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CRISPR: A New Era In Molecular Biology

Review Article

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Summary

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system has been seized upon with a fervor enjoyed previously by small interfering RNA (siRNA) and short hairpin RNA (shRNA) technologies and has enormous potential for high-throughput functional genomics studies. Editing via (CRISPR)–CRISPR-associated (Cas) constitutes a next-generation method for programmable and highthroughput functional genomics. CRISPR–Cas systems are readily reprogrammed to induce sequence-specific DNA breaks at target loci, resulting in fixed mutations via host-dependent DNA repair mechanisms. Bacteria and archaea acquire resistance to invading viruses and plasmids by integrating short fragments of foreign nucleic acids at one end of the CRISPR locus. CRISPR loci are transcribed and the long primary CRISPR transcript is processed into a library of small RNAs that guide the immune system to invading nucleic acids, which are subsequently degraded by dedicated nucleases.

I. Introduction

Microbes have devised various strategies that have helped them to survive exposure to foreign genetic elements. Considerable proportions of bacterial and archaeal genomes consist of genes derived by horizontal genetransfer among related or unrelated species via transduction, conjugation, and transformation[1]. This has forced microbes to establish an array of defense mechanisms that allows them to recognize and distinguish foreign DNA from self DNA. These systems maintain genetic integrity, yet occasionally allow exogenous DNA uptake and conservation of genetic material that gives an advantage for the bacteria to adapt in an environment. Recently, an adaptive microbial immune system has been identified as clustered regularly interspaced short palindromic repeats (CRISPR) that protects bacteria against foreign genomes[2].

Clustered regularly interspaced short palindromic repeats are segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a bacteriophage virus or plasmid. The latest analysis shows that CRISPR loci are composed of 21- to 48-base pair (bp) direct DNA repeats interspersed with nonrepetitive nucleotides of 26 to 72 bp called spacers. Repeats and spacers are conserved within a given loci. Each CRISPR locus is defined by the sequence of the repeat and the typical repeat is defined as the most frequent within a CRISPR locus. The number of repeats within a CRISPR varies between strains of the same microbial species. Two to 375 repeats have been found per locus. The number and the sequence of the spacers also vary between strains of the same microbial species[3]. There are no open reading frame is present within CRISPR loci. Upstream of the CRISPR locus is a leader region containing 20 to 534 bp which contain high adenine and thymine content[4]. Within this leader region lies a promoter which helps in the transcription of the CRISPR. Finally, a few CRISPR-associated (*cas*) genes are almost always found in the vicinity of the CRISPR region . Adjacent to these CRISPR repeats and the spacer DNA is a set of conserved CRISPR-associated (cas) genes that encode the Cas proteins[5].

A CRISPR/Cas system, also named CRISPR-associated system, usually contains between 4 and 20 different *cas* genes These genes are found both upstream or downstream of the repeat/spacer region. CRISPR along with Cas proteins, forms the CRISPR/Cas systems. Six "core" cas genes have been identified. Besides the cas1 to cas6 core genes, subtype-specific genes and genes encoding "repeat-associated mysterious proteins" (RAMP) have been identified and grouped into subtypes functionally paired with particular CRISPR repeat sequences[6].

II: CRISPR as an adaptive immunity in prokaryotes:

Prokaryotes belonging to the most varied groups contain a peculiar type of DNA, repetitive in nature which was recognized in 2000 as a family[7], distinguished by the regular spacing of the recurrent motif and consequently defined as short regularly spaced repeats (SRSR). Clustered regularly interspaced short palindromic repeats (CRISPRs) are a novel class of repetitive DNA[8] that has been identified in 88% of the archaea bacterial genomes and 39% of the eubacterial genomes that has been sequenced so far[9].

It has been found that some of the spacer sequences match with the fragments of extra-chromosomal elements (mainly from plasmids and virus genomes) and thus has led to the hypothesis that the CRISPR–Cas system might be a novel defense system that is able to protect a host cell against invading alien nucleic acid[10,11]. As reviewed here, over the course of the past few years this hypothesis has been confirmed experimentally[12], and thus

three distinct stages are recognized in the CRISPR defense mechanism: (i) adaptation of the CRISPR via the integration of short sequences of the invaders as spacers; (ii) expression of CRISPRs and subsequent processing to small crispr guide RNAs; and (iii) interference of target DNA by the crRNA guides. Recent analyses of key Cas proteins indicate that, despite some functional analogies, this fascinating prokaryotic immune system shares no phylogenetic relation with the eukaryotic RNA interference system[13].

A: Acquisition of new spacer sequences

The acquisition of new spacers either viral or plasmid fragments occurs at the leader (L) side of the CRISPR[14]. The leader sequence includes a binding site for proteins (probably Cas proteins) that are responsible for repeat duplication and/or spacer acquisition[15]. Both sense and anti-sense spacer sequence orientation turned out to be functional , however, only the leader strand is transcribed. This indicates that the mechanism at the level of mRNA does not operate via classical anti-sense mechanism, suggesting that DNA is the target. After disruption of the gene encoding, acquisition of new spacers seemed to be affected strongly suggesting its role in CRISPR adaptation[16].

It has been suggested that the spacer acquisition can also be due to nonhomologous recombination. It was also reported that the mRNA were the source of the new spacers. The presence of a gene coding for a putative reverse transcriptase (RT) the lies within the vicinity of the *cas* genes explained the presence of spacers that correspnds to coding and noncoding strands. However, many CRISPR/Cas loci lack such a gene[17]. Thus, the most likely source for the new spacers is thought to be dsDNA[18,19].

Adaptation to plasmids and predatory phages by spacer acquisition has been shown to occur readily in several species. In the course of studies of phage therapy, M102 phages were introduced into rats , for the prevention of tooth decay to eliminate *Streptococcus mutans*, which is the mainly responsible for dental cavities. Following this, bacteriophage insensitive mutants were isolated that had added an M102 matching spacer sequence to one of the two CRISPR arrays in this species[20]. Similar adaptation can be induced in *S. thermophilus* cultures by challenging the cultures by phage[21].

The studies have revealed that the new spacers are inserted at the leader end of the CRISPR array and most of the integrations happens to be at the first position in the cluster. The loss of one or more repeat spacer sequences has also been observed, which suggests that CRISPRs do not grow unchecked[22]. Commonly single repeat spacer unit gets added, but up to four new units have been detected[23].

B: CRISPR expression

Expression of the CRISPR regions was first described small RNA profiling studies of the archaea *Archaeoglobus fulgidus* and *S. solfataricus*[24,25]. It has been suggested that longer transcripts (termed pre-crRNA) of repeats and spacers , potentially covering the entire CRISPR, were processed to small crRNAs. It has been observed that in E. coli K12, the leader strand of the CRISPR region is transcribed and processed[12]. In the expression studies of CRISPRs from the bacterium *Staphylococcus epidermidis*[26], and the archaeon *P. furiosus*[27] similar type of observations has been recorded.

In contrary to this, it has been reported that the transcription has been observed not only for the leader strand but also for the complementary strand of CRISPRs in the archaeal genus Sulfolobus. CRISPR transcription initiates at the end of the locus that contains the leader sequence, and the CRISPR promoter might even reside within the leader itself. Since transcription is constitutive and unidirectional in nature but one possible exception to unidirectionality has been reported[28].

The first biochemical insight into the molecular mechanism of CRISPR processing was obtained by analyzing E. coli strain K12[12]. This bacterium possesses a single cluster of cas genes that encodes eight proteins of which three are well-conserved proteins, Cas1 (integrase/ nuclease), Cas2 (nuclease) and Cas3 (helicase/nuclease). The other five-proteins viz; Cse1–Cse2–Cse4– Cas5e–Cse3 are more variable in nature. These variable proteins are also referred to as CasABCDE and constitutes a Cascade. It has been observed that in *Thermus thermophilus,* cyclic AMP regulator protein upregulates *cas* genes[29,30] and thus the CRISPR response may be regulated by cAMP signal transduction. In *S. mutans*, increased expression of *cas* genes has been reported following the analysis of the transcriptome of a *clpP* protease mutant, which suggests the regulation of CRISPR loci[31].

C: CRISPR Interference

It has been seen that the interference stage of the CRISPR/Cas system somewhat resembles the eukaryotic RNA interference (RNAi)[32]. But it is a misguided hypothesis that CRISPR mediates microbial immunity via RNA interference[17]. RNAi silences the foreign invading nucleic acid sequence before or after it integrates into the eukaryotic host chromosome, and/or prevent cellular processes through a small interfering RNA guide[33]. On contrary to this, CRISPR-encoded immunity involves the enzymatic machinery. Although both the mechanisms involve a guide RNA in an inhibitory ribonucleoprotein complex, only Dicer, Slicer, and the RNA-induced silencing complex (RISC) may have analogous counterparts[34].

CRISPR systems, together with cas genes shows high diversity in mechanisms of adaptable immunity used by many bacteria and archaea in protecting themselves from invading viruses, plasmids, and any other foreign nucleic acids[35]. CRISPR interference occurs differently in different CRISPR systems. In one system, Cas6e/ Cas6f cut at the junction of single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) formed by hairpin loops. In other systems,trans-activating (tracr) RNAs are used to form dsRNA which are cleaved by Cas9 and RNaseIII. In one of the systems, Cas6 homolog is used for cleaving of the direct repeats and hence do not require hairpin loop[36].

As a general theme of different CRISPR systems, there is biogenesis of crRNAs in which there is site specific cleavage of precrRNA precursors in the repeat sequences by Cas proteins followed by 3′ splicing events. As a result, crRNAs have a welldefined 5′ end that begins with ~8 nt of the upstream repeat sequence and a more heterogeneous 3′ end[26]. In one of the CRISPR systems, sequences are incorporated from invading DNA between CRISPR repeat sequences encoded as arrays within the bacterial host genome . CRISPR repeat arrays are transcribed and subsequently processed into CRISPR RNAs

(crRNAs), each containing a variable sequence transcribed from the foreign invading DNA, known as the "protospacer" sequence and part of the CRISPR repeat. Each crRNA hybridizes with a second RNA, known as the transactivating CRISPR RNA (tracrRNA)[37] which then forms a complex with the Cas9 nuclease[38]. Twenty nucleotides at the 5′ end of the protospacer portion of the crRNA direct Cas9 to a specific target DNA site using standard RNA-DNA complementarity base-pairing rules, if they are adjacent to short sequences known as protospacer adjacent motifs (PAMs). Some CRISPR systems that recognizes other PAM sequences in different species of bacteria employs different crRNA and tracrRNA sequences have also been utilized for targeted genome editing[39,40,41].

III: CRISPR/Cas as a genome editing tool

CRISPR/Cas9 has proved to be an efficient tool for genome editing of human cells. The "humanized" versions of *S. pyogenes* Cas9 and *S. thermophilus* Cas9 were coexpressed with custom-designed crRNAs or with tracrRNA coexpressed with custom-designed crRNAs in human embryonic kidney, chronic myelogenous leukemia, or induced pluripotent stem cells as well as in mouse cells[42]. The end results in the target DNA were observed, demonstrating RNA-guided Cas9 had stimulated gene editing by non-homologous end joining repair or gene replacement by homology directed repair. Multiplexing i.e. targeting with multiple crRNAs was also achieved successfully[43].

The therapeutic potential of CRISPR-Cas has already been demonstrated in many cases. In bacteriology, Cas9 has been employed as an antimicrobial agent and has been used to target antibiotic resistant in highly virulent strains of bacteria[17]. A patient with cystic fibrosis showed functional repair of the CFTR gene in vitro in cultured intestinal stem cell organoids using CRISPR-Cas[44]. hydrodynamic injection of CRISPR components could correct a defective gene causing hereditary tyrosinemia in mice. This led to an expansion of mutation-corrected hepatocytes in vivo and resulted in a rescued phenotype in adult mice[45]. Germ line mediated editing of mice with Duchenne muscular dystrophy prevented the mice from the disease[46].

CRISPR-Cas9 is evolving as a promising technology in the field of engineering and synthetic biology. A multiplex CRISPR approach referred to as CRISPRm was developed to facilitate directed evolution of biomolecules[47]. CRISPRm generates quantitative gene assembly and DNA library insertion into the fungal genomes by optimizing CRISPR/Cas9, thus providing a strategy to improve the activity of biomolecules. It has also been reported that short DNA oligonucleotides containing PAM sequences activates the enzymes by inducing Cas9 to bind ssRNA in a programmable fashion and thus providing ways to target transcripts without undergoing any prior affinity tagging[48].

While CRISPR technology moves genome editing from the realm of the practically impossible to the possible but on the contrary moving from difficult to easy is still not achieved. Majority of applications require a pure population of cells i.e. clones of single cell which is a labor and time intensive process. So, before planning any gene editing experiment all the requirements of the application should be taken care of. As reported by Mohr et al. in 2016[49], ideal ways of designing guide RNA (gRNA) for a particular event depends a great deal upon the downstream purpose.

IV: CRISPR with benefits

The key potential of the CRISPR-Cas system lies in its ability to genetically modify an organism, leaving no foreign DNA behind and in its versatility and simplicity of programming. Unlike ZFNs and TALENs, which requires reprogramming i.e. editing of DNA-interacting domains located at different sites on the DNA-binding scaffolds, execution of the changes made by CRISPR-Cas systems only applies to recombinant RNA sequences[50,51]. Ease of use, low cost, high speed, multiplexing potential and equal or higher specific DNA targeting ability have increased its popularity at the scientific global level[52].

Engineering within cells using different combinations of mutations that reflect various stages of neoplastic disease can be done as multiple gene-targeting events within the same cell are possible using CRISPR/Cas9[53,54]. So to identify stage-specific syntheticlethal interactions cell lines so produced could then be used in combination with siRNA or shRNA. Such combinatorial screens have already been performed in yeast and Drosophila[55,56].

The most eye catching property of CRISPR is the ability to flexibly and precisely target Cas9 to essentially any genomic location. For DNA targeting, Cas9 must recognise short PAM sequence and also there is requirement of base-paring of the 20 nt target sequence with the spacer region of the guide RNA (gRNA)[57]. The strongest gRNAs (i.e., those resulting in highest fold repression by dCas9) have high level of complementarity to PAM-adjacent target, but targets where complementary factor is little less, may still be bound by the dCas9- gRNA complex[58,59]. Repressive effect will be decreased if multiple mismatches occurs between the sgRNA and a potential target[60]. By targeting different positions within the gene, repressive effect can also be adjusted[61].

The most important current application of CRISPR interference is the production of phage-resistant strains of bacteria for the dairy industry[62]. There is disruption in the generation of normal fermentation cycles once the dairy starter culture is infected with phage which ultimately leads to the low quality finished product[63]. It has been observed that in *S. thermophilus,* the acquisition of new spacers is an exceptional tool for the control of phage infection in the dairy industry, as it helps in differentiating strains that are resistant to multiple bacteriophages but still retaining the same starter culture properties.

An important advantage of CRISPRi over RNAi is the absence of CRISPR/Cas systems in eukaryotes for various applications in which competition with the endogenous pathways becomes difficult. In contrast to RNAi, which mostly to knocks down gene expression, CRISPR/Cas9, on the other hand, permanently alter the genetic code and up or down regulate gene expression either transcriptionally or at the posttranscriptional level[64].

V: Concluding Remarks

Unlike other moments in scientific history, the CRISPR/Cas system has opened an era of changes, which may span from groundbreaking therapeutic applications to daunting fears of irreversible perturbation of human evolution. The prokaryotic CRISPR/Cas system is somewhat different as DNA rather than RNA is the prime target for interference. Although much more is still to be

discovered, still it has become quite clear that the CRISPR/Cas system is a specific technology and is as useful as the model to which it is applied and thus enabling diverse organisms to serve as models and accelerating their manipulation is yet another reason why CRISPR technology is so powerful.

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