A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells

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ABSTRACT

Homologous gene targeting is the ultimate tool for reverse genetics, but its use is often limited by low efficiency. In a number of recent studies, site-specific DNA double-strand breaks (DSBs) have been used to induce efficient gene targeting. Engineering highly specific, dedicated DNA endonucleases is the key to a wider usage of this technology. In this study, we present two novel, chimeric meganucleases, derived from homing endonucleases. The first one is able to induce recombination in yeast and mammalian cells, whereas the second cleaves a novel (chosen) DNA target site. These results are a first step toward the generation of custom endonucleases for the purpose of targeted genome engineering.

INTRODUCTION

Genome engineering has become one of the major challenges of the post-genome era, and the development of gene targeting in the 1980s (1–3) has provided the means to perform precise genome surgery. However, classical methods can prove to be very inefficient, depending on the target locus and the organism. Meganuclease-induced gene targeting is a technology wherein homologous recombination is induced by a site-specific DNA cleavage. In mammalian cells, meganuclease-induced targeted double-strand breaks (DSBs) enhanced gene targeting yields by several orders of magnitude (4–8) [up to 10^2 of the total transfected cells can in fact be targeted (8)]. DSBs induced by meganucleases also allow efficient targeting in plants and insects (9–12).

Meganucleases are endonucleases, which recognize large (12–45 bp) DNA target sites (13). In the wild, meganucleases are essentially represented by homing endonucleases, a large family of DNA nucleases found in phages, bacteria, archaeabacteria and various eukaryotes (14–16). Homing endonucleases are often encoded by introns or inteins behaving as mobile genetic elements. They recognize sites that usually correspond to intron-free or intein-free genes, where they produce a DSB. Eventually, DSB repair by homologous recombination with an intron- or intein-containing gene results in the insertion of the intron or intein where DSB occurred. Early studies on HO-induced mating type switching in budding yeast (17,18), and the I-SceI (19–22) and I-TevI (23–26) homing endonucleases have paved the way for meganuclease-induced gene targeting (4–8).

Meganuclease-induced gene targeting has, however, one major limitation: the target locus must contain a meganuclease cleavage site. Therefore, as a first step in all current applications, it is required to introduce that cut site. Wider uses of natural meganucleases as universal tools for gene targeting applications would depend on the possibility of finding enzymes whose target sequences are already contained at the target loci. This is most unlikely, since the natural repertoire of homing endonucleases is currently limited to about 300 proteins, most of them still hypothetical or uncharacterized. It thus becomes necessary to resort to novel, artificial meganucleases, for which specificity has been tuned according to target choice. One approach is to focus on structure-based, rational design.

Detailed three-dimensional structures have been obtained for four proteins of the LAGLIDADG family (27–36), the largest of the four homing endonucleases families. The hallmark of these proteins is a well conserved LAGLIDADG peptide motif, termed (do)decapetide, found in one or two copies. Homing endonucleases with only one such motif, such as I-CreI (37) or I-CeuI (38), function as homodimers. In contrast, larger proteins bearing two (do)decapetide motifs, such as I-SceI (19), PI-SceI (39) and I-DmoI (40), are single chain proteins. Despite an apparent lack of sequence conservation (overall pairwise sequence homology below 25%), structural comparisons indicate that LAGLIDADG proteins, should they cut as dimers or single chain proteins, adopt a similar active conformation. In all structures, the LAGLIDADG motifs are central and form two packed α-helices where a 2-fold (pseudo-)symmetry axis separates two monomers or apparent domains. On either side of the LAGLIDADG α-helices, a four-stranded β-sheet provides a DNA binding interface that drives the interaction of the protein with a half site of the target DNA sequence [clearly shown by structures of I-CreI with target DNA (28,29)].
In order to challenge the modularity of the interaction between DNA and domains of LAGLIDADG endonucleases, we designed a first series of novel meganucleases by swapping or fusing different homing endonuclease domains. I-CreI was chosen as the template, as this protein remains active in a broad range of temperature conditions (37) and its interaction with DNA is well characterized (28,29). A single chain version of I-CreI (scl-CreI) was modeled and engineered. scl-CreI cleaves its cognate DNA substrate \textit{in vitro} and induces homologous recombination both in yeast and mammalian cells. In addition, we created a chimeric endonuclease (DmoCre) by fusing to I-CreI one of the two I-DmoI domains. DmoCre is functional, and cleaves the expected hybrid DNA target. These results and other recent work (41) demonstrate that functional novel meganucleases can be designed by domain swapping or fusion of homing endonuclease domains, and suggest that many other nucleases with novel specificities could be designed in this way for the purpose of genome engineering.

**MATERIALS AND METHODS**

**Modeling**

All modeling and drawings of protein structures were realized using ICM (42). The structures from I-CreI and I-DmoI correspond to PDB entries 1g9y and 1b24, respectively. Residue numerations in the text always refer to these structures, except for residues in the second I-CreI protein domain of the homodimer (residue numbers were set as for the first domain).

**Gene synthesis**

PCR-based assembly of a series of overlapping oligonucleotides was used to construct a gene coding for I-DmoI, while the single chain synthetic I-CreI gene was designed and sent for synthesis to Entelechon GmbH. An I-CreI gene was then obtained by PCR amplification of a fragment corresponding to the second protein domain within the single chain I-CreI gene. The DmoCre hybrid gene was built by PCR-assembly using a pair of overlapping primers corresponding to the swap region. All genes were written with codons favoring protein expression in prokaryotic systems (I-DmoI and I-CreI sequences correspond to the protein sequences in structures 1b24 and 1g9y, respectively). Sequences can be obtained upon request.

**Protein expression and purification**

His-tagged proteins were over-expressed in \textit{Escherichia coli} BL21 (DE3) cells using pET-24d (+) vectors (Novagen). Induction with IPTG (1 mM) was performed at 25°C. Cells were sonicated in a solution of 25 mM HEPES (pH 8) containing protease inhibitors (complete EDTA-free tablets, Roche) and 5% (v/v) glycerol. Cell lysates were centrifuged twice (15 000 \textit{g} for 30 min). His-tagged proteins were then affinity-purified, using 5 ml Hi-Trap chelating columns (Amersham) loaded with cobalt. Several fractions were collected during elution with a linear gradient of imidazole (up to 0.25 M imidazole, followed by plateau at 0.5 M imidazole and 0.5 M NaCl). Protein-rich fractions (determined by SDS-PAGE) were concentrated with a 10 kDa cut-off centrifprep Amicon system. The resulting sample was eventually purified by exclusion chromatography on a Superdex75 PG Hi-Load 26-60 column (Amersham). Fractions collected were submitted to SDS-PAGE and analysed for cleavage activity (for I-CreI and I-DmoI). Selected protein fractions were concentrated and dialyzed against a solution of 25 mM HEPES (pH 7.5) and 20% (v/v) glycerol.

**Far-UV circular dichroism and temperature-induced equilibrium unfolding**

Circular dichroism measurements were performed on a J-810 spectropolarimeter using a 0.1 cm path length quartz cuvette. Equilibrium unfolding was induced increasing temperature at a rate of 1°C/min (using a programmable Peltier thermostoic). Samples were prepared by dialysis against 25 mM potassium phosphate buffer, pH 7.5, at protein concentrations of 17, 11, 37.5 and 41 \textmu M for I-CreI, scl-CreI, I-DmoI and DmoCre, respectively.

**In vitro cleavage assays**

pGEM plasmids with single meganuclease DNA target cut sites (see Fig. 5A for the sequences) were first linearized with XmnI. Cleavage assays were performed at 37 or 65°C in 12.5 mM HEPES (pH 8), 2.5% (v/v) glycerol and 10 mM MgCl₂. The concentrations were 10 nM for the substrate, 0.7 \mu M for I-DmoI, scl-CreI and DmoCre, 1.5 \mu M for I-CreI. Reactions were stopped after 1 h by addition of 0.1 vol of 0.1 M Tris–HCl (pH 7.5), 0.25 M EDTA, 5% (w/v) SDS and 0.5 mg/ml proteinase K and incubation at 37°C for 20 min. Reaction products were examined following separation by electrophoresis in 1% agarose gels.

**On-line cleavage experiments**

The method of Eisenschmidt \textit{et al.} (43) was adapted to monitor cleavage of double-stranded I-CreI target DNA. Quenched fluorescent DNA substrate was prepared by hybridization of the following three oligonucleotides (purchased from Eurogentec): (i) fluorescent-( linker)-5’-TCA- AAACGTCTCGAGACGCTTGAAGCTTATGCTTAGTTG-3’; (ii) 5’-CCAACATAGCATAAGCTTCAACTCAGCTTTGTGCTGCT-3’ and (iii) 5’-AGGCCGACCCGCGCATC-3’-(linker)-Dark-Quencher (the I-CreI target site is underlined). Cleavage reactions were carried out at 37°C with excess enzyme over substrate (single turnover kinetics) (44), in the wells of a flat bottom 96-well microtiter plate. Fluorescence was acquired during cleavage reactions using a fluorescence-plate reader (Fluoroskan Ascent from Thermolabsystem) with excitation and emission wavelength, respectively, set at 485 and 538 nm. Reactions were initiated by addition of substrate DNA and magnesium to enzyme samples. Final samples contained 12.5 mM HEPES (pH 7.5), 10 mM MgCl₂ and 21.8 nM substrate DNA (total sample volumes were 110 \textmu l). For each enzyme concentration, at least three separate experiments were used to obtain \textit{k}_\text{obs} values (we assume that cleavage is a first-order process) by modeling fluorescence curves data with equation 1:

\[
\text{fluorescence} = a + bt + c \left[1 - \exp(-k_{\text{obs}}t)\right]
\]

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where \( t \) is the reaction time (min), \( a \) the initial sample fluorescence (a.u.), \( c \) the fluorescence increase upon total product formation and the term \( bt \) is used to correct for an apparent non-specific linear increase in fluorescence. Eventually, modeling the dependence of \( k_{\text{obs}} \) on enzyme concentrations \([E]\) with equation 2 allowed determining apparent \( k_{\text{cat}}^* \) and \( K_m^* \):

\[
k_{\text{obs}} = \frac{(k_{\text{cat}}^* [E])}{(K_m^* + [E])}
\]

### Mapping of the cleavage sites

After cutting with \( XmnI \) and I-CreI, I-DmoI, scI-CreI or \( \text{DmoCre} \), the digestion products were run on agarose gels. In each case, the two \( XmnI \)-meganuclease bands were excised and purified from the gel, and sequenced with SP6 and T7 primers. The sequences obtained in this way corresponded to the sequence of the top strand, 5′ of the cleavage site, and the sequence of the bottom strand, 3′ of the cleavage site. In each case, the sequence was found to be swiftly interrupted at a position that we inferred to correspond to the cleavage site.

### Yeast colorimetric assay

Our yeast transformation method has been adapted from previous protocols (45). For staining, we used a classic qualitative X-Gal Agarose Overlay Assay. Each plate was covered with 2.5 ml of 1% agarose in 0.1 M sodium phosphate buffer, pH 7.0, 0.2% SDS, 12% dimethyl formamide, 14 mM \( \beta \)-mercaptoethanol, 0.4% X-Gal, at 60°C. Plates were incubated at 37°C.

### Mammalian cells assays

COS cells were transfected with Superfect transfection reagent according to the manufacturer’s (Qiagen) protocol. Seventy-two hours after transfection, cells were rinsed twice with \( 1\times \) PBS and incubated in lysis buffer (Tris–HCl 10 mM pH 7.5, NaCl 150 mM, Triton X-100 0.1%, BSA 0.1 mg/ml, protease inhibitors). Lysate was centrifuged and the supernatant used for protein concentration determination and \( \beta \)-galactosidase liquid assay. Typically, 30 μl of extract was combined with 3 μl Mg 100× buffer (MgCl\(_2\) 100 mM, \( \beta \)-mercaptoethanol 35%), 33 μl ONPG 8 mg/ml and 234 μl sodium phosphate 0.1 M pH 7.5. After incubation at 37°C, the reaction was stopped with 500 μl of 1 M Na\(_2\)CO\(_3\) and OD was measured at 415 nm. The relative \( \beta \)-galactosidase activity is determined as a function of this OD, normalized by the reaction time and the total protein quantity.

### RESULTS

#### Design of the single chain I-CreI meganuclease

I-CreI from *Chlamydomonas reinhardtii* (Fig. 1A) is a small LAGLIDADG homing endonuclease that has a single (do)decapetide motif and dimerizes into a structure similar to that of larger, single chain LAGLIDADG proteins. To engineer a single chain version of I-CreI (scI-CreI), two I-CreI copies were fused. This required placing a linker region between the two domains, and a significant part of the I-CreI protein had to be removed at the end of the domain preceding the linker (Fig. 2).

The three-dimensional structure of I-DmoI from *Desulfuroccocus mobilis* (Fig. 1B) is comparable with that of I-CreI, with the exception that I-DmoI comprises a linker region that leads from one apparent domain to the other. The boundary of that linker finely matches related main chain atoms of the I-CreI dimer. In the first domain, residues 93–95 from the third \( \alpha \)-helices of I-CreI and I-DmoI (prior to the linker) are structurally equivalent and at the beginning of the second LAGLIDADG \( \alpha \)-helix (second domain), I-DmoI residues 104–106 correspond to I-CreI residues 7–9 (compare Fig. 1A and B). In addition, Leu95 and Glu105 from I-DmoI have conserved identities in I-CreI, and I-DmoI residue Arg104 aligns with another basic residue in I-CreI (Lys7).

Thus, the scI-CreI was designed by inserting the I-DmoI region from residue 94–104 (sequence MLERIRLFNMR) between a first I-CreI domain (terminated at Pro93) and a second I-CreI domain (starting at Glu8), as shown in Figure 2. Detailed structural analysis of how the new linker connects the scI-CreI protein domains (in a modeled structure) revealed no potential incompatibility (not shown). For example, the side chains of non-polar amino acids taken from I-DmoI, Met94, Ile98 and Phe101 point inside fitting cavities of I-CreI. A single mutation was made (P93A), however, to promote regularity of the backbone in the \( \alpha \)-helix prior to the linker region.
Design of the DmoCre hybrid meganuclease

Superimposition of the main chain atoms from the LAGLIDADG sequence motifs from I-DmoI and I-CreI results in low r.m.s. deviation (0.66 Å, Fig. 3A and B). This is a strong indication of the similarity between the two LAGLIDADG packing interfaces. Such similarity could be sufficient to allow swapping DNA binding domains between I-DmoI and I-CreI, thereby creating proteins that cleave hybrid DNA targets. We thus replaced the C-terminal I-DmoI sequence (2nd domain) with that of I-CreI, starting at the second LAGLIDADG motif.

The resulting hybrid protein, which we called DmoCre, corresponds to I-DmoI up to Phe109 followed by I-CreI from residue Leu13 (Fig. 3C). Compared with the connection created between scI-CreI domains, the DmoCre linker region retains more of I-DmoI. This preserves to some degree the packing interface between the first and second LAGLIDADG α-helices. For the remainder of the interface, the LAGLIDADG motif from I-CreI (sequence LLGLIIGDG) is now packed along the first of the two motifs from I-DmoI (sequence LAGFVDGDG). As a consequence of sequence homology, substituting the I-DmoI motif for that of I-CreI should not disrupt considerably the structural fit between the central α-helices. However, detailed examination of the residues at and around the packing interface indicates that a few residues may not allow proper interaction between the two protein domains (not shown). Ile107 from I-DmoI may clash (steric repulsion) with Phe94 from I-CreI. Residue Ile107 was thus mutated to leucine, the corresponding amino acid for that position in I-CreI. Also, Leu47, His51 and Leu55 from I-DmoI are too close to Lys96 and Leu97 from I-CreI. The three residues were then mutated to alanine, alanine and aspartic acid, respectively, in order to avoid strains between side chains (additionally, a salt-bridge may be formed between the novel aspartic acid and Lys96).

Biochemical and biophysical properties of the single chain I-CreI protein

A synthetic gene corresponding to the new enzyme was engineered and the scI-CreI protein over-expressed in E.coli.

The engineered protein was found to be well-folded (data not shown) and stable at temperatures up to 50°C, which is comparable with I-CreI. Both proteins unfold above 50°C (Fig. 4), unfolding of scI-CreI being apparently complete at
65°C. In contrast, the denaturation profile for I-CreI indicates that a population of folded proteins remains until temperature has reached 80°C. However, under the conditions used to induce unfolding, precipitates appear. Thus, folding properties of both I-CreI and scI-CreI at high temperature could not be obtained with confidence, i.e. melting points ($T_m$) could not be compared.

The ability of purified scI-CreI to cleave DNA substrates in vitro was tested, using linearized plasmids bearing a copy of the I-CreI homing site. Similarly to parent I-CreI (37), the novel enzyme cleaves an I-CreI target site at 37°C (Fig. 5B). To determine the cleavage site, we used a method based on the sequencing of the cleaved fragments (see Materials and Methods). When used to determine the cleavage sites of I-CreI and I-DmoI, this method confirmed the results obtained previously (40,46). Cleavage with scI-CreI appeared to result in two staggered single-strand nicks, non-distinguishable from what is obtained with I-CreI (data not shown). Our method would not allow the detection of minor cleavage sites 5' or 3' of each nick, and we cannot exclude that scI-CreI might be less precise than I-CreI. Nevertheless, scI-CreI appears to behave mostly as its wild type counterpart.

Catalytic properties of scI-CreI and I-CreI were examined and compared. Double-stranded DNA cleavage reactions were monitored on-line, following the conversion of quenched fluorescent substrates into fluorescent products (43). Apparent $K_m$* and $k_{cat}$* were determined analyzing how the observed reaction rates ($k_{obs}$) depend on enzyme concentrations (Fig. 5D and E) under single turnover conditions (with excess enzyme) (44). We found that the apparent catalytic rate ($k_{cat}$*) of scI-CreI (~0.03 min$^{-1}$) is about three times lower than that of I-CreI (~0.09 min$^{-1}$). Our I-CreI $k_{cat}$* (determined at 37°C) is also three times higher than the value quoted by Chevalier et al. (41). For both proteins, $K_m$* were found to be in the order of, or above, the highest enzyme concentrations (182 nM) used in our experiments.

The DmoCre hybrid, as parent I-DmoI, is functional at high temperature

DmoCre was engineered and tested similarly to scI-CreI. DmoCre, which is well-folded at 20°C (data not shown), is the least stable of the four proteins tested in this work. Above that temperature, the hybrid protein unfolds progressively with a thermal denaturation profile (Fig. 4) suggestive of reduced folding co-operativity (compared with either I-DmoI or I-CreI). Again, under the conditions used to induce unfolding, the apparition of precipitated materials unfortunately precludes further characterization of folding properties.

We found that I-CreI and I-DmoI homing sites are resistant to (in vitro) cleavage by DmoCre, and that the engineered enzyme has, as expected, a novel DNA cutting specificity (Fig. 5C). Combinations of either of the two I-DmoI homing half-sites (D1 and D2) with either of the two I-CreI homing half-sites (C1 and C2) result in four possible hybrid DNA substrates (Fig. 5A). Only vectors bearing either the C1D2 or the C2D2 combination could be efficiently cleaved at 65°C (Fig. 5C). Chevalier et al. obtained similar results earlier (41). As they pointed out, cleavage of substrates bearing the D2 half-site, by E-DreI or DmoCre, indicates that the N-terminal and C-terminal I-DmoI domains bind the second (D2) and first (D1) half-sites, respectively.

We sequenced the fragments resulting from cleavage of the C1D2 site. Interestingly, cutting appeared to result again from two staggered nicks, leaving two 4 bp 3' overhangs. As
A

I-CreI:
5’ TCAAAACGTGGAGAAGCTTTTG  3’
3’ AGTTTGTGCAAGCTTCGTTGAC  5’

I-DmoI:
5’ GCCCTGGGCGTAAAGTTCGCGCG  3’
3’ CGGAAAGGGCCCATTTCAAGCGCCCG  5’

C1D2:
5’ TCAAAACGTGGAGAAGCTTTTG  3’
3’ AGTTTGTGCAAGCTTCGTTGAC  5’

C2D2:
5’ CCAACAGTGTCAGTTCCGCGCG  3’
3’ GTTTTGAAGAGGTTCTCAAGCGCCCG  5’

D1C2:
5’ GCCCTGGGCGTAAAGTTCGCGCG  3’
3’ CGGAAAGGGCCCATTTCAAGCGCCCG  5’

D1C1:
5’ GCCCTGGGCGTAAAGTTCGCGCG  3’
3’ CGGAAAGGGCCCATTTCAAGCGCCCG  5’

B

Enzyme | DmoCre | 1-CreI | 1-DmoI
Target | 1-CreI | 1-DmoI | 1-DmoI |
Cleavage 37°C 65°C 37°C 65°C - 65°C

C

Enzyme | DmoCre
Target | C1D2 | C2D2 | D1C2 | D1C1
Cleavage - 65°C 37°C - 65°C 37°C - 65°C 37°C

D

I-CreI

Fluorescence (a.u.)

Time (min)

scl-CreI

Fluorescence (a.u.)

Time (min)
pointed out for scI-CreI, the method we use does not allow detection of minor cleavage sites. Nevertheless, we can infer from the results that in DmoCre, the I-CreI and I-DmoI moieties have kept most of their cleavage specificities, the nick in the I-CreI and I-DmoI parts of the site being positioned exactly as in the wild type sites cut by the wild type proteins (see Fig. 5A).

As for wild type I-DmoI, cleavage activity for both C1D2 and C2D2 targets was barely detectable at 37°C (Fig. 5C). In addition, our on-line fluorescence detection method and procedure would not allow monitoring on-line cleavage reactions at high temperatures. Therefore, we have not been able so far to characterize further the biochemical properties of DmoCre.

**Single chain I-CreI cleaves its DNA substrate in living cells**

In order to test the functionality of scI-CreI in vivo, we designed an assay to monitor meganuclease-induced homologous recombination in yeast and mammalian cells. In yeast, Xenopus oocytes and mammalian cells, DNA cleavage between two direct repeats is known to induce a very high level of homologous recombination between the repeats (47–54). The recombination pathway, often referred to as single-strand annealing (SSA), removes one repeat unit and all intervening sequences (Fig. 6A) (55). We thus constructed a SSA reporter vector, with two truncated, non-functional copies of the bacterial LacZ gene and an I-CreI cut site within the intervening sequence, in a yeast replicative plasmid. Cleavage of the cut site should result in a unique, functional LacZ copy that can be easily detected by X-gal staining.

The reporter vector was used to transform yeast cells. A small fraction of cells appeared to express functional LacZ (not shown), probably due to recombination events during transformation (F. Pâques and J.E. Haber, personal communication). Co-transformation with plasmids expressing either I-CreI or scI-CreI, in contrast, resulted in blue staining for all plated cells (Fig. 6B, lower row). These results could be confirmed by a quantitative assay (not shown) similar to the one used in mammalian cells (see below). Even in non-induced conditions (glucose), the residual level of protein was enough to induce SSA, suggesting that scI-CreI, as much as I-CreI, is highly efficient in yeast cells. Furthermore, SSA induction was truly dependent on cleavage of the target cut site by I-CreI proteins, as vectors devoid of that site display no increase in β-galactosidase activity compared with background levels (Fig. 6B, upper row). scI-CreI, though, does not affect cell growth, whereas upon over-expression (on galactose plates) of wild-type I-CreI only a small number of slow growing colonies (all of them blue) is observed. Dose-dependent toxicity of I-CreI had been observed previously in bacteria (56) and flies (12) where it seemed to result from non-specific cleavage in rDNA. However, lack of toxicity of scI-CreI could be due to a different level of expression or stability.

Our SSA assay was modified for tests in mammalian cells. The promoter and termination sequences of the reporter and meganuclease expression plasmid were changed, and plasmid recombination was evaluated in a transient transfection assay. Similar levels of induced recombination (2–3-fold increase) were observed with either scI-CreI or I-CreI. A typical experiment is shown in Figure 6C. As in the yeast experiment, recombination depends on an I-CreI cut site between the repeats, for no increase of the β-galactosidase was observed in the absence of this site.

Another recombination assay, based on recombination between inverted repeats, was also used to monitor meganuclease-induced recombination in COS cells (Fig. 6D). While direct repeats can recombine by SSA, homologous recombination between indirect repeats requires a gene conversion event (see Fig. 6D) (55). Similar stimulation of gene conversion (3–4-fold) was observed with either scI-CreI or I-CreI. A typical experiment is shown in Figure 6E. As expected for a true homologous recombination event (see Fig. 6D), no enhancement was observed in the absence of a homologous donor template.

We have shown that DmoCre, our other artificial meganuclease, is functional at high temperature. As a probable consequence, DmoCre could not induce any detectable recombination in our yeast assay carried out at 30°C. Attempts to perform the assay at higher (non-lethal) temperatures gave no better result.

**DISCUSSION**

In this study, we present hybrid endonucleases derived from homing endonucleases through domain swapping. Domain swapping is a powerful strategy to create hybrid enzymes (57).
The simplest approach is to assemble distinct protein moieties, identified in their natural context (the protein they belong to) as functionally and structurally independent domains. Following this strategy, nucleases with new specificities and large cut sites have recently been engineered. Protein fusions combining a DNA binding domain (58–61) or a DNA binding protein (62–65) to the non-specific catalytic domain of FoI, a Class IIS restriction enzyme, were successfully tested in in vivo assays for cutting (62–65), mutagenesis (61) and even recombination (60). Chimeric meganucleases derived from the HO yeast endonuclease represent another interesting case (66). HO is an atypical LAGLIDADG protein, with two LAGLIDADG motifs, and a C-terminal zinc finger domain. Nahon and Raveh could change the specificity of HO by swapping the zinc finger domain (66).

However, typical LAGLIDADG homing endonucleases correspond to a more complex situation, and the functionality of artificial hybrid enzymes is not a trivial issue. First, in the natural proteins, the catalytic, active site is surrounded by two elements of their DNA binding motif. Linkage between catalysis and specific binding is thereby complete. Thus, in natural proteins, the catalytic, active site is surrounded by two binding domains that can be recombined, and that these domains could induce recombination between direct repeats, which could be the precursors of artificial, designed endonucleases. LAGLIDADG proteins belong to the largest (and best characterized) family of homing endonucleases. Hence, domain swapping between LAGLIDADG enzymes would provide a great number of novel proteins. Obtaining specific nucleases for each desired locus, however, will require additional strategies. We have shown that the two DNA binding domains behave rather independently. This will be essential, for instance if directed molecular evolution strategies are to be used to select from mutagenized libraries of either natural or chimeric homing endonucleases, for each domain could be modified independently.

There are many possible applications for novel endonucleases recognizing such large cleavage sites, but the first one is certainly their use in genome engineering, and more precisely, in meganuclease-induced gene targeting. In this case the artificial novel proteins have to be functional in living cells. sac-CrelI was shown to induce homologous recombination in yeast and mammalian cells (our assays enable the testing of proteins that are active in the 30–37°C range). We could induce recombination between direct repeats, which most often occurs by SSA, but also between inverted repeats, which usually occurs by gene conversion (55). Gene conversion, which underlies all homologous targeting strategies, is enzymatically more demanding (55), and this experiment represents a further step toward genome surgery with engineered meganucleases. The detectable levels of sac-

![Figure 6.](https://academic.oup.com/nar/article-abstract/31/11/2952/1220264/A-novel-engineered-meganuclease-induces-homologous)
Crel-induced recombination in both kinds of assay confirm our in vitro results, but they also illustrate for the first time that man-made proteins derived from homing endonucleases will be useful for DNA targeting applications.

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