 1 illustrates their duplex → cruciform transition. The oligomers consist of a BamHI or HindIII 5′-sticky end for positional cloning into the multiple cloning region of the pUC19 plasmid, followed by a 16-bp alternating A-T sequence, then a 4-bp loop-forming segment, and finally another 16-bp alternating T-A stretch. This arrangement provided a highly efficient self-depurinating loop on one of the duplex strands (SD-1), i.e. 5′-GTGG-3′ flanked by a T-A first stem base pair (9, 10) on one of the duplex strands (top), whereas the complementary strand (SD-2), with a 5′-CCAC-3′ loop had no self-depurinating ability. The control insert sequence, with the same sticky ends and alternating A-T stretches, which had instead 5′-TTTT-3′ on the loop-forming segment of one strand and 5′-AAAA-3′ on its complement, totally lacked self-catalyzed depurination.

2 The abbreviations used are: AP, apurinic; MB, mung bean.
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In fact, consistent with well known problems associated with cloning cruciform-forming sequences, after transforming E. coli with standard ligation reaction products (27) of both self-depurinating and control cruciform inserts with the linearized and dephosphorylated plasmid vector, the number of resulting clones was 50–100 times less than for a similarly sized but non-self-complementary insert used as a control of ligation and transformation conditions. However, there was also a reproducibly significant difference between the behavior of self-depurinating and control cruciform-forming inserts. Thus, several cloning attempts yielded no clones with the self-depurinating insert, but 10–20 clones with the non-self-depurinating T-loop cruciform under comparable standard transformation and ligation conditions. Reasoning that such a consistent failure may be due to self-depurination of one of the insert strands while in the stem-loop conformation during the ligation reaction, we sought to further shift its duplex → hairpin equilibrium by adding non-self-complementary flanking sequences to both strands (SD-3 and SD-4; Table 1). This strategy proved successful, and the cloning of such a modified insert resulted in 10 clones. Nevertheless, subsequent restriction digestion and sequence analysis revealed a high rate of insert loss and/or acquired mutations in the self-depurinating loop. These results are summarized in Table 2. To ensure the presence of the self-depurinating insert with the correct loop

depurination capacity (10). When cloning difficulties for the self-depurinating insert were encountered with the original design just described, non-self-complementary flanks were added to the SD-1 and SD-2 sequences; this resulted in SD-3 and SD-4 oligomers that eventually were successfully cloned and used in plasmid experiments. The alternating A-T stems were selected for these experiments because similar cruciform-forming sequences had been shown previously (16) to readily extrude in supercoiled plasmid DNA under physiological conditions in vitro. Single-stranded oligomers Tloop-1, Tloop-2, and SD-2 with loop sequences 5’-TTTTT-3’, 5’-AAAAA-3’, and 5’-CCAC-3’, respectively, showed no evidence of backbone cleavage by the piperidine treatment employed to reveal AP sites. In contrast, oligomer SD-2 did give such evidence of self-depurination, with a rate of $4 \times 10^{-4}$ min$^{-1}$ (Fig. 2). The addition of the noncomplementary flanking sequences, as in SD-3, slightly reduced this value (not shown).

Cloning of the Cruciform-forming Sequences—For kinetic reasons, palindromic deoxyoligonucleotide sequences are prone to form self-complementary hairpins instead of annealing with their complements to form the duplexes with cohesive ends that are required for successful cloning. To minimize such hairpin formation, high strand concentrations ($10^{-5}$ M) of the potential duplexes were annealed while cooling from 70 to 4 °C over 12–16 h. Such slow annealing leads to attainment of the thermodynamically favored duplex state. In fact, PAGE analysis revealed at least 50% duplex formation by mobility shift assay (not shown).

Cruciform-forming palindromic or inverted repeat sequences have been found to be unstable in E. coli and prone to spontaneous deletions (24). Nevertheless, we selected potential A-T-rich stem sequences for our self-depurination trials in plasmids because they are known to readily extrude cruciforms in the supercoiled plasmid environment (16, 26) and at the same time make relatively stable plasmid inserts.
sequence in all the experiments, every large-scale plasmid preparation was followed by sequencing.

**Confirmation of Cruciform Extrusion in Supercoiled Plasmids by Mung Bean Endonuclease Digestion of the Cruciform Loops**—Cruciform extrusion under superhelical stress should result in the formation of two single-stranded loops at the center of the inverted repeat, one on each strand of the DNA duplex; such loops should be susceptible to single-strand-specific endonuclease cleavage (26, 28, 29). Previously, we employed mung bean endonuclease to demonstrate the stem-loop structure of the catalytic intermediate for self-depurination (9); we now used a similar approach to detect cruciform extrusion. In this connection, it is important to note that not all the supercoiled plasmid molecules will have the extruded cruciform; rather, only those topoisomers in which the supercoil density reaches a critical level sufficient to provide the energy for a transition to a nonequilibrium state (26). It is important to know what fraction of the plasmid population contains extruded cruciform at any one time and therefore may be capable of self-catalyzed depurination in one of its cruciform loops; this information is crucial in estimating the rate of self-catalyzed depurination in the sample.

Mung bean endonuclease digestion of a plasmid with extruded cruciform should lead to a site-specific double-stranded break that can be detected by the appearance of extra fragments in a restriction endonuclease digest. BssSI restriction endonuclease converts pUC19 into three fragments of ~1200, 1000, and 300 bp (Fig. 3A). The 1000-bp fragment contains the cruciform insert; its digestion by MB endonuclease should lead to the appearance of fragments of 533 and 466 bp. As Fig. 3B shows, these fragments only appear when the plasmid is predominantly supercoiled; neither fully linearized, nor predominantly relaxed plasmids display any detectable additional fragments. Up to 50% of the supercoiled plasmid fraction contains extruded cruciform at any one time, as revealed by quantitation of the kinetics of MB endonuclease digestion followed by BssI restriction analysis (not shown). This proportion of extruded cruciform is sufficient to enable unambiguous detection of self-catalyzed depurination in the extruded stem-loop.

**Detection of Self-catalyzed Depurination on Agarose Gel Using PCR Amplification**—Several assays illustrated in Fig. 4, involving PCR amplification and primer extension, were employed to detect self-catalyzed depurination in the double-stranded plasmid DNA with extruded cruciform. Two sets of primers encompassing the insert region at position 420 were used for these experiments, at positions 24–501 and 190–678 (the numbers correspond to the start position of the primer sequence in the pUC19 plasmid). When a cruciform extrudes from the double-stranded DNA, both strands form a stem-loop structure, and both loops are digestible with MB endonuclease (Fig. 3). However, only one of these stem-loops is capable of self-catalyzed depurination that creates an apurinic site in one of the loops, and only this loop (but not the one on the complementary strand) can be cleaved by AP endonuclease. When a substantial fraction of the PCR template is digested by one of these nuclease, truncated DNA copies will be produced from such a template (along, of course, with full-size products from intact templates); these truncated copies will hybridize with full-size complementary strands to yield faster moving bands on agarose gel electrophoresis. As shown in Fig. 5, the appearance of such a band in the “self-depurinating” plasmid (Fig. 5, lane 6), but not in the vector or in nondepurinating plasmid (lanes 4 and 1) suggests the presence of such truncated templates and therefore occurrence of self-catalytic depurination within the supercoiled plasmid DNA under physiological conditions. As expected, if cruciform extrusion occurs, a similar size band appears when stem-loop-forming plasmids are treated with mung bean single strand-specific endonuclease that digests the loops on both complementary strands (lanes 2 and 3).

**Detection of Self-catalyzed Apurinic Sites by Analyzing PCR Amplification Products on Denaturing PAGE**—Only the stem-loop formed by the coding strand of the plasmid used in these experiments should be susceptible to digestion by AP endonuclease as a result of self-catalyzed depurination. The experiments described below were aimed at detecting and identifying PCR products that arise as a result of self-catalyzed depurination in extruded stem-loops and subsequent strand breakage or digestion at the resultant apurinic sites. This site-specific breakage of only the coding template strand should lead to truncated primer extension products of a specific size with the reverse primer but not with the direct one. However, the reality tends to be more complicated because the mere presence of stem-loop-forming sequences and stem-loop formation during PCR amplification can also lead to truncated products as a result of the polymerase “pausing,” dissociating, or “skipping” the self-paired stem-loop region (30, 31). Obviously, with the strand breakage at the apurinic site occurring only in a minority of the substrate molecules, the truncated products may be difficult to detect because of the exponential nature of the PCR amplification that “drowns” minor templates.

Asymmetric PCR amplification (32, 33) with the excess primer labeled provides a means for identification and analysis of such truncated primer extension products, at the same time circumventing most of the above-mentioned problems. Unambiguous identification of the truncated products arising as a consequence of self-catalyzed depurination was achieved by comparing results of asymmetric PCR utilizing several primer pairs (illustrated in Fig. 4), primer extension without amplification, and the effect of treating the template with AP endonuclease. Fig. 6 shows a typical asymmetric PCR amplification pattern of the BssSI-digested plasmid along with a schematic

**TABLE 2**

| Insert | Nondepurinating T-loop | Self-depurinating GTGG loop |
|--------|------------------------|-----------------------------|
| Clones obtained | 20 | 10 |
| Clones analyzed | 10 | 10 |
| Insert present | 10 | 5 |
| Insert sequenced | 5 | 5 |
| Loop mutations | 0 | 3 |

A strong negative effect of self-depurinating ability on cloning efficiency is apparent.
array of BssSI digestion fragments and various PCR products obtained with BssSI-digested plasmids as templates. The amplified region is between nucleotides 198 (direct) and 678 (reverse) of the pUC19 plasmid. Taking into account the sizes of the inserts, the sizes of the main amplification products are 488 bp (pUC19), 494 bp (pUC-T loop), and 506 bp (pUC-SD). These size differences, albeit small, are nevertheless sufficient to afford discernable differences in electrophoretic mobility. The BssSI restriction site at 2670 nt determines the size of the run-off PCR products, i.e., 694, 700, and 712 bp, respectively, for the three plasmids; their band intensity is quite high because of the asymmetric PCR design, with labeled primer in significant excess. Clearly, the products for the pUC19 vector are in full agreement with these predictions, with virtually no minor bands. However, both SD and control Tloop plasmids with cruciform inserts show additional bands, one of ~650 nt and, to a lesser extent, another one of ~520 nt. These bands are apparently the results of cruciform “skipping” (~650 nt) in the run-off product and polymerase “slippage” at the alternating A-T region (~520 nt) in the main amplification product. The pattern is very similar for both direct and reverse primers. However, when the reverse primer is labeled, a prominent band of ~250 nt appears only for the self-depurinating template, but not for the control. Its size corresponds reasonably well to the position of the self-depurinating site (expected size is 230 nt). Moreover, treatment with AP endonuclease significantly enhances its intensity (shown in Fig. 7), consistent with its being the result of template strand cleavage at the apurinic site as a result of self-depurination. Thus, the foregoing results provide evidence that self-depurination does occur in the negatively

FIGURE 3. Digestion of the pUC19-SD plasmid with the extruded cruciform by MB endonuclease and AP endonuclease. A, schematic representation showing recognition sites of the BssSI restriction enzyme used to confirm the location of the cruciform and the AP site. B, a PAGE analysis of cruciform extrusion in pUC-SD plasmid, as revealed by MB endonuclease and/or BssSI restriction endonuclease digestion of supercoiled and linearized plasmid fractions. Lin, linear plasmid; SC, supercoiled plasmid; OC, open circular plasmid.
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supercoiled plasmid containing the consensus sequence for self-depurination.

Estimation of the Rate of Self-catalyzed Depurination in Supercoiled Plasmids—Although apurinic sites in the extruded stem-loops apparently accumulate during plasmid growth and purification, it is possible to control their formation in vitro and even estimate its rate by cleaving the backbone at the already existing sites with piperidine treatment and then looking at the accumulation of new ones. Supercoiled plasmids so freed of AP sites were prepared (see “Experimental Procedures”). The presence of the extruded cruciform in the purified fraction was confirmed by sequential mung bean endonuclease and BssSI endonuclease digestion. Asymmetric PCR with the reverse (678 nt) primer labeled was used to confirm the absence of AP sites in the preparation and to reveal the newly accumulated AP sites (Fig. 7). The self-depurinating plasmid freed of AP sites was digested with MB and BssSI or only with BssSI. The 210-nt band was absent in the sample digested only with BssSI, but a very similar band appeared after MB digestion of the loop region that produced a template truncated at the same position or within 2 nt of the site of self-catalyzed depurination. After quantitation, such results were used to estimate the fraction of the truncated template in the mix. The 210-nt bands are the products of primer extension without amplification; the bands in the 700-nt range result from similar extension of the original labeled primer of 678 nt and to some extent, from extension of the PCR products containing the 678-nt primer, either truncated or full size, that are annealed to the full-size BssSI restriction fragment. Hence, the full-size run-off product fraction may be artificially increased, but that increase can be moderated if the excess of the 678-nt primer and low number of PCR cycles makes annealing of that primer to the template more probable than annealing of the longer, secondary PCR products. Comparing the intensities of the 210 nt and 700 nt fragments provides the lower limit of the fraction of the truncated template in the mix. The validity of this approach can be tested by comparing the numbers obtained in such a fashion to the fraction of the MB endonuclease-digested plasmid fraction measured by direct quantitation of ethidium bromide fluorescence on the agarose gel (Fig. 3B). In fact, the agreement between the two numbers is quite reasonable, i.e. 25 and 30%, respectively.

Kinetics of Self-catalyzed Depurination in Extruded Cruciforms—Supercoiled plasmids incubated at pH 5 (see “Experimental Procedures”) and digested with AP endonuclease and BssSI were used as templates in asymmetric PCR, with the reverse primer (678 nt) labeled. Comparison of the intensities of the truncated 210 nt band and the 700 nt run-off product gives an estimate of the AP site content in the original incubated samples. Fig. 8 shows a fraction of the truncated primer extension product in the PCR mix increasing with the time of incubation. Because AP endonuclease, the agent used to reveal the AP sites, required 1 h of incubation at 37 °C, the shorter time points are blurred, presumably because self-catalyzed depurination does not stop during the AP endonuclease digestion. Still, by 24 h of incubation,

FIGURE 4. Schematic representation of the detection of an apurinic site using PCR amplification and primer extension. Two pairs of primers for the pUC19 plasmid are shown on either side of the cruciform-forming duplexes inserted at position 449. The BssSI 1000-bp fragment containing the inserts and primer binding sites serves as a template in PCRs. These inserts replace the pUC19 region 426–452 (HindIII-BamHI sites) with stem-loop-forming inserts of 58 bp for the pUC19-SD plasmid and 40 bp for the pUC-Tloop plasmid. Hence, the PCR fragments produced using any pair of pUC19 primers that surround the insertion sites will slightly differ in length when the pUC19 vector, the pUC19-Tloop (−26 + 40 = +14 bp), or the pUC19-SD (−26 + 58 = +22 bp) are used as templates. This variation in length effectively prevents any experimental mix-ups and allows unambiguous identification of the template. Self-catalyzed depurination at position 449 can only occur on one of the strands, that containing the self-depurinating TGTGGA loop plus first base pair residues. When the AP site results in strand breakage (either by β-elimination with piperidine or by AP endonuclease digestion), shortened templates give rise to truncated PCR products with reverse primers 501 and/or 678. When both strands are digested with MB nuclease, extension of both direct and reverse primers should yield truncated copies, whose length will similarly vary with the primer location.

FIGURE 5. Detection of self-catalyzed depurination by agarose gel electrophoresis. PCR amplification of region 24–501 of the pUC19 vector, with or without stem-loop-forming inserts at position 449, after treatment with AP endonuclease (that digests an AP site) or MB endonuclease, each specific for single-stranded DNA. Strand break(s) at the depurination site lead to truncated PCR products, which, in turn, upon annealing with primer extensions to the full-size complementary strands, produce faster moving partially double-stranded duplexes. Such an additional band is seen when the plasmid self-depurinating insert (GTGGG loop of pUC19-SD) is treated with AP endonuclease (lane 6). This band is the result of primer extension truncation at the AP site, which was converted to strand breaks. A similar endonuclease band appears when both insert-containing plasmids are treated with MB endonuclease (lanes 2 and 3) that converts single-stranded loops of extruded cruciform into strand breaks on both complementary strands. No extra bands appear when the vector is treated with either enzyme (lanes 4 and 5) or after AP treatment of nondepurinating plasmid pUC19-Tloop (lane 1). Note the slight difference in the mobility of the full-size amplification fragments, consistent with the differences in the template size of the three plasmids.
20% of the plasmid is digested by the AP endonuclease to produce the truncated PCR template. Taking into account that the cruciform-containing fraction of the supercoiled plasmid is also ~20%, one can conclude that most of the potential self-depurinating sites in the plasmid are converted into AP sites in less than 24 h.

**DISCUSSION**

In this study, we have sought insight into the possible biological relevance of site-specific self-catalyzed depurination, which was discovered in a single-stranded stem-loop formed by a short deoxyoligonucleotide segment of the coding strand of the human β-globin gene surrounding the site of the sickle cell mutation (9). It is the case that self-catalyzed depurination is inhibited when such a stem-loop-forming sequence is annealed with its complementary strand to form a linear duplex. However, when strand segments of a double-stranded genome separate during transcription or replication, there are significant opportunities for transient formation of single-stranded stem-loops of the size required for self-depurination by inverted repeat sequences; such inverted repeat sequences are, in fact, overrepresented in many genomes, have been implicated in some diseases, and are well known to trigger genomic instability (15, 34). Another opportunity for stem-loop formation is provided by extrusion of cruciforms from double-stranded DNA. Although extrusion of cruciforms from linear duplex DNA is thermodynamically unfavorable, so that such structures should be short-lived, in negatively supercoiled DNA, relatively stable cruciforms can extrude when the superhelical density of a closed circular DNA fragment exceeds some critical level. Such cruciform extrusion has been extensively studied in DNA plasmids and was also recently demonstrated in genomic DNA as well. In fact, cruciform extrusion has been directly visualized in the sequence of human chromosome 11 by atomic force microscopy (15), and the same cruciform-forming sequences were demonstrated to be the source of frequent chromosomal rearrangements (34–36). Formation of relatively stable extruded secondary structures has also been shown to be greatly facilitated by transcription (37–39), and such structure formation apparently underlies somatic mutations in some genes, e.g. in Tp53 (39), and plays a role in somatic hypermutation in B cell immunoglobulin gene mutation (38).

Our approach in this investigation has been to insert a cruciform-forming inverted repeat with a loop sequence capable of efficient self-catalyzed depurination in vitro into the pUC19 plasmid and thereby to demonstrate that a significant fraction of such a plasmid does, in fact, contain the extruded cruciform. A pH of 5 has been used to accelerate the self-depurination in the supercoiled plasmid. However, as shown in the accompanying paper (10), the rate at pH 7, though lower, is still above background, making the present observations biologically significant.

If self-catalyzed depurination occurs in one of the cruciform loops (the other loop, with a complementary sequence, cannot self-depurinate), the resultant apurinic site can be converted into a strand break that leads to a shorter template and therefore truncated primer extension or PCR amplification products. Hence, the results presented enable us to unambiguously attribute some of the truncated primer extension products to such strand breakage at the apurinic site. It is also significant that the rate of self-catalyzed depurination in such extruded cruciforms was found to be similar to that in a single-stranded stem-loop structure.

Previously, inverted repeats and hairpin-forming sequences had been associated with mutational hot spots and genomic instability (40, 41). Stem-loop-mediated self-catalyzed depurination may be one of the mechanisms underlying that association, especially because bypass of an apurinic site by DNA
repair polymerases (as opposed to AP site excision and subsequent repair) is almost always mutagenic, and such bypass events apparently occur (at least in in vitro experiments) with impressive 10–20% frequency (42).

In sum, self-catalyzed depurination leads to highly mutagenic apurinic sites with a potential to give rise to substitutions and short deletions or additions. Such self-depurinating sequences are, in fact, very widely distributed in the human genome; they are grossly overrepresented in many genes whose functional loss can be associated with various diseases, and they appear to function in other genes as a means of creating sequence diversity (43). The present study showing that self-catalyzed depurination of G residues can occur in a supercoiled plasmid takes what was previously an apparent peculiar in vitro reaction inherent in DNA a significant step closer to its direct demonstration in vivo.

FIGURE 7. Accumulation of apurinic sites starting with AP-free plasmids. Supercoiled fractions of the piperidine-treated plasmids (piperidine treatment converts AP sites into nicks and so converts supercoiled plasmids into open circular ones), after digestion with either AP endonuclease or MB endonuclease, followed by digestion with BssSI, were used as templates in asymmetric PCR with labeled primer 678 to reveal the presence (or absence) of strand breaks at the apurinic site that results in a 250 nt product. The 190–678 PCR products are —500 nt; the full-size runoff primer extension products (determined by the site of BssSI digestion) are —700 nt. Although no short products are observed for the pUC19 vector and for BssSI-only treated pUC19-Tloop and pUC19-SD templates, MB endonuclease treatment results in 250 nt products for both cruciform-containing plasmids. Quantitative comparison of the 250 nt band (primer extension product truncated at the AP-generated strand break) to the 713 nt run-off band (full-length primer extension plus products of the annealing and extension of the 500 nt PCR fragment), which can only be smaller than the true fraction, not larger, and therefore provides a lower limit to the fraction of AP sites. The initial slope of the kinetic plot gives a rate of 0.8 × 10–2 min–1, which is slightly less than the depurination rate of the insert by itself. This is consistent with the quantitation methodology and the fact that not all the supercoiled plasmids contain an extruded cruciform.

FIGURE 8. Kinetics of self-catalyzed depurination in a supercoiled plasmid. Generation of AP sites in a supercoiled plasmid freed of AP sites at time zero (see “Experimental Procedures” and Fig. 7 legend) was monitored (after digestion with AP endonuclease) on denaturing PAGE of asymmetric PCR products with the 678 primer labeled (the appearance of strand breaks results in accumulation of truncated template for this primer). The fraction of AP-containing plasmid was calculated as a ratio of the intensities of the 250 nt band (primer extension product truncated at the AP-generated strand break) to the 713 nt run-off band (full-length primer extension plus products of the annealing and extension of the 500 nt PCR fragment), which can only be smaller than the true fraction, not larger, and therefore provides a lower limit to the fraction of AP sites. The initial slope of the kinetic plot gives a rate of 0.8 × 10–2 min–1, which is slightly less than the depurination rate of the insert by itself. This is consistent with the quantitation methodology and the fact that not all the supercoiled plasmids contain an extruded cruciform.

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REFERENCES
1. De Bois, R., and van Larebeke, N. (2004) Mutagenesis 19, 169–185
2. Lindahl, T., and Nyberg, B. (1972) Biochemistry 11, 3610–3618
3. Loeb, L. A., and Preston, B. D. (1986) Annu. Rev. Genet. 20, 201–230
4. Chakravarti, D., Mailander, P. C., Cavalieri, E. L., and Rogan, E. G. (2000) Mutat. Res. 456, 17–32
5. Guillet, M., and Boiteux, S. (2002) EMBO J. 21, 2833–2841
6. Simonelli, V., Narciso, L., Dogliotti, F., and Fortini, P. (2005) Nucleic Acids Res. 33, 4404–4411
7. Tornaletti, S., Maeda, L. S., and Hanawalt, P. C. (2006) Chem. Res. Toxicol. 19, 1215–1220
8. Boiteux, S., and Guillet, M. (2004) DNA Repair 3, 1–12
9. Amosova, O., Coulter, R., and Fresco, J. R. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 4392–4397
10. Amosova, O., Smith, A., and Fresco, J. (2011) J. Biol. Chem. 286, 36316–36321
11. Minton, A. P. (2001) J. Biol. Chem. 276, 10577–10580
12. Zheng, K. W., Chen, Z., Hao, Y. H., and Tan, Z. (2010) Nucleic Acids Res. 38, 327–338
13. Dai, X., Greierstein, M. B., Nadas-Chinni, K., and Rothman-Denes, L. B. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 2174–2179
14. Dai, X., Kloster, M., and Rothman-Denes, L. B. (1998) J. Mol. Biol. 283, 43–58
15. Kurahashi, H., Inagaki, H., Yamada, K., Ohye, T., Taniguchi, M., Emanuel, B. S., and Toda, T. (2004) J. Biol. Chem. 279, 35377–35383
16. Dayn, A., Malkhosyan, S., Duzhy, D., Lyaminchev, V., Panchenko, Y., and Mirkin, S. (1991) J. Bacteriol. 173, 2658–2664
17. Cunningham, L. A., Coté, A. G., Cam-Ozdemir, C., and Lewis, S. M. (2003) Mol. Cell. Biol. 23, 8740–8750
18. Pearson, C. E., Ruiz, M. T., Price, G. B., and Zannis-Hadjopoulos, M. (1994) Biochemistry 33, 14185–14196
19. Alvarez, D., Novac, O., Callejo, M., Ruiz, M. T., Price, G. B., and Zannis-Hadjopoulos, M. (2002) J. Cell. Biochem. 87, 194–207
20. Shlyakhtenko, L. S., Hsieh, P., Grigoriev, M., Potaman, V. N., Sinden, R. R.,
Self-depurination of G Residues in Supercoiled Plasmids

and Lyubchenko, Y. L. (2000) J. Mol. Biol. 296, 1169–1173
21. Potaman, V. N., Sbyakhtenko, L. S., Oussatcheva, E. A., Lyubchenko, Y. L., and Soldatenkov, V. A. (2005) J. Mol. Biol. 348, 609–615
22. Wright, B. E., Reimers, J. M., Schmidt, K. H., and Reschke, D. K. (2002) Cancer Res. 62, 5641–5644
23. Wright, B. E., Reschke, D. K., Schmidt, K. H., Reimers, J. M., and Knight, W. (2003) Mol. Microbiol. 48, 429–441
24. Havas, K., Flaus, A., Phelan, M., Kingston, R., Wade, P. A., Lilley, D. M., and Owen-Hughes, T. (2000) Cell 103, 1133–1142
25. Chen, Z., and Ruffner, D. E. (1996) BioTechniques 21, 820–822
26. Panyutin, I., Lyamichev, V., and Mirkin, S. (1985) J. Biomol. Struct. Dyn. 2, 1221–1234
27. Sambrook, J., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Vol. 2, 1–84, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Sheflin, L. G., and Kowalski, D. (1985) Nucleic Acids Res. 13, 6137–6154
29. Asakura, Y., Kikuchi, Y., and Yanagida, M. (1985) J. Biochem. 98, 41–47
30. Viswanathan, V. K., Krcmarik, K., and Cianciotto, N. P. (1999) BioTechniques 27, 508–511
31. Svoboda, P. (2009) Cold Spring Harb. Protoc. 2009, pdb.ip64
32. Gyllensten, U. B., and Erlich, H. A. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 7652–7656
33. Pierce, K. E., Sanchez, J. A., Rice, J. E., and Wangh, L. J. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 8609–8614
34. Inagaki, H., Ohye, T., Kogo, H., Yamada, K., Kowa, H., Shaikh, T. H., Emanuel, B. S., and Kurahashi, H. (2005) Hum. Mutat. 26, 332–342
35. Inagaki, H., Ohye, T., Kogo, H., Kato, T., Bolor, H., Taniguchi, M., Shaikh, T. H., Emanuel, B. S., and Kurahashi, H. (2009) Genome Res. 19, 191–198
36. Kato, T., Inagaki, H., Yamada, K., Kogo, H., Ohye, T., Kowa, H., Nagaoka, K., Taniguchi, M., Emanuel, B. S., and Kurahashi, H. (2006) Science 311, 971
37. Krasilnikov, A. S., Podtelezhnikov, A., Vologodskii, A., and Mirkin, S. M. (1999) J. Mol. Biol. 292, 1149–1160
38. Wright, B. E., Schmidt, K. H., Minnick, M. F., and Davis, N. (2008) Mol. Immunol. 45, 3589–3599
39. Wright, B. E., Schmidt, K., Hunt, A., Lodmell, J. S., Minnick, M. F., and Reschke, D. (2011) Carcinogenesis, in press
40. Kolb, J., Chuzhanova, N. A., Högel, J., Vasquez, K. M., Cooper, D. N., Bacolla, A., and Kehrer-Sawatzki, H. (2009) Chromosome Res. 17, 469–483
41. Zhao, J., Bacolla, A., Wang, G., and Vasquez, K. M. (2010) Cell Mol. Life Sci. 67, 43–62
42. Kokoska, R. J., McCulloch, S. D., and Kunkel, T. A. (2003) J. Biol. Chem. 278, 50537–50545
43. Fresco, J., Amosova, O., Wei, P., Alvarez-Dominguez, J., Glumcher, D., and Torres, R. (2011) in Evolutionary Biology: Concepts, Biodiversity, Macroevolution and Genome Evolution (Pontarotti, P., ed) pp. 3–19, Springer-Verlag, Berlin