

# Generation of Highly Site-Specific DNA Double-Strand Breaks in Human Cells by the Homing Endonucleases I-PpoI and I-CreI

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**We have determined the ability of two well-characterized eukaryotic homing endonucleases, I-PpoI from the myxomycete *Physarum polycephalum* and I-CreI from the green alga *Chlamydomonas reinhardtii*, to generate site-specific DNA double-strand breaks in human cells. These 18-kDa proteins cleave highly conserved 15- or 24-bp rDNA homing sites in their respective hosts to generate homogeneous 4-base, 3' ends that initiate target intron transposition or "homing." We show that both endonucleases can be expressed in human cells and can generate site-specific DNA double-strand breaks in 28S rDNA and homing site plasmids. These endonuclease-induced breaks can be repaired *in vivo*, although break repair is mutagenic with the frequent generation of short deletions or insertions. I-PpoI and I-CreI should be useful for analyzing DNA double-strand break repair in human cells and rDNA.** © 1999 Academic Press

DNA double strand breaks (DSBs) are a common and important form of DNA damage that can be generated by exogenous agents such as ionizing radiation, and by endogenous agents such as reactive oxygen species. DSBs are also generated during nucleic acid metabolism. These "physiologic" DSBs play roles in the resolution of replication products and in meiotic recombination and immunoglobulin or T-cell receptor gene rearrangement. Unrepaired DSBs from any of these sources can promote mutagenesis, cell cycle arrest or cell death (1–4).

DSB repair is best understood in bacteria and yeast, where homologous recombination is required for efficient repair (4–6). Mammalian cells, in contrast, rely more heavily upon non-homologous end joining for

DSB repair (1–4). A better mechanistic understanding of DSB repair in mammalian cells will require detailed molecular information on the processing of repair intermediates in cells with different DSB repair capacities. These data can be most easily obtained from analyses of the repair of highly site-specific DSBs. This approach has been developed and exploited in budding yeast, where the mating type switch (*HO*) endonuclease has been used to generate site-specific DSBs in heterotopic copies of the 24-bp *HO* DNA target site (6). A comparable approach using the budding yeast homing endonucleases I-*SceI* and PI-*SceI* has been developed for plant and animal cells (7).

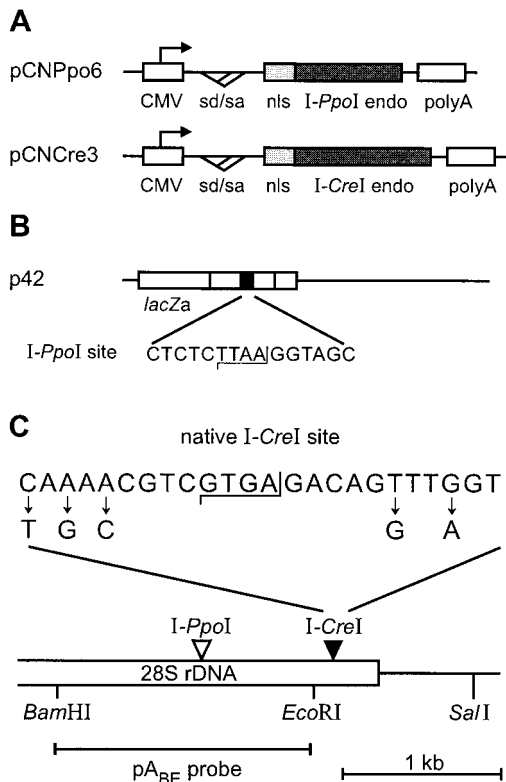
We show here that two additional, well-characterized eukaryotic homing endonucleases, I-PpoI from the myxomycete *Physarum polycephalum* (8, 9) and I-CreI from the green alga *Chlamydomonas reinhardtii* (10–12), can generate highly site-specific DNA double strand breaks in human cells. Both I-PpoI and I-CreI function as homodimers that bind and cleave highly conserved 15 or 24-bp rDNA homing sites, respectively, to generate 4 base, 3' extended DNA ends that initiate and target intron transposition or "homing" (13). We have recently determined the homing site sequence degeneracy and X-ray crystal structures of both of these endonucleases (14–17). I-CreI and the budding yeast homing endonucleases I-*SceI* and PI-*SceI* are all members of the LAGLIDADG family of homing endonucleases (13). I-PpoI, in contrast, is a member of the His-Cys box family of homing endonucleases that have not been expressed before in mammalian cells.

## MATERIALS AND METHODS

**Cell culture.** Human HT-1080 fibrosarcoma cells (18) were grown in Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 u/ml penicillin G sodium and 100 µg/ml streptomycin sulfate (GibcoBRL) in a humidified 37°C, 7% CO<sub>2</sub> incubator. *In vivo* cleavage assays were performed by CaPO<sub>4</sub>-mediated transfection of 2–4 × 10<sup>5</sup> cells with 0–20 µg/10 cm dish of CsCl gradient-purified plasmid DNA(s) (19). The toxicity of both endonucleases was determined by transfecting

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**FIG. 1.** Structure of endonuclease expression plasmids and homing site target DNAs. (A) *I-PpoI* and *I-CreI* expression plasmids pCNPpo6 and pCNCre3. (B) pBluescript-derived *I-PpoI* homing site plasmid p42. (C) Location of *I-PpoI* and *I-CreI* homing sites in the human 28S rDNA gene (open box). Sequence differences between the native *C. reinhardtii* *I-CreI* homing site (top) and the human 28S rDNA *I-CreI* site (bottom) are indicated at the arrows. Restriction endonuclease cleavage sites in the 28S rDNA gene and adjacent intergenic spacer (line to the right of the 28S rDNA gene) used in blot hybridization analyses are indicated below the gene. The region detected by the pA-derived human rDNA hybridization probe is indicated by the bracketed line below the 28S rDNA gene. Labels: CMV, cytomegalovirus immediate early gene promoter/enhancer; sd/sa, splice donor/splice acceptor; nls, SV40 large T-antigen nuclear localization signal; *I-PpoI* endo, *I-PpoI* endonuclease gene; *I-CreI* endo, *I-CreI* endonuclease gene; polyA, SV40 polyadenylation signal; *lacZα*, *lacZα* segment of bacterial *lacZ* gene (open box) separated by pBlueScriptII polylinker (vertical lines within open box); *I-PpoI* site, native *I-PpoI* homing site; *neo*, neomycin/G418 resistance gene. Staggered lines in the *I-PpoI* and *I-CreI* homing site sequences indicate positions of endonuclease cleavage to generate 4 base, 3' extended ends.

cells ( $1-2 \times 10^5/10$ -cm dish) with pSV2*neo* plasmid (0.5  $\mu$ g/dish) and 0, 0.5, or 5  $\mu$ g of endonuclease plasmid. Growth medium was supplemented with G418 (500  $\mu$ g/ml; GibcoBRL) 24 h after transfection, and G418-resistant colonies were crystal violet-stained and counted after 15–18 days additional growth.

**Plasmids.** *I-PpoI* and *I-CreI* endonuclease expression vectors (Fig. 1) were constructed in pCMV $\beta$  (20), a constitutive mammalian expression vector. The *I-PpoI* expression vector pCNPpo6 was constructed from a cloned *Physarum* intron (pI3-941; 9). The *I-PpoI* ORF was PCR-amplified, digested with *HhaI* and then ligated to an oligonucleotide adapter to add a new ATG start codon, an in-frame, N-terminal SV40 large T-antigen nuclear localization signal (nls)

and flanking restriction cleavage sites. The resulting nls-*PpoI* fragment was ligated into the *NotI* site of pCMV $\beta$  to generate pCNPpo6 (Fig. 1A). The *I-CreI* expression vector pCNCre3 was constructed from plasmid pI-*CreI* (21). The *I-CreI* open reading frame was amplified, cleaved with *HhaI* and then ligated to the adapter oligonucleotide described above. The resulting nls-*CreI* fragment was ligated into the *NotI* site of pCMV $\beta$  to generate pCNCre3 (Fig. 1A). The *I-PpoI* homing site plasmid p42 (9) contained a 38-bp insert of the 15-bp *I-PpoI* homing site and 23 bp of flanking DNA into the *PstI* and *EcoRV* sites of pBluescriptII KS(-) (Stratagene).

**Western analysis of *I-PpoI* production.** Cell pellets were lysed in 100  $\mu$ l of 100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue and 20% (v/v) glycerol followed by heating to 100°C for 10 min. The resulting lysates were cleared by centrifugation (11,000g for 5 min at 20°C). Western analyses were performed by electroblotting protein from SDS-PAGE gels onto nitrocellulose membrane (Nitro-bind, MSI) prior to blocking with 10% nonfat milk in 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Tween 20 for 30 min at 20°C. Rabbit polyclonal anti-*PpoI* antiserum (kindly supplied by V. Vogt) was diluted 1:500 into the above buffer containing 1% nonfat milk prior to incubation for 16 h at 4°C. Bound antibody was detected with a peroxidase-conjugated anti-rabbit secondary antibody (Vector). Bound secondary antibody was visualized by adding 0.3% H<sub>2</sub>O<sub>2</sub>, 0.5 mg/ml diaminobenzidine and 0.4% NiCl<sub>2</sub> (w/v) in 50 mM Tris-HCl, pH 7.4, for 5 min at 20°C.

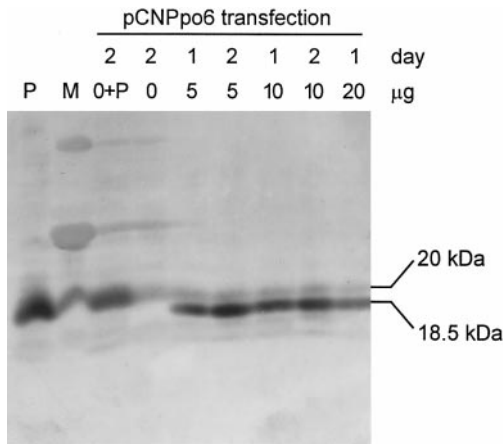
**Southern analyses of target cleavage.** DNA preparations, probe labeling and Southern blot hybridization analyses were performed as previously described (22). *I-PpoI* digestions were performed using enzyme and buffer supplied by Promega. *I-CreI* digestions were performed in 20 mM Tris-HCl, pH 9, 10 mM MgCl<sub>2</sub> using purified recombinant *I-CreI* (15, 21). *I-CreI* digests were incubated at 37°C, and stopped by adding 0.25 vol of 50 mM Tris-HCl, pH 7.4, 125 mM EDTA, 2.5% SDS. Hybridization probes consisted of pBlueScriptII KS(+) plasmid DNA and a 1619-bp *EcoRI/BamHI* restriction fragment from the 3' end of the human 28S rDNA gene (23). Quantitative image analyses were performed on a Molecular Dynamics Phosphorimager system (Dept. of Microbiology, Univ. of WA).

**Isolation and characterization of DSB repair products.** DNA (500 ng) from cells transfected with pCNPpo6 and p42 was digested with 20 U of *EcoRI* and 105 U of *I-PpoI* (Promega) for 16 hr at 37°C. Digests were heat-inactivated, then desalted on Microcon 50 spin columns (Amicon) prior to electroporation into *E. coli* TB-1 cells. Plasmid DNA from carbicillin-resistant colonies was checked for *I-PpoI* cleavage sensitivity as described above prior to DNA sequence analysis of the homing site region (24).

## RESULTS

**Endonuclease expression and toxicity in human cells.** Western blot analysis with a rabbit polyclonal anti-*PpoI* antiserum was used to determine whether *I-PpoI* could be expressed in human cells. Newly synthesized *I-PpoI* endonuclease of the correct molecular weight (18.5 kDa) was detected 24 and 48 h following transfection. We did not observe low molecular weight degradation or partial synthesis products (Fig. 2). No immunoreactive protein was observed in mock-transfected cell extracts, and detection of the 20-kDa "long form" *I-PpoI* (Promega; 9) added to mock-transfected HT-1080 cells indicated that extract preparation did not destroy *I-PpoI* antigenicity (Fig. 2).

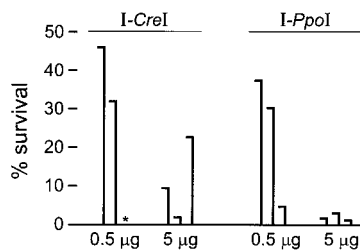
We cotransfected HT-1080 cells with pSV2*neo* plasmid and increasing amounts of the endonuclease plasmids pCNPpo6 or pCNCre3 to determine whether con-



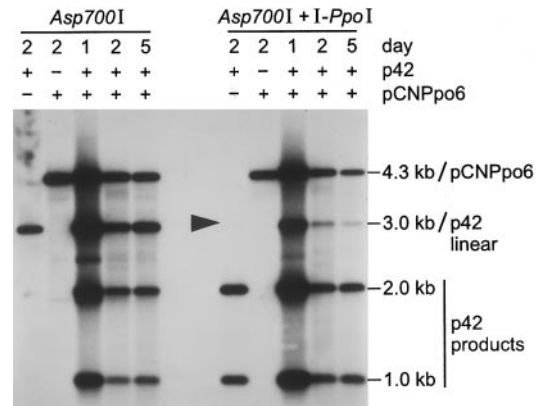
**FIG. 2.** Western blot analysis of I-PpoI expression in human cells. Cell lysates prepared 1 or 2 days after transfection of HT-1080 cells with the I-PpoI expression vector pCNPp06 were used in Western analysis with a rabbit polyclonal I-PpoI antisera. P, positive control 20-kDa recombinant I-PpoI protein (lane 1); M, molecular weight/size standard (lane 2); mock-transfected HT-1080 cells with no (0 + P, lane 3) or added (0, lane 4) recombinant I-PpoI protein; HT-1080 cells transfected with differing amounts of pCNPp06 plasmid and harvested 1 or 2 days following transfection (lanes 5–9). The day of harvest (day) and amount of transfected pCNPp06 ( $\mu\text{g}$ ) are indicated above lanes 3–9. Molecular weights are indicated in kDa to the right of the photo.

stitutive I-PpoI or I-CreI expression was toxic. Transfection of up to 10  $\mu\text{g}$  of pCMV vector without an endonuclease open reading frame did not suppress G418-resistant colony formation in HT-1080 or in several additional human cell lines (unpublished results). In contrast, we observed endonuclease plasmid dose-dependent suppression of G418-resistant colony formation (60–70% at a 1:1 ratio to  $\geq 90\%$  at a 10:1 ratio of endonuclease:pSV2neo plasmid; Fig. 3).

**Cleavage of plasmid and rDNA targets in human cells.** Homing site and endonuclease coding plasmids were transfected into HT-1080 cells to determine



**FIG. 3.** Constitutive I-CreI and I-PpoI expression reduces survival of human cells. HT-1080 cells were transfected with 0.5  $\mu\text{g}$  pSV2neo plasmid DNA and 0–5  $\mu\text{g}$  of endonuclease plasmid. Cell survival, measured by the number of G418-resistant colonies formed after 15–18 days growth in the presence of G418, is shown for three independent experiments (vertical lines). In each case cell survival was normalized to a within-experiment control consisting of pSV2neo plasmid alone. No colonies were observed in mock-transfected cells. \*, not determined.

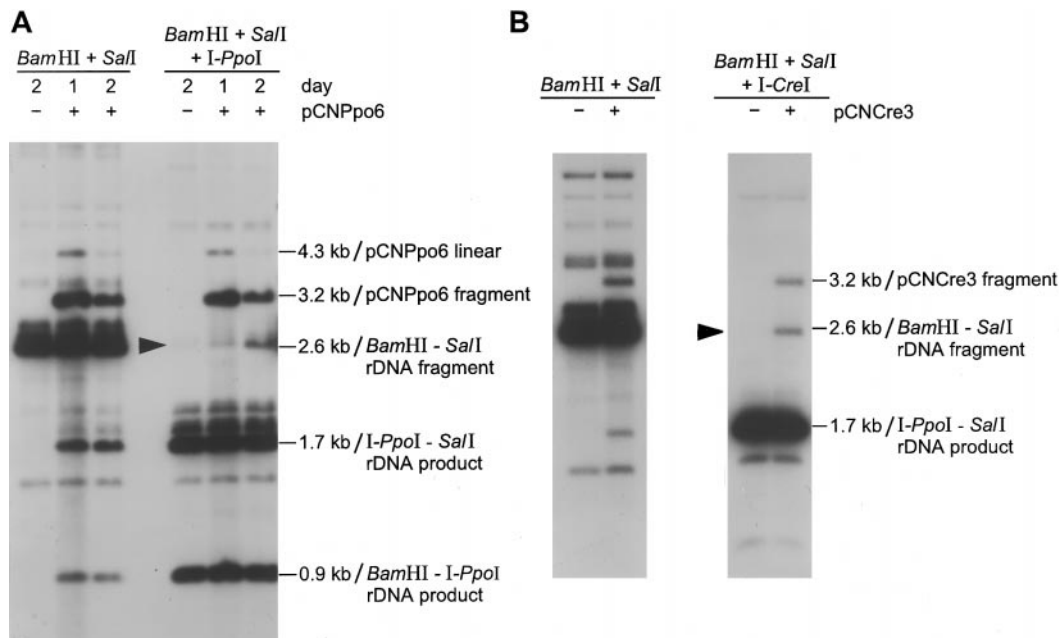


**FIG. 4.** Site-specific cleavage of I-PpoI plasmid substrate *in vivo*. Cells were transfected with p42 target plasmid, or with p42 and pCNPp06 endonuclease plasmid, prior to DNA isolation on the indicated post-transfection day. DNA was digested with *Asp700I* to linearize the target and endonuclease plasmids (five left *Asp700I* lanes), or with *Asp700I* followed by I-PpoI (five right *Asp700I* + I-PpoI lanes) prior to Southern analysis to reveal I-PpoI-resistant, linear p42 molecules (▶). The hybridization probe detected both target and endonuclease plasmids. Fragment sizes (in kb) and identities are shown at the right of each panel.

whether I-PpoI could cleave a plasmid-borne homing site *in vivo*. The I-PpoI homing site of p42 plasmid DNA was not altered by transfection: p42 DNA isolated from HT-1080 cells was fully I-PpoI-sensitive (Fig. 4). In cells cotransfected with p42 and pCNPp06, *in vivo* cleavage of the p42 homing site was observed up to five days following transfection. Approximately 40% of the p42 plasmid substrate was cleaved at each time point (Fig. 4). Comparable results were obtained in cotransfection experiments using I-CreI endonuclease and homing site plasmids (additional results not shown).

When HT-1080 cells were transfected with an I-PpoI or I-CreI endonuclease plasmid alone, homing site-specific cleavage of 28S rDNA was observed after 24 and 48 h (Fig. 5). All of the potential I-PpoI and I-CreI homing sites in 28S rDNA in HT-1080 cells were cleavage sensitive prior to endonuclease exposure. Approximately 10% of the I-PpoI homing sites in 28S rDNA were cleaved *in vivo* 24 and 48 h after the transfection of pCNPp06 (Fig. 5A). We obtained comparable results when HT-1080 cells were transfected with pCNCre3 ( $\leq 5\%$  of 28S I-PpoI homing sites cleaved at 24 h; Fig. 5B).

**Generation of endonuclease-resistant plasmid and rDNA copies *in vivo*.** We observed the generation of endonuclease-resistant plasmid and 28S rDNA substrates in conjunction with homing site-specific cleavage products in the experiments described above (see ▶ indicated fragments, Figs. 4 and 5). The most abundant I-PpoI-resistant p42 plasmid species was full length (3 kb), represented up to 5% of plasmid substrate at early time points (Fig. 4) and could be detected up to 5 days following transfection. These



**FIG. 5.** Analysis of *I-Ppo*I and *I-Cre*I cleavage of 28S rDNA in human cells. Cells were transfected with the pCNPpo6 (A) or pCNCre3 (B) prior to DNA isolation. DNAs were digested with *Bam*HI and *Sal*I prior to hybridization (left *Bam*HI + *Sal*I lanes in each panel) or with *Bam*HI and *Sal*I followed by *I-Ppo*I (A) or *I-Cre*I (B; right lanes in each panel) prior to Southern analyses with a 28S rDNA probe. A small amount of plasmid probe was included to allow detection of the endonuclease plasmids. *I-Ppo*I and *I-Cre*I-resistant rDNA fragments seen only after endonuclease expression are indicated by the arrows (▶).

*I-Ppo*I-resistant p42 plasmids could not be cleaved with up to a 1000-fold excess (units/ $\mu$ g total cellular DNA) of *I-Ppo*I (data not shown). A second *I-Ppo*I-resistant fragment, of 2.4–2.5 kb (Fig. 4), may have arisen from either p42 or pCNPpo6. The major *I-Ppo*I and *I-Cre*I-resistant 28S rDNA species was full length (2.6 kb), and could be detected up to 48 h after transfection (Fig. 5A).

We isolated and characterized *I-Ppo*I-resistant p42 plasmid molecules in order to determine the molecular basis for endonuclease-resistance. DNA samples from transfected cells were cleaved with *I-Ppo*I and *Eco*RI prior to electroporation into *E. coli* to linearize—and suppress the transformation efficiency of—native p42 and *Eco*RI-sensitive pCNPpo6 plasmids, thus promoting the selective recovery of *I-Ppo*I-resistant p42. DNA from cells transfected with p42 alone was used as a control.

Carbenicillin-resistant colonies were recovered at ~1000-fold higher frequency, when corrected for the plasmid DNA content of samples, from DNA isolated from HT-1080 cells transfected with p42 and pCNPpo6, as opposed to p42 alone. Twenty-nine of 33 carbenicillin-resistant colonies contained *I-Ppo*I-resistant p42 DNA derivatives that included 18 different *I-Ppo*I homing site mutations. A majority of these mutant homing sites (16/18, or 89%) contained deletions, although we also identified single base insertions at the ends of the 4 base overhangs created by *I-Ppo*I cleavage (Fig. 6). The four remaining plasmids in-

cluded 2 native p42 plasmids and 2 contained grossly rearranged plasmid DNAs that were not further characterized.

## DISCUSSION

The ability of *I-Ppo*I and of *I-Cre*I to generate highly site-specific, chemically homogeneous DNA double strand breaks should make both endonucleases useful for analyses of DNA double strand break repair in human cells. We observed the efficient generation of homing site-specific cleavage products from both co-transfected plasmids and endogenous 28S rDNA genes at levels comparable to or higher than those previously reported for *I-Sce*I (25–27). The detection of cleavage products up to 5 days after transfection may indicate that repeated cycles of cleavage and rejoining are occurring, or that cleavage products can be protected from nuclease degradation *in vivo*.

The ability of both *I-Ppo*I and *I-Cre*I to cleave 28S rDNA may explain part or all of the toxicity of *I-Ppo*I and *I-Cre*I in human cells and the toxicity of *I-Ppo*I in budding yeast (28). The budding yeast homing endonucleases *I-Sce*I and *PI-Sce*I, in contrast, apparently lack endogenous rDNA cleavage sites and are less toxic when expressed in mammalian cells (7). Additional human chromosomal DNA cleavage sites outside 28S rDNA may exist for *I-Ppo*I, *I-Cre*I and the budding yeast endonucleases. These additional sites have not been directly identified, but can be predicted from the

clones (n)	sequence	bp altered
p42(wildtype)	ATAATTAGTAACTATGACTCTCTCTTAAGGTAGCCAAATGCCTGCAGCCCGG	
4	ATAATTAGTAACTATGACTCTC.TAAGGTAGCCAAATGCCTGCAGCCCGG	-1
3	ATAATTAGTAACTATGACTCTC...GGTAGCCAAATGCCTGCAGCCCGG	-4
2	ATAATTAGTAACTATGACTCTCT...GGTAGCCAAATGCCTGCAGCCCGG	-3
3	ATAATTAGTAACTATGACTCTCTT...GGTAGCCAAATGCCTGCAGCCCGG	-2
2	ATAATTAGTAACTATGACTCTCTTA..GTAGCCAAATGCCTGCAGCCCGG	-2
1	ATAATTAGTAACTATGACTCTCTTAA.GTAGCCAAATGCCTGCAGCCCGG	-1
1	GCTGC.(40).....(126).CGTTA	-127
1	TTACG.(82).....GCAGCCCGG	-124
1	ATAATTA.....(45).TTTCGC	-89
1	ATAATTAGTAACTA.....TGCTGCAGCCCGG	-22
1	ATAATTAGTAACTATGAC.....CTGCAGCCCGG	-21
1	ATAATTAGTAACTATGACT.....ATCCA	-34
1	ATAATTAGTAACTATGACT.....AGCCAAATGCCTGCAGCCCGG	-10
1	ATAATTAGTAACTATGACTC.....CCGG	-26
2	ATAATTAGTAACTATGACTC.....CGG	-27
1	ATAATTAGTAACTATGACTCTCTTAA.....ATGCCTGCAGCCCGG	-9
1	ATAATTAGTAACTATGACTCTCTTAAaGGTAGCCAAATGCCTGCAGCCCGG	+1
2	ATAATTAGTAACTATGACTCTCTTTAAGGTAGCCAAATGCCTGCAGCCCGG	+1

**FIG. 6.** DNA sequence analysis of *I-PpoI*-resistant p42 target plasmids. The sequence of the native p42 homing site (wild type) is shown at top, with the *I-PpoI* homing site in bold and indicated below by vertical lines. The cleaved 4-bp region is shaded. The number of *I-PpoI*-resistant plasmids containing each observed mutation is given to the left (n), and the number of altered nucleotides to the right (bp altered), of each mutant homing site. Deleted nucleotides are indicated by periods (.), inserted nucleotides are shown in lower case. Underlined nucleotides at junctions may have originated from the left or right half of the homing site.

limited homing site sequence degeneracy that can be tolerated by these four endonucleases (15, 28–31). It should be possible to limit *I-PpoI* or *I-CreI* toxicity in human and yeast cells by regulated expression or the incorporation of amino acid substitutions that attenuate catalytic activity (15, 16, 32).

Site-specific DSBs generated by *I-PpoI* or *I-CreI* in human cells may be repaired by one of several different pathways (1–6). The short deletions and insertions we observed in *I-PpoI*-resistant plasmids, where there is little homologous sequence flanking the homing site, are likely to have been generated by DNA end joining. Comparable results have been seen in plasmid recircularization and gene deletion or rearrangement junctions, and in experiments where site-specific DNA double strand breaks were generated with either *I-SceI* or *PI-SceI* in mammalian cells (1–4, 33–40). Our results with plasmid substrates indicate that end-joining may be mutagenic, as judged from the 1000-fold increase in endonuclease-resistant plasmid DNAs after *I-PpoI* cleavage and rejoining *in vivo*. Alternative explanations for this increase, such as over-replication of rare, preexisting mutant plasmids or the generation of mutations in *E. coli*, are unlikely: our plasmid substrates lacked eukaryotic replication origins, and the increase in mutant plasmids was observed only in DNA samples from cells co-transfected with target *and* endonuclease plasmids (Fig. 4).

The human 28S rDNA genes consist of ~300 copies distributed among five chromosome arms (23, 41, 42). DNA repair can occur in mammalian rDNA (43–47),

and homologous recombination, gene conversion or single-strand annealing could all be used in addition to end joining to repair DSBs in rDNA (1–6). It may be possible to identify which of these potential pathways are used to repair DSBs in human rDNA by further characterizing endonuclease-resistant rDNA generated during *in vivo* cleavage experiments, and by determining how rDNA copy number or the number of copies of different rDNA sequence variants (see, e.g., 48) change during DSB repair. The ability of *I-PpoI* and *I-CreI* to generate DSBs in human and yeast rDNA should also facilitate comparative analyses of roles of the human Werner syndrome and budding yeast Sgs1p helicases. Recent experiments indicate that both of these RecQ helicases are localized to the nucleolus, and may play roles in rDNA metabolism, stability and repair (reviewed in 49).

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