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Engineering DNA processing enzymes for the postgenomic era

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DNA has been a main focus of biomedical research ever since its discovery as the hereditary molecule. This discovery laid the foundation for scientists to begin both to elucidate and to manipulate its function. Since then many DNA processing enzymes have been discovered and many technologies have been developed to process and manipulate DNA with these enzymes. The sequencing of entire genomes has increased the demand for sophisticated DNA processing enzymes. This need is being addressed by engineering DNA processing enzymes using rational and evolutionary approaches to improve or design novel properties. It now appears feasible that engineered DNA processing enzymes will find utility in molecular medicine as tools for advanced genome engineering.

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Introduction

After the description of the DNA structure as a double stranded helix that encodes the hereditary information of living systems in 1953 [1], the late 1950s and the decade of the 1960s witnessed enormous breakthroughs in DNA enzymology. It was during this time that DNA polymerase, DNA ligase, and the first sequence-specific nucleases were identified, just to name a few of the most important discoveries. The combination of these DNA processing enzymes enabled the development of recombinant DNA technology [2], which made it possible to join DNA isolated from different sources and laid the foundation for the first generation of genetic engineering techniques. The expectations toward sophisticated enzymatic processing of DNA have grown with the increased knowledge in genomics and hence, the demand for advanced DNA processing enzymes is high. In this review, I highlight some of the recent advances made to generate DNA processing enzymes to address the challenges put forward in the postgenomic era.

Improving DNA polymerases for advanced applications

Because of the pivotal role in DNA synthesis, DNA polymerases play a central role among the DNA processing enzymes. Besides its fundamental role in biology, the use of DNA polymerases in biotechnology is staggering. One of the most used technologies in molecular biology is the polymerase chain reaction (PCR). PCR allows the amplification of DNA from a template and is used for a large number of applications such as DNA sequencing, DNA cloning, genotyping, and expression monitoring. It is therefore not surprising that engineering of DNA polymerases has been extensive to improve the properties for any given application. To identify DNA polymerases with desired properties, sophisticated molecular evolution schemes, and selection methods have been developed [3,4]. One focus to increase the usefulness of DNA polymerases has been to widen their specificity to incorporate unnatural or modified nucleic acids [5]. Another focus has been to improve the fidelity and the processivity of the enzyme to avoid errors during the amplification and to increase the length of DNA fragments that can be amplified (see [6] for a recent example). However, for some applications the opposite can be advantageous. A nice example for this has recently been presented by d'Abbadie *et al.* [7•]. The authors used molecular breeding to derive a DNA polymerase, which extended multiple mismatches, something that one would like to avoid for most PCR applications. However, this polymerase turned out to be useful to amplify ancient DNA. Ancient DNA samples typically accumulate lesions over time, and are hence difficult to amplify with standard polymerases. Most polymerases pause when they encounter a mismatch in the DNA and therefore no amplification product is obtained. The evolved polymerase, however, was able to read through the mismatches and amplified the desired DNA fragments. Because of this property this DNA polymerase should be useful for genetic analyses in paleontology, archaeology, and forensic medicine.

The engineering of DNA polymerases will likely continue and expand in the near future, as the requirements for next generation sequencing technologies and the field of synthetic biology develop further.

Modifying restriction enzymes

One of the first DNA processing enzymes that found widespread use were restriction endonucleases (restriction enzymes, or REases). These enzymes are found in many different bacteria, where they bind and cleave-specific short DNA sequences. In order to prevent cleavage of their own DNA, the bacteria also express a DNA

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methylase that methylates the recognition sequence of the endonuclease, thereby protecting it from cleavage. Any foreign DNA entering the cell, however, will likely not be methylated at these sequences and will hence be cleaved and subsequently destroyed. Over 3000 REases have been identified in different bacterial strains, and more than 600 of these (mainly type II enzymes), targeting almost 300 different sequence motives, are commercially available for biotechnological applications such as plasmid vector construction, gene cloning, and restriction fragment length polymorphism analyses.

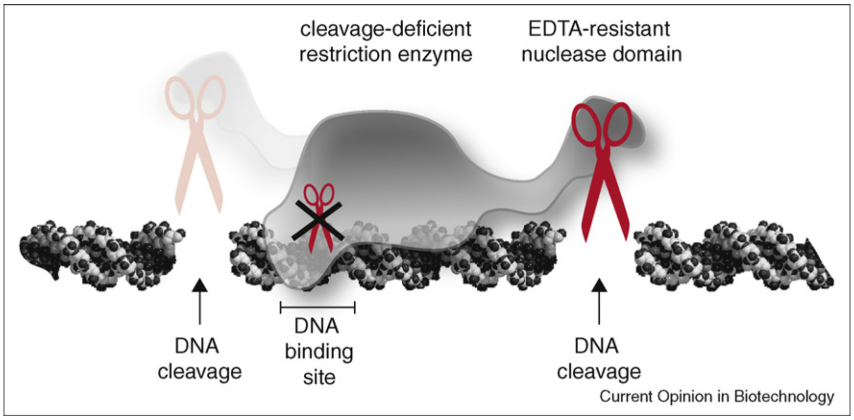
The demand for novel recognition sequences that can be cleaved is high and hence the search for new enzymes is ongoing. The alteration of DNA specificity of existing REases is a valuable alternative to the isolation of novel enzymes from nature. Here, the coding sequence of an existing restriction enzyme is mutated to recognize and cleave a new target site. Increasing structural information [8] aids in the rational design of REases with altered specificities. Successes to change the DNA specificity of endonucleases have indeed been reported [9]. However, our current understanding of how DNA binding specificity can be changed by rational design is still rather limited. Improved selection schemes and enhanced directed evolution approaches [10] have helped to accelerate the speed in which desirable mutants can be identified with, or without prior knowledge of structural information [11,12]. Nevertheless, it remains challenging to change the DNA specificity of restriction enzymes, and many engineered enzymes maintain a considerable activity toward sequences other than the desired target.

An increasingly popular way to designing REases with altered cleavage properties has been the generation of chimeric endonucleases [13]. One approach relies first on the isolation of mutant enzymes, which retain DNA-specific binding without catalytic activity. These cleavage-deficient variants are then fused to protein domains that possess nonspecific nuclease activity (Figure 1). These artificial endonucleases bind specifically to the original sequence, but cleave the DNA outside the target sequence and are called neoschizomers [14•]. Another successful example for a functional chimeric endonuclease has recently been described, which possesses previously undescribed DNA specificity [15••]. Here, different target recognition domains from type IIB enzymes were fused together to form chimeric molecules. Surprisingly, this domain swapping generated many functional enzymes, which now cleaved hybrid recognition sites with high activity. The authors speculate that the combinatorial reassortment of the 18 known type IIB enzymes would greatly expand the list of available restriction enzymes. If this were the case, the number of sequences that can be cleaved could double.

Engineering DNA specificity of rare-cutting endonucleases

Restriction enzymes have been instrumental to cut and paste DNA sequences into vectors in bacteria to start and decipher the language of the genetic code. However, the complete sequence of multicellular organisms has resulted in a demand for enzymes that allow specific manipulation of DNA in these complex genomes. Unfortunately, the relatively short recognition target sequences of the commonly used REases make them unsuitable for this exercise, as they would cut many times in the genome. For this reason, the search for DNA cleaving enzymes, which recognize longer DNA sequences has increased with the growing number of sequenced genomes.

Figure 1



Schematic presentation of chimeric endonucleases. The mutation in the restriction enzyme is illustrated by a crossed out scissor. The fused nuclease domain is shown by the red scissor. Arrows show the DNA cleavage sites. The second cleavage site is illustrated by a fainted nuclease domain, which likely occurs because of the flexibility of the nuclease domain in the fusion protein.

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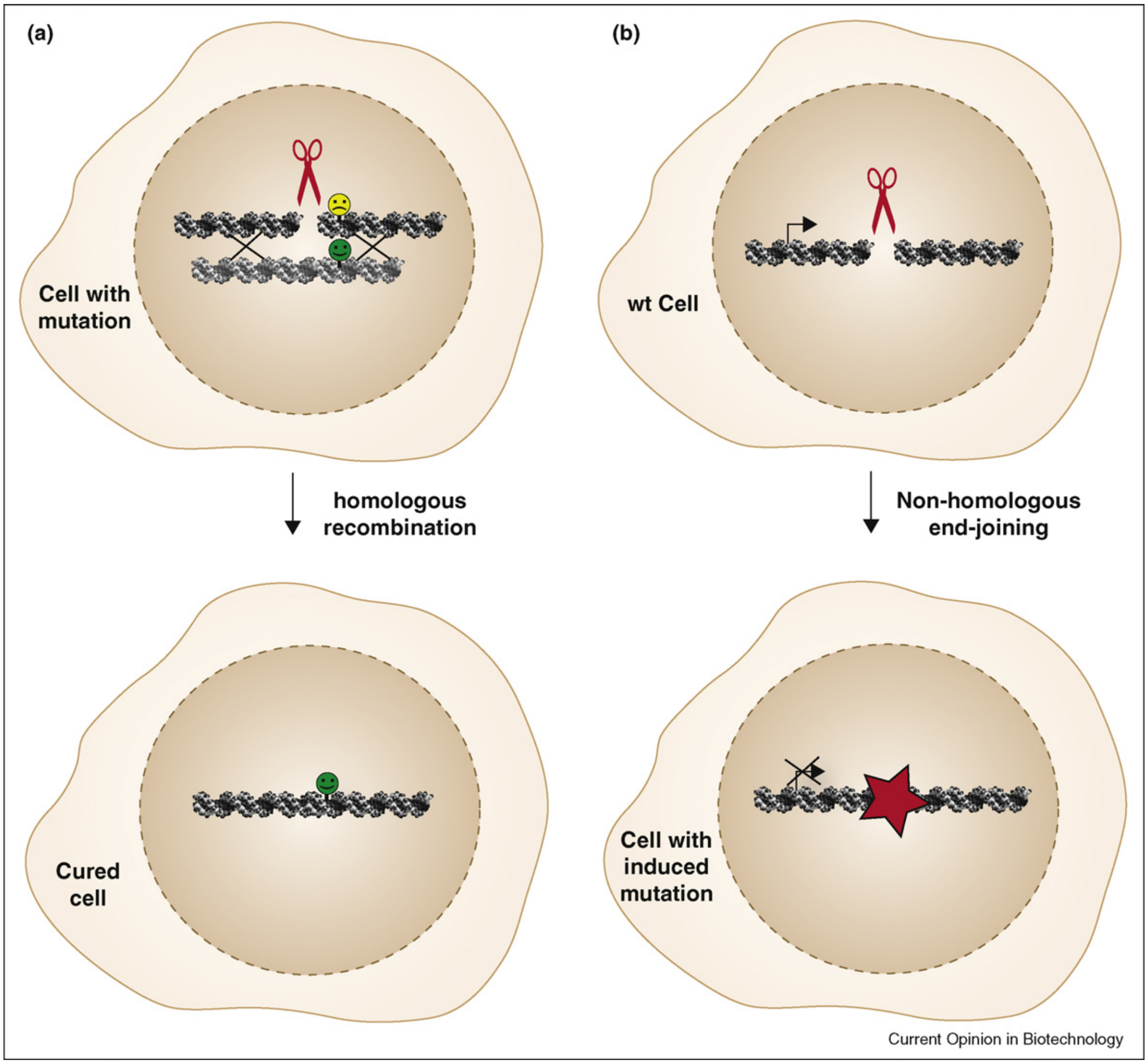
Site-specific endonucleases that recognize and cleave longer target sequences indeed exist in nature. Homing endonucleases (HEGs) are enzymes encoded by mobile introns or inteins [16,17] that recognize DNA sequences normally bigger 12 basepairs. Because they typically provide for their own propagation while conferring no benefit on the host, they are considered examples of selfish DNA. The HEGs I-CreI and I-SceI are examples of the LAGLIDADG family of homing endonucleases that have been extensively used to develop novel biotechnological applications. They are currently the best-studied enzymes of its kind and crystal structures for both enzymes bound to its native recognition site have been published [18,19]. Their utility rests on their long recognition motives (14–30 basepairs), which are typically absent in heterologous organisms. Hence, the introduction of a recognition target site of these enzymes into the genome of an organism, followed by expression of the HRG introduces a single cut at the site in the genome [20•]. The development of this technology has made important contributions to various fields of biology such as DNA repair [21] and DNA methylation [22] and the enzymes have demonstrated their utility in many different organisms, such as *Xenopus* [23], *A. gambiae* [24], *Drosophila* [25], Zebrafish [26], plants [27], and mammalian cells [28]. To extend the usefulness of HRGs for advanced genome editing and manipulation, considerable efforts have concentrated on engineering these enzymes

Figure 2



In vivo DNA engineering with HGEs and ZNFs. **(A)** Homology-directed DNA repair correcting a mutation (yellow circle). The engineered HGE or ZNF cutting in the vicinity of the mutation is shown as a red scissor. The introduced cleavage stimulates the homologous recombination pathway and the introduced DNA fragment without the mutation (green circle) is used to correct the mutation. **(B)** Generation of a gene knockout using an engineered HGE or ZNF. The introduced cut within a gene is repaired via nonhomologous end joining, which typically results in a loss of genetic material. The deletion is illustrated by a red star. The transcription start-site of the gene is shown as an arrow.

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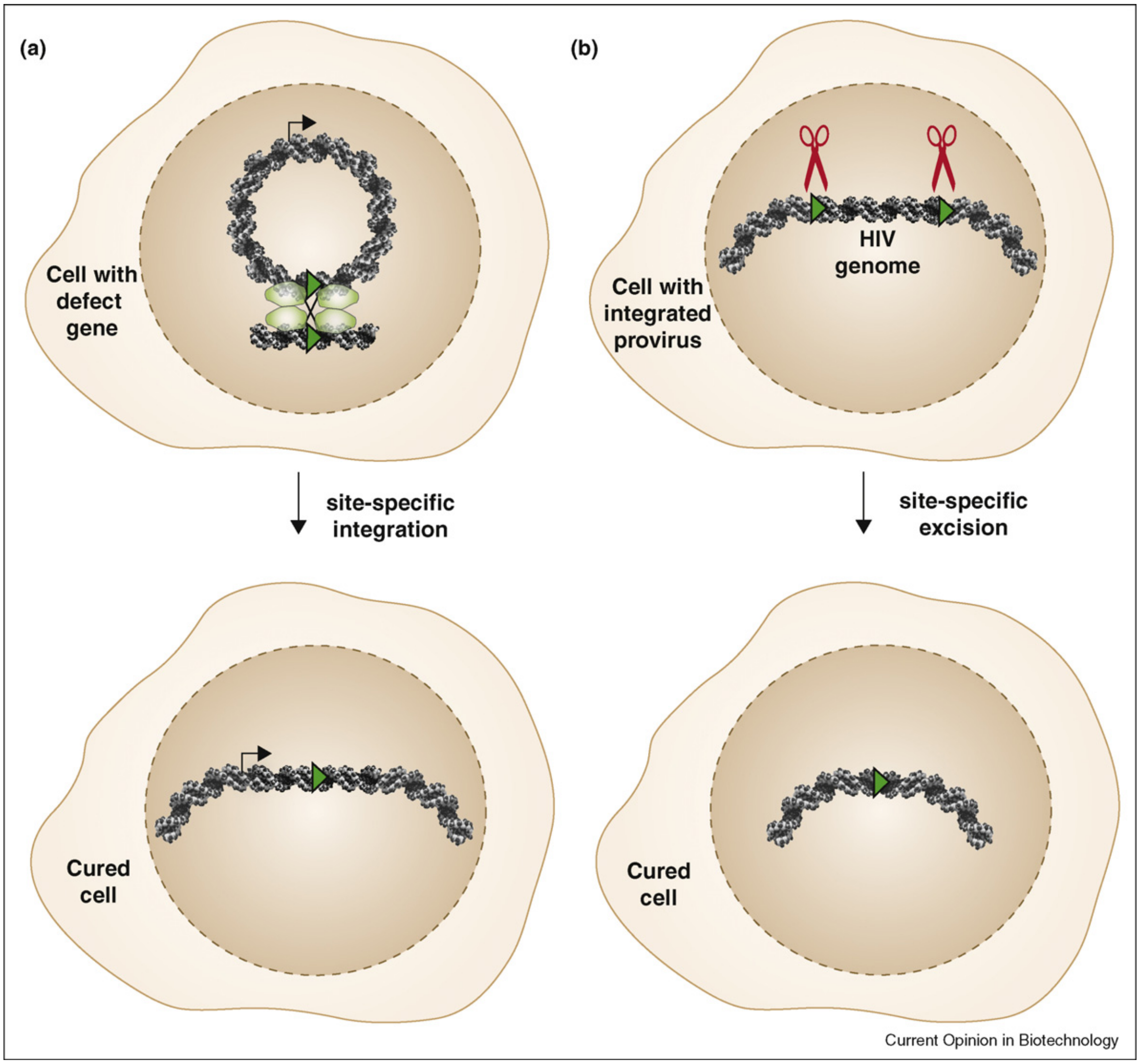
to derive novel specificities. Several groups have reported successful alteration of DNA binding specificities of these enzymes using rational and directed evolution approaches [29–32]. An impressive example for the potential medical utility of such enzymes has recently been described by Redondo *et al.* [33••]. The authors described the development of an engineered I-CreI meganuclease that cleaves a sequence within the human XPC gene, a gene mutated in xeroderma pigmentosum patients. Providing a wild-type DNA fragment together with the engineered I-CreI enzyme in cells carrying the xeroderma pigmentosum mutation stimulated homologous recombination mediated DNA repair and resulted in the isolation of cells, which now carried the wild-type XPC allele. This example demonstrated the promising possibilities of engineered HGEs as molecular scalpels for genome engineering and gene therapy (Figure 2A).

An alternative method to obtaining sequence-specific endonucleases with long recognition sites has been the development of zinc-finger nucleases (ZNFs) [34]. ZNFs combine the nonspecific cleavage domain of the FokI restriction enzyme with DNA binding domains of zinc-finger proteins (ZFPs) [35•]. Because the recognition specificities of ZFPs can be manipulated experimentally, and extended recognition sequences can be generated by adding different ZFP-domains to form an engineered protein recognizing 18 or more basepairs, ZNFs offer an elegant way to designing custom rare-cutting

Figure 3



Recombinases and integrases as tools for genome surgery. **(A)** Site-specific integration of a DNA circle encoding a gene (transcription start-site indicated by an arrow). The integrase (green spheres) finds the recognition target sites in the genome and the DNA circle (green triangles) and recombines the circle into the genome of the cell. **(B)** Site-specific excision of an integrated provirus from a cell. The engineered site-specific recombinases (red scissors) recognize sequences within the LTR (green triangles) and recombine the sequences at these sites to excise the integrated provirus.

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endonucleases [36••]. These engineered enzymes can be used to generate gene knockouts or gene replacements via homologous recombination in cells (Figure 2). The development of multi-finger array technology [37] has accelerated the identification of functional proteins with desirable properties and many successful gene targeting and gene replacements have been reported in different organisms utilizing this technology [38–44].

Development of recombinases and integrases for advanced DNA engineering

Another class of DNA processing enzymes that have gained increasing popularity over the years are site-specific recombinases, such as the Cre/loxP and FLP/FRT system, and site-specific integrases, such as the phiC31 integrase. Site-specific recombinases and integrases are enzymes that recognize DNA sequences of around 30–40 basepairs, which makes them suitable for the engineering of DNA in genomes of heterologous organisms. The recombination of two recognition sites in a cell can induce deletions [45], inversions [46], integration [47], or chromosomal translocations [48,49] depending on the experimental design. Because of their ability to specifically and effectively remove DNA segments from the genome of living systems (conditional knockout), their role in the functional annotation of genes in complex organisms and modeling of human diseases has been instrumental [50]. A limitation of their utility has been the restricted number of sequences that they can recombine. Hence, enzymes with different specificities increase the repertoire for these enzymes and newly identified recombinases with useful properties have indeed been identified [51], and characterized [52], recently.

As for the endonucleases, the engineering of target site specificity would greatly enhance the utility of recombinases and integrases and would allow precise engineering of genomes for medical applications (Figure 3). Consequently, the engineering toward higher efficiencies and novel specificities for both integrases [53] and recombinases [54,55] has been intense. First examples of therapeutically relevant applications have also been demonstrated, where the phiC31 integrase was used to promote site-specific integration of the dystrophin gene into the patient's own myogenic precursor cells [56••] and by evolving the Cre derived recombinase Tre, an enzyme that was able to excise an integrated HIV-provirus from infected cells [57••].

Opportunities and challenges for future DNA processing enzymes

Engineered polymerases, restriction enzymes, recombinases, integrases, HGEs, ZNFs, and other DNA processing enzymes promise to provide excellent tools for sophisticated DNA manipulations. The prospects for these enzymes are bright and manifold. For instance, the astonishing recent advances in DNA sequencing technologies call for new, tailored DNA polymerases [58] and the emerging field of synthetic biology will greatly benefit from designer enzymes that help to engineer complex biological systems [59]. Furthermore, the technological advances in engineering DNA processing enzymes appear to make clinical applications feasible. First practical therapeutic applications of these enzymes will likely involve *ex vivo* therapies using a patient's own stem cells. After editing the stem cell genome, the cells could be expanded in culture and reinserted into the patient to produce differentiated cells with corrected functions. Before this scenario can become reality, extensive analyses of potential pitfalls, such as cytotoxicity, immunogenicity, tumorgenicity, and other unwanted side-effects have to be thoroughly investigated. Undesired effects after overexpression of DNA processing enzymes in cells have indeed been reported [60–62] despite their successful application in many living systems. If, however, these issues can be solved molecular surgery will become possible in the future.

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