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Bovine Genome Analysis to Unravel the Location and Feature of Target Sites of RNA-Guided Hyperactivated Recombinase Gin with Spacer Length Six

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ABSTRACT

Background: Programmable nucleases are very promising tools of genome editing (GE), but they suffer from limitations including potential risk of genotoxicity which led to the exploration of safer approach of GE based on RNA-guided recombinase (RGR) platform. RNA-guided recombinase (RGR) platform operates on a typical recognition or target site comprised of the minimal pseudo-core recombinase site, a 5 to 6-base pair spacer flanking it and whole this central region is flanked by two guide RNA-specified DNA sequences or Cas9 binding sites followed by protospacer adjacent motifs (PAMs).

Methods: The current study focuses on analysis of entire cattle genome to prepare a detailed map of target sites for RNA-guided hyperactivated recombinase Gin with spacer length six. For this, chromosome wise whole genomic sequence data was retrieved from Ensembl. After that search pattern for recombinase Gin with spacer length six was designed. By using this search pattern, RGR target sites were located by using dreg program of Emboss package.

Result: Total number of RGR target sites identified in bovine genome for recombinase Gin was 677 with spacer length six. It was also investigated that whether these RGR target sites are present with in any gene or not and it was found that RGR target sites lies in both genic and intergenic region. Besides this, description of genes in context with these target sites was identified.

Key words: Cattle genome, Gene description, Gin, Hyperactivated recombinase, RNA-guided, Spacer length.

INTRODUCTION

Recombinases are powerful tools for genetic modifications, but often require complex directed-evolution experiments to retarget specificity and are limited by lack of user-programmability. Hence, to address these constraints, modified site-specific recombinases which have 'relaxed' substrate specificity called as hyperactivated recombinases have been developed. To address the limitation of lack of user-programmability, RNA-guided recombinase platform (RGR) has been developed. This platform operates on a typical recognition site which envisages a minimal pseudo-core recombinase site, a 5 to 6-base pair spacer flanking it and whole this central region being flanked by two guide RNA-specified DNA sequences or Cas9 binding sites followed by protospacer adjacent motifs (PAMs).

Though only one RGR platform based on hyperactivated recombinase Gin (β) is available currently, more hyperactivated recombinases have been evaluated as part of ZFR platforms (Gaj *et al*., 2013; Sirk *et al*., 2014). In case of Gin itself, four more (α, γ, δ, ε and ζ) hyperactivated versions have been evaluated (Gaj *et al*., 2013). Sirk *et al.* (2014) described functionality of ZFR platforms based on β and Sin hyperactivated recombinases. Information may pour in for some new hyperactivated recombinases in the future. This will be useful since more number of hyperactivated recombinases will increase the overall targeting capacity of recombinase-based or RGR platform based genome editing. RGR target sites for the RGR platforms based on the hyperactivated recombinases other than Gin (β) can be easily discerned by only replacing the central 20 bppseudo-recognition sequence in the target sequence with that for the other hyperactivated recombinase (Chaikind *et al*., 2016).

RNA guided recombinase platform may also be utilized for study or treatment of genetic diseases similar to RNA guide endonuclease based platform. A candidate SNP allele for African swine fever virus resilience T1591C of p65 has been introgressed from warthog to the genome of conventional swine cells (Palgrave *et al*., 2011). Isoleucyl-tRNA synthetase (IARS) syndrome is a recessive disease of Japanese Black cattle caused by a single nucleotide substitution. Ikeda *et al.* (2017) have reported correction of disease mutation using CRISPR/Cas9-assisted genome editing.

RNA-guided recombinase (RGR) platform, having its ability to carry out efficient, reliable and safe genome editing, has a great potential in genome modifications for animal

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breeding and may become a tool of choice for this purpose in the future. However, application of genome editing in animal breeding through RGR will become reality only after we overcome two critical challenges. These include accurate information about the Quantitative trait nucleotides (QTNs) for a particular trait or set of traits and the detailed map of RGR target sites throughout the genomes of livestock species. It is certain that the first challenge would be overcome because of the current era of genomics and ongoing GWAS studies as well as application of GS in animal breeding since we will come to know various QTNs through this. Overcoming the second challenge pertaining to RGR target sites would include preparation of detailed maps for target sites of available RGR platforms as well as RGR platforms that can be constructed in future based on other evaluated hyperactivated recombinases in genomes of different livestock species. The present study would contribute in addressing the second challenge wherein a detailed map of target sites for hyperactivated recombinases Gin with spacer length six was prepared and genes in context to RGR target sites were identified in entire cattle genome (*Bos taurus*).

MATERIALS AND METHODS

The experiment was conducted during Ph. D research session 2017 -18 and 2018-19 at Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh. Chromosome wise whole genomic sequence data was retrieved from Ensembl (Bos_taurus.UMD3.1.dna. chromosome). Search pattern was designed depending on structure of RGR target site which includes degenerate sequence of 20 bp core recombinase recognition sites (NNNNAAASSWWSSTTT NNNN) flanked by spacer (6 bases), guide RNA binding site (25 bases) and PAM sequences at both the ends. Designed search pattern was as follows:

CC[ATCG]AAA[TCG][ATCG][AT][AT][ATCG][ACG]TTT[ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][ATCG][

Table 2: Location and features of important target sites identified in genic region of bovine genome for hyperactivated recombinase gin with spacer length six.

Chr. no.	Start position	End position	RGR target site sequence	Gene description
1	75184537	75184614	CCTGGGAAGCCCTCAGTGGGTTAAAAG AGCATTAAACATTGATTTGCTCATAAAGT TTCTTGGAATCAGGTGTTTGGG	Protein MB21D2
2	36257786	36257863	CCAGAGCTCATGGAGGTGATCTTTTCTC AAGTTAAACATACGTTTAATTTCAGTATC ATAGTTCTATTCCATCTGGGG	Integrin beta-6 isoform X1
2	86895761	86895838	CCAATCCCGGTCCTCAGAGAGCTTCAG TTTCTGAAACTTAAGTTTATTTCTATGATA TCTTGTTCCTGTAGGCACAGG	Inactive phospholipase C-like protein 1
5	1.17E+08	1.17E+08	CCCCAAAAGCCAGTAAACAGGGCAGTT TGGTGGAAAGGAAAGTTTGCTGTATTTT TGGTGCCTGCAACTGGACAGGGG	Fibulin-1 precursor
8	38011499	38011576	CCAATTTTTCCAGGGCTAGAGACCTTCT TCTTTAAAGAAATCTTTAAACCAAACACT GAAGGCTTGGGTTTTCCCAGG	Lysine-specific demethylase 4C
10	49024876	49024953	CCATGTATGTTCATCTCTGTGCAAGTAG CTCCAAAAGGAATATTTTTTTAATTTTAA AAATTTATTTTTAATTGAAGG	Nuclear receptor ROR-alpha
11	82979748	82979825	CCAAGTAAATACAGAACTTTGAGGTACT AAACAAAACGTTAGTTTTGTTTCAAGGT TAAACATGTTTGTCACAGTAGG	Neuroblastoma-amplified sequence
12	76804827	76804904	CCCAAGCTGGCTTGTTTTTTTTAAGTTT TTTTAAAATGAAGATTTGATGGATAGCAT GAGAGAAAGGGAGGGGTGTGG	Claudin-10 isoform b
14	9134518	9134595	CCTCTTTTCCTGCATCATCTCACTTAACC CTCAAAACAATCCTTTACAAGAGGTATTG TTGGCATCCTTGTTGAAGGG	Protein NDRG1
14	9368738	9368815	CCTAAAGTGACTTCATGCTTTAAGGGGG AAAAAAAATCTTCCTTTATTTGCTCTGAT TGTCAGCTATTGCATTTAAGG	Thyroglobulin precursor
15	57904828	57904905	CCTTTTTAGTGCTCTGTGTCTCTATGCT GCATGAAATATTAATTTTTTTGGAAAATG AGCTACTTATTATTTTATTGG	Anoctamin-3
16	67622051	67622128	CCTATTTATACTAAAGAGAAAACAAAGTA GAAAAAACTTAACTTTTCTGAGTGGTAA GTATAAACCTAGCTTCCATGG	TRMT1-like protein
17	17477131	17477208	CCTACAGGAACATGATTAAAGGATTAAA TGAAAAAAGTATTATTTCATGAACATAAA TGATATAAATAAGGGACGAGG	GRB2-associated-binding protein 1 isoform X1
18	29716848	29716925	CCATGCACAACAACAAAGACAAAGCAC AGCCAAAAATAAAGCTTTTAAAAAAATT AAAAATATTTGAAAATCACATGG	Cadherin-8 isoform X1
18	36983798	36983875	CCCTTGAGATTTTGTTGGAATCCTGACG TATGTAAAGTTTCCTTTATGGAGAATTTC ATACTGATTCTTCCTACACGG	NEDD4-like E3 ubiquitin-protein ligase WWP2
20	31891235	31891312	CCTGCTGGTGTAATGTCGCTTACCTGGG CATAAAAATCAATGTTTGCCAATGAACTT GGATTGCTGAGCTGTGTATGG	Growth hormone receptor precursor

Table 2: Continue.....

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20	62038715	62038792	CCTATCTATTTTGAGTTACTATGGGGTTG GCCAAAACGTTCATTTGATGGTACCAAA AAACCCAAACGAGCCTTTTGG	Catenin delta-2 isoform X1
24	8045598	8045675	CCCTTCTTCCTACTTAAGTAAAAAAGGG AAAGGCAAAGGAATCTTTATTTCCCTTG GTATCAATGTAAAAGTAACTTGG	Docking protein 6
25	26700954	26701031	CCACATGTGCCGCACCTAAGACCCTGA GCAGCCAAATGAATATTTAAAAATGGGG CCAGGGGGCTCACCTTTTCCTGG	Kinesin-like protein KIF22
26	34327821	34327896	CCTTTTCAATTATTCCTACCATTTTCTTAA AAAAATATTCATTTATTTATTTGGTTGTGC TGGGTCTTAGTTGGGG	Hyaluronan-binding protein 2 precursor

hyperactivated recombinase Gin with spacer length six. It was found that RGR target sites are located in several important protein coding genes which regulates important biological proteins like Mab-21 domain containing 2, integrin beta-6 isoform X1, fibulin-1 precursor, thyroglobulin precursor, kinesin-like protein KIF22, Raftlin protein, inactive phospholipase C-like protein 1, docking protein 6, growth hormone receptor precursor, lysine-specific demethylase 4C, TRMT1-like protein, GRB2-associated-binding protein 1 isoform X1, cadherin-8 isoform X1, hyaluronan-binding protein 2 precursor, claudin-10 isoform b, nuclear receptor ROR-alpha, neuroblastoma-amplified sequence, protein NDRG1, anoctamin-3, NEDD4-like E3 ubiquitin-protein ligase WWP2, catenin delta-2 isoform X1 *etc.*

A RGR site in gene MB21D2 coding for Mab-21 domain containing 2 was located on chromosome 1. Olivieri *et al.* (2016) studied to identify genomic regions and metabolic pathways associated with dry matter intake, average daily gain, feed efficiency and residual feed intake in an experimental Nellore cattle population and found the association of MB21D2 gene with average daily gain. An *et al.* (2018) identified loci and candidate genes for internal organ weights in Simmental beef cattle by genome-wide association study. They found that polymorphism in MB21D2 gene is associated with kidney weight.

A RGR site in gene ITGB6 coding for integrin beta-6 was located on chromosome 2. The encoded macromolecule forms a dimer with alpha v chain and this heterodimer may bind to ligands like fibronectin and transforming growth factor beta one. These can function as receptors for foot-and-mouth disease virus (FMDV) in epithelium. Berryman *et al.* (2005) have shown that 'foot-and-mouth disease virus (FMDV) infection mediated by the integrin takes place through clathrin-dependent endocytosis however not caveolae or alternative endocytic pathways that rely upon lipoid rafts. A RGR site in gene coding for fibulin-1 precursor was located on chromosome 5. Fibulin-1 may play a role in cell adhesion and migration along protein fibers within the extracellular matrix (ECM). It might be vital for development related processes and contribute to the supramolecular organization of basement membranes. Debber *et al.* (2002) reported that fibulin-1 gene (FBLN1) is disrupted in a reciprocal chromosomal translocation t (12;22) which is associated with a complex type of synpolydactyly.

A RGR site in gene coding for thyroglobulin precursor was located on chromosome 14. Thyroid hormones regulates metabolism and affect the homeostasis of fat deposition. The sequence coding iodoprotein (TG), producing the precursor for thyroid hormones, has been proposed as a positional and functional candidate gene for a QTL with an effect on fat deposition. Gan *et al.* (2008) identified 6 novel SNPs at the 3' flanking region of the TG gene. The SNP marker association analysis indicated that the SNP markers were significantly associated with marbling score. They prompt that theTG-gene-specific SNP is also a helpful marker for meat quality traits in future marker-assisted selection programmes in *Bos taurus*. A RGR site in gene KIF22 encoding kinesin-like protein KIF22 was located on chromosome 25. It is involved in spindle formation and the movements of chromosomes during mitosis and meiosis. It binds to microtubules and to DNA. A RGR site in gene coding for raftlin isoform X3 was located on chromosome 1. Raftlin protein is pivotal for maintenance of lipid rafts and may be involved in regulation of B cell antigen receptor mediated signaling (Saeki *et al.*, 2003). Raftlin promotes binding of double stranded RNA, activations of B cell receptors and toll like receptor 3 signaling pathways and is involved in IL 17 production to release pro inflammatory cytokines. Qu *et al.* (2017) investigated differentially expressed membrane proteins of pulmonary alveolar macrophages infected with highly pathogenic porcine reproductive and respiratory syndrome virus and its attenuated strain by label free quantitative proteomic analysis and found higher abundance of raftlin in the HP PRRSV group compared to the AP PRRSV and control groups which explain the more severe inflammation triggered by HP PRRSV.

A RGR site in gene PLCL1 coding for inactive phospholipase C-like protein 1 was located on chromosome 2. It is involved in an inositol phospholipid-

based intracellular signaling cascade. It regulates the turnover of receptors and thus contributes to the maintenance of GABA-mediated synaptic inhibition. Its aberrant expression might contribute to the genesis and progression of respiratory organ malignant neoplastic disease. Asano *et al.* (2014) investigated the role of phospholipase C-related catalytically inactive protein (PRIP) in insulin granule exocytosis using Prip-knockdown mouse insulinoma (MIN6) cells and demonstrated that PRIP regulate KIF5B-mediated insulin secretion. It was found that insulin release from Prip-knockdown MIN6 cells was higher than that from control cells.

A RGR site in gene KDM4C coding for lysine-specific demethylase 4C was located on chromosome 8. The encoded super molecule could be a trimethylation-specific demethylase and converts specific trimethylated histone residues to the dimethylated type. This enzymatic action regulates gene expression and chromosome segregation. KDM4C is induced during adipocyte differentiation and depletion of KDM4C is sufficient to block this kind of cellular differentiation (Lu *et al.*, 2012). KDM4C is amplified or upregulated in several cell lines derived from esophageal squamous carcinomas, medulloblastoma and breast cancer (Yang *et al.*, 2000, Cloos *et al.*, 2006, Ehrbrecht *et al.*, 2006, Liu *et al.*, 2009 and Uimonn *et al.*, 2014). In addition, in agreement with a contribution of KDM4C to tumor development, inhibition of KDM4C expression reduces cell proliferation (Cloos *et al.*, 2006).

A RGR site in gene coding for growth hormone receptor precursor was located on chromosome 20. Growth hormone receptor (GHR) is receptor for pituitary gland growth hormone involved in regulating postnatal body growth. On ligand binding, it activates the JAK2/STAT5 pathway. Rahmatalla *et al.* (2011) studied effect of non-synonymous single nucleotide polymorphism (SNP) in exon 8 which leads to a phenylalanine to tyrosine amino acid substitution (F279Y) in the receptor. They predicted the consequences of the F279Y mutation on milk yield, fat, protein, casein, lactose yield and content as well as somatic cell score (SCS), in a German Holstein dairy cattle population. They found that tyrosine variant occurred as the minor allele (16.5%) but its substitution effects were 320 kg (305 d), 0.02 kg per day and 0.07 kg per day for milk, casein and lactose yields, respectively. The same allelomorph had negative effects on fat, protein and casein contents. Besides this, the high-milk-yield tyrosine allele was also associated with lower SCS ($p<0.05$). They suggested that F279Y polymorphism possess high potential as a marker for the improvement of milk traits in selection programs. Qin *et al.* (2007) identified polymorphism in exon 10 of GHR in three cattle breeds (Nanyang cattle, limousin and galloway) and found correlations between GHR gene polymorphism and body size indexes in cattle. A RGR site in DOK6 gene coding for docking protein 6 was located on chromosome 24. DOK proteins are enzymatically inert scaffolding proteins. They provide a tying up platform for the assembly of multimolecular signal complexes. DOK6 promotes Ret-mediated neurite growth and may affect brain development and maintenance.

RNA-guided recombinase target sites for hyperactivated recombinase beta in bovine genome has been also explored previously in which 436 RGR target sites were identified in bovine genome for recombinase Beta with spacer length five (Pathak *et al.*, 2019).

CONCLUSION

As RGR target sites are located in several important protein coding genes, RGR platform may expand and revolutionize our ability to explore and alter the genome of livestock and hold great promise for exiting developments in the near future. Current or future generations of RNA guided recombinase may prove useful tools to cleanly delete or integrate DNA for the study or treatment of genetic diseases, or to mediate the precise exchange of genetic material during animal breeding.

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Conflict of interest

All authors declare that they have no conflict of interest.

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