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Chromatin structure analysis enables detection of DNA insertions into the mammalian nuclear genome

Challise J. Sullivan [1], Erik D. Pendleton [1], Rachel E. Abrams [1], David L. Valente, Michelle L. Alvarez, Richard H. Griffey, John Dresios *

Leidos Inc., 10260 Campus Point Drive, San Diego, CA 92121, United States

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ABSTRACT

Background: Genetically modified organisms (GMOs) have numerous biomedical, agricultural and environmental applications. Development of accurate methods for the detection of GMOs is a prerequisite for the identification and control of authorized and unauthorized release of these engineered organisms into the environment and into the food chain. Current detection methods are unable to detect uncharacterized GMOs, since either the DNA sequence of the transgene or the amino acid sequence of the protein must be known for DNA-based or immunological-based detection, respectively.
Methods: Here we describe the application of an epigenetics-based approach for the detection of mammalian GMOs *via* analysis of chromatin structural changes occurring in the host nucleus upon the insertion of foreign or endogenous DNA.
Results: Immunological methods combined with DNA next generation sequencing enabled direct interrogation of chromatin structure and identification of insertions of various size foreign (human or viral) DNA sequences, DNA sequences often used as genome modification tools (*e.g.* viral sequences, transposon elements), or endogenous DNA sequences into the nuclear genome of a model animal organism.
Conclusions: The results provide a proof-of-concept that epigenetic approaches can be used to detect the insertion of endogenous and exogenous sequences into the genome of higher organisms where the method of genetic modification, the sequence of inserted DNA, and the exact genomic insertion site (s) are unknown.
General significance: Measurement of chromatin dynamics as a sensor for detection of genomic manipulation and, more broadly, organism exposure to environmental or other factors affecting the epigenomic landscape are discussed.

1. Introduction

Recombinant DNA technology allows the modification of particular characteristics of animals, plants, or microbes by introducing selected segments of genetic material from other, sometimes non-related, organisms. According to the World Health Organization, genetically modified organisms (GMOs) are defined as organisms in which the DNA has been altered in a way that does not occur naturally by mating and/or natural recombination [24]. Genetically engineered animals represent a pioneering technology with various applications in biomedicine, through the production of various proteins, drugs, vaccines, and tissues for human use; in agriculture, through the generation of more efficient and disease-resistant livestock; and in diet, through enhancement of the quality and reduction in the cost of food production [25]. Given these benefits of GMOs, it is necessary to develop accurate and sensitive methods to detect, track, and assess the authorized and unauthorized release of GMOs into the environment and into the food chain [7,19]. Additionally, the development of such detection methods is a prerequisite for reliable identification and control of engineered organisms that create risks to the food supply and to human health (*e.g.* agroterrorism).

Commonly used methods cannot detect uncharacterized genetically engineered organisms, since either the DNA sequence of the transgene or the amino acid sequence of the protein must be available for DNA- (*e.g.* PCR, probe magnetic capture/spectroscopy, microarrays) or immunochemical- (*i.e.* monoclonal and polyclonal antibodies) based detection, respectively [15,17,18,2,22,28,5,9]. Similarly, designing DNA microarrays for GMO surveillance would be challenging given the extreme diversity of genomic sequences among organisms and the great variety of molecular tools that can

* Corresponding author.
E-mail address: john.dresios@leidos.com (J. Dresios).
[1] These authors contributed equally to this work.

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be used for production of GMOs [8].

We propose that insertion of DNA sequences into a host genome causes remodeling of the chromatin structure by altering the interactions between histone proteins and DNA sequences around the inserted elements. Such chromatin structure changes could influence gene expression by modifying both short- and long-range regulatory interactions, therefore leading to alterations in protein expression, and eventually to a desired physiological outcome. To this end, we applied genome-wide chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) technology to characterize DNA–histone interactions for the identification of molecular signatures corresponding to insertion of endogenous or exogenous DNA elements into the mouse genome. The results provide a proof of concept that chromatin mapping technologies can be used to detect the insertion of DNA sequences into the genome of a higher mammalian organism.

2. Materials and methods

2.1. Sample selection

Muscle tissue samples from wild-type and genetically modified mice (Jackson Laboratory, Bar Harbor, ME, USA) were of FVB/NJ genetic background, were all male, and were ~8 weeks old. Wild-type (stock# 001800) and three GMO samples were selected for testing. Genome alterations present in the GMO mouse samples included insertions of various size endogenous (mouse) or foreign (*e.g.* human, viral) DNA elements that were incorporated into the host genome using various genome modification tools (*e.g.* viral sequences, transposons) and engineering methods (*e.g.* embryonic stem cells transformation or pronuclear injection). A list of the samples used in this study along with their genomic modifications and size of DNA insertion is shown in Table 1. The composition of transgenic insertions is illustrated in Fig. 1.

GMO sample 1 (stock# 018304; [4]) was created *via* the microinjection method and contained an inserted transgene holding the human alpha-skeletal actin (*ACTA1*) promoter sequence, the full-length human tropomyosin-3 cDNA sequence (*TPM3*) and a cassette containing the simian virus 40 (SV40) small t-antigen (tAg) intron and 3′UTR (Fig. 1A). GMO sample 2 (stock# 012460; [6]) was created *via* the microinjection method and contained an insertion of 40 copies of a DNA sequence cassette consisting of the entire coding region of the mouse GTP-binding proteins class Gq protein subunit Gaq (*Gnaq*) gene, under control of a mouse alpha-myosin heavy chain (*Myh6*) promoter, followed by a SV40 intron and a polyadenylation signal (polyA) (Fig. 1B). GMO sample 3 (stock# 017594; [32]) was created *via* co-injection of two transgenes using the Sleeping Beauty (SB) transposon approach. The first transgene contains a left and right inverted repeat/direct repeat sequence (IR/DR) known as the SB transposon recognition site, a mouse tyrosinase (*Tyro*) enhancer sequence, and Tyro minigene (*TyBS*). The second transgene has the mouse protamine 1 (*Prm1*) promoter, a 25 bp linker, SB10 gene, and a rabbit β-globin splice/polyA sequence (Fig. 1C).

2.2. Chromatin immunoprecipitation (ChIP-seq)

ChIP-seq experiments were performed in agreement with the guidelines set forth by the ENCODE project [14]. Three histone antibodies were used for the chromatin immunoprecipitation experiment, specifically: H3K4me3 (Millipore, MA) which binds to active promoters, H3K36me3 (AbCam, MA) which binds to active exon boundaries, and H3K4me1 (AbCam, MA) which binds to active enhancers [11]. An optimized protocol for isolation of nuclei from skeletal muscle tissue was developed and was based on a previously published method [26]. Briefly, minced skeletal muscle was cross-linked with formaldehyde and nuclei were prepared for chromatin immunoprecipitation according to the chromatin shearing (sonication) method described by [21]. Chromatin samples were incubated with each histone antibody and immunoprecipitated using Protein A/G Magnetic Beads (Thermo Fisher Scientific, MA) according to the product specifications. Samples were reverse cross-linked and purified using a PCR Purification Kit (Qiagen, MD), followed by quantification using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, CA). The DNA fragment size range was determined using the Bioanalyzer High Sensitivity ChIP (Agilent Technologies, CA).

Validation of the DNA–histone immunoprecipitation reactions was conducted by quantitative PCR using SYBR FAST qPCR Master Mix (KAPA Biosystems, MA) and positive control primers (IDT, IA) designed to bind specific genomic regions known to be immunoprecipitated by the three tested antibodies. Specifically, amplification of the following gene regions was verified before sequencing was initiated: Actg1 and Actb for H3K4me3, Actg1 and Elf1 for H3K36me3, and Actg1, Elf1, and Gapdh for H3K4me1.

Samples were prepared for multiplex sequencing following the Illumina ChIP-seq Library Prep Kit (Illumina, CA). Sequencing libraries were quantified with Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, CA) and Illumina/Universal Quantification Kit (KAPA Biosystems, MA), and DNA fragment sizes were determined using the Bioanalyzer High Sensitivity ChIP (Agilent Technologies,

Table 1
Wild-type and genetically modified organism (GMO) mice samples used in this study.

Sample	Genetic background	Gender	Age	Engineering approach	Endogenous (mouse) insertions	Exogenous (human, viral, etc.) insertions	Total insertion size (~kb)
Wild-type ($n=4$)	FVB/NJ	Male	8 wks	N/A	N/A	N/A	N/A
GMO sample 1 (#018304)	FVB/NJ	Male	8 wks	Microinjection	N/A	Human *ACTA1* promoter Human *TPM3* cDNA SV40 tAg Intron & 3′UTR	40
GMO sample 2 (#012460)	FVB/NJ	Male	8 wks	Microinjection	Mouse *Myh6* promoter Mouse *Gnaq* cDNA	SV40 Intron & poly(A)	200
GMO sample 3 (#017594)	FVB/NJ	Male	8 wks	Sleeping Beauty transposon	Transgene A: Mouse *Tyro* enhancer Mouse *Tyro* minigene Transgene B: Mouse *Prm1* promoter	Transgene A: Left and right IR/DR Transgene B: Linker SB10 gene Rabbit β-globin splice/poly (A)	8 2