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Efficient isolation of specific genomic regions by insertional chromatin immunoprecipitation (iChIP) with a second-generation tagged LexA DNA-binding domain

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ABSTRACT

Comprehensive understanding of mechanisms of epigenetic regulation requires identification of molecules bound to genomic regions of interest *in vivo*. We have developed a novel method, insertional chromatin immunoprecipitatin (iChIP), to isolate specific genomic regions retaining *in vivo* molecular interaction in order to perform non-biased identification of interacting molecules. Here, we developed a second-generation tagged LexA DNA-binding domain, 3×FNLDD, for the iChIP analysis. 3×FNLDD consists of 3 × FLAG tags, a nuclear localization signal (NLS), the DNA-binding domain (DB) and the dimerization domain of the LexA protein. Expression of 3×FNLDD can be detected by immunoblot analysis as well as flowcytometry. We showed that iChIP using 3×FNLDD is able to consistently isolate more than 10% of input genomic DNA, several-fold more efficient compared to the first-generation tagged LexA DB. 3×FNLDD would be a useful tool to perform the iChIP analysis for locus-specific biochemical epigenetics.

Keywords: Insertional Chromatin Immunoprecipitation; iChIP; LexA; FLAG Tag

1. INTRODUCTION

Epigenetic regulation of eukaryotic genomic regions is mediated by molecular complexes in the context of chromatin [1]. We recently developed insertional chromatin immunoprecipitation (iChIP), which is a method to biochemically isolate a genomic region of interest which retains molecular interaction [2]. The scheme of iChIP is as follows: 1) A repeat of the recognition sequence of an exogenous DNA-binding protein such as LexA is inserted into the genomic region of interest in the cell to be analyzed. 2) The DNA-binding domain (DB) of the exogenous DNA-binding protein is fused with a tag(s) and a nuclear localization signal (NLS)(s) and expressed into the cell to be analyzed. 3) The resultant cell is stimulated, if necessary, and crosslinked with formaldehyde or other crosslinkers. 4) The cell is lysed, and the crosslinked DNA is fragmented by sonication. 5) The complexes including the exogenous DB are immunoprecipitated with an antibody against the tag. 6) The isolated complexes retain molecules interacting with the genomic region of interest. Reverse crosslinking and subsequent purification of DNA, RNA, proteins, or other molecules allows their identification and characterization. By using iChIP, we succeeded in directly identifying protein and RNA components of an insulator, which functions as boundaries of chromatin domains [3], showing that iChIP is a powerful tool for elucidation of molecular mechanisms of epigenetic regulation. In order to easily identify molecular interaction *in vivo*, however, more efficient isolation of specific genomic regions of interest is necessary.

To this end, here, we developed a second-generation tagged LexA DNA-binding domain, 3×FNLDD, to perform the iChIP analysis more efficiently. 3×FNLDD consists of 3 × FLAG tags, an NLS, DB and the dimerization domain of the LexA protein. Expression of 3×FNLDD can be detected by immunoblot analysis as well as flowcytometry. We showed that iChIP using 3×FNLDD is able to consistently isolate more than 10% of input of specific genomic regions, several-fold more efficient compared to the first-generation tagged LexA DB. 3×FNLDD would be useful for the iChIP analysis of specific genomic regions to perform their biochemical analysis.

2. MATERIALS AND METHODS

2.1. Plasmid Construction

To construct 3×FNLDD/pCMV-7.1, the DNA sequence encoding NLS-LexA DB was cleaved from FCNLD/pMIR [2] with *Bam*H I and *Not* I, blunted, and inserted

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into p3XFLAG-CMV-7.1 (Sigma-Aldrich) that was cleaved with *Bgl* II and blunted to generate 3×FNLD/pCMV-7.1. Subsequently, the DNA sequence encoding DB and the dimerization domain of LexA was amplified with the LexA-N (26081) (5'-ccctttCCTGAGGGAATG AAAGCGTTAACG-3') and LexA-C w/D (26628) (5'-aa atgtcgaCTACAGCCAGTCGCCGTTGCG-3') primers using pBTM116 [4] as template. The PCR product was cleaved with *Mlu* I and *Sal* I and inserted into *Mlu* I- and *Sal* I-cleaved 3×FNLD/pCMV-7.1 to generate 3×FNLDD/pCMV-7.1.

To construct pMXs-I2, the coding sequence of enhanced green fluorescent protein (EGFP) of pMXs-IG [5] was replaced with that of human CD2 antigen (hCD2) [6].

To construct 3×FNLDD/pMXs-I2, the DNA sequence encoding 3×FNLDD was cleaved with *Sac* I and *Sal* I, blunted, and inserted into the pMXs-I2 vector that was cleaved with *Eco*R I and *Not* I and blunted.

All PCR-derived DNA sequences were verified by DNA sequencing.

2.2. Cell Lines

293T was maintained in DMEM supplemented with 10% fetal calf serum (FCS). Ba/F3 [7]-derived cells were maintained in RPMI-1640 supplemented with 10% FCS, 10 mM Hepes (pH 7.2), 1 × non-essential amino acid, 1 mM sodium pyruvate, 5 nM 2-mercaptoethanol, 1 ng/ml interleukin-3.

1×10^7 of Ba/F3 were transfected with *Mlu* I-digested pGL3C-Neo-cHS4c × 24-LexA × 2 [3] (100 μg) together with the hygromycin resistance gene (3 μg) by electroporation using Gene Pulser II (Bio-Rad) at 250 V, 975 μF. The transfected cells were selected in the presence of hygromycin (1 mg/ml) to establish the cHS4-core-1.2k cell line. Subsequently, 1×10^7 of cHS4-core-1.2k were transfected with *Sca* I-digested 3×FNLDD/pCMV-7.1 (100 μg) or *Hind* III-digested FCNLD/pEF (100 μg) together with the puromycin resistance gene (3 μg) by electroporation. The transfected cells were selected in the presence of hygromycin (1 mg/ml) and puromycin (0.6 μg/ml) to establish the 3×FNLDD/cHS4-core-1.2k or FCNLD/cHS4-core-1.2k cell line.

2.3. Immunoblot Analysis

Nuclear extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Immunoblot analysis was performed as described before [6]. Anti-FLAG M2 Ab was purchased from Sigma-Aldrich.

2.4. Flowcytometry

2 μg of pMXs-I2 or 3×FNLDD/pMXs-I2 was transfected into 1×10^6 of 293T cells with Lipofectamine 2000 (Invitrogen). Two days after transfection, cells were harvested and stained with phycoerythrin (PE)-conjugated anti-hCD2 Ab (BD Biosciences). Subsequently, cells were intracellularly stained with fluorescein isothiocyanate (FITC)-conjugated anti-FLAG M2 (Sigma-Aldrich) using the Fixation/Permeabilization and Permeabilization buffer set (eBioscicence). Flowcytometric analysis was performed on FACS Calibur (BD Biosciences), and data was analyzed with FlowJo software (TreeStar).

2.5. Chromatin Preparation and iChIP

Cells (4×10^6) were fixed with 1% formaldehyde at 37˚C for 5 min. The chromatin fraction was extracted and fragmented (2 kbp-long on average) by sonication and subjected to iChIP as described previously [3]. The DNA purified by phenol-chloroform extraction and ethanol precipitation was used as a template for real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) using the Applied Biosystems 7900HT Fast Real-Time PCR System. PCR cycles were as follows: heating at 50˚C for 2 min followed by 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The primers used in this experiment are LexA-N2 (26572) (5'-ttctctatcgataggtacctcg-3') and LexA-C (26573) (5'-tct attcagcggatctcgagcg-3').

3. RESULTS AND DISCUSSION

3.1. Generation of the Expression System of 3×FNLDD

For iChIP analysis, we have used the FCNLD protein [2] consisting of 2 × FLAG tags, a tobacco etch virus (TEV) protease cleavage site, calmodulin-binidng peptide, the NLS of SV40-T-antigen, and LexA DB. To generate more efficient tagged LexA proteins for iChIP analysis, we constructed a plasmid expressing the 3×FNLDD protein consisting of 3 × FLAG tags, the NLS of SV40-T-antigen, and DB as well as the dimerization domain of LexA (**Figure 1(a)**). To increase efficiency of immunoprecipitation, 3 × FLAG tags were used instead of 2 × FLAG tags. In addition, we removed the TEV protease cleavage site and calmodulin-binding peptide because efficiency of cleavage by TEV was low in crosslinked chromatin (data not shown).

The pMXs-I2 vector expressing hCD2 or 3×FNLDD/pMXs-I2 bicistronically expressing 3×FNLDD and hCD2 was transfected into 293T cells. Nuclear extracts were prepared and subjected to immunoblot analysis using anti-FLAG Ab. As shown in **Figure 1(b)**, expression of 3×FNLDD was detected.

Next, we examined expression of 3×FNLDD in individual cells by flowcytometry. 293T cells transfected with pMXs-I2 or 3×FNLDD/pMXs-I2 were stained with PE-conjugated anti-hCD2 Ab and subsequently intracel-

lularly stained with FITC-conjugated anti-FLAG Ab. As shown in **Figure 1(c)**, expression of 3×FNLDD was clearly detected on hCD2 (+) cells.

3.2. Efficient Isolation of Specific Genomic Regions by iChIP with 3×FNLDD

Next, we examined efficiency of iChIP with 3×FNLDD. To this end, we first established a cell line, cHS4-core-1.2k, by transfection of the pGL3C-Neo-cHS4c × 24-LexA × 2 plasmid possessing 2 copies of the cHS4c × 12-LexA cassette, in which 8 × repeats of the LexA DNA-binding sequence was flanked at each side by six copies of the core sequence of chicken HS4 insulator (cHS4-core) (**Figure 2(a)**). The cHS4-core-1.2k cell line retained about 100 copies of the cHS4c × 12-LexA cassette in the genome (data not shown), thus about 1200 copies of the cHS4-core sequence are integrated in the genome. We subsequently transfected the FCNLD/pEF or the 3×FNLDD/pCMV-7.1 plasmid into the cHS4-core-1.2l cell line to express FCNLD or 3×FNLDD, respectively. The chromatin fraction was prepared from the stable cell lines expressing FCNLD or 3×FNLDD and subjected to iChIP for isolation of cHS4-core as a target region. Isolation efficiency of cHS4-core was evaluated by detection of the LexA-binding elements in real-time PCR (**Figure 2(a)**). Multiple clones expressing FCNLD or 3×FNLDD were analyzed to obtain clones showing high iChIP efficiency. **Figure 2(b)** shows % input of the LexA-binding elements purified by iChIP using a representative FCNLD- or 3×FNLDD-expressing clone. iChIP with anti-FLAG Ab using the FCNLD-expressing clone showed 2.9% of the input DNA, which is consistent with the data we reported previously [2]. In contrast, the 3×FNLDD-expressing clone showed 11.3% of the input, which is several-fold higher than the FCNLD-expressing clone. We observed consistent results using clones established independently (data not shown). In contrast, % input of the promoter region of the *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) gene, a negative control genomic region, was less than 0.1% in 3×FNLDD-expressing clones (data not shown), showing that backgrounds of iChIP with 3×FNLDD are low. These data indicate that 3×FNLDD would immunoprecipitate the target sequence more efficiently than FCNLD and be useful for efficient isolation of specific genomic regions by iChIP. Increase in the number of the FLAG tag sequence may contribute to increase in efficiency of immunoprecipitation. In addition, addition of the dimerization domain of LexA would help form stable dimers to increase binding affinity/avidity to the LexA elements.

4. CONCLUSIONS

In this study, we generated a second generation tagged

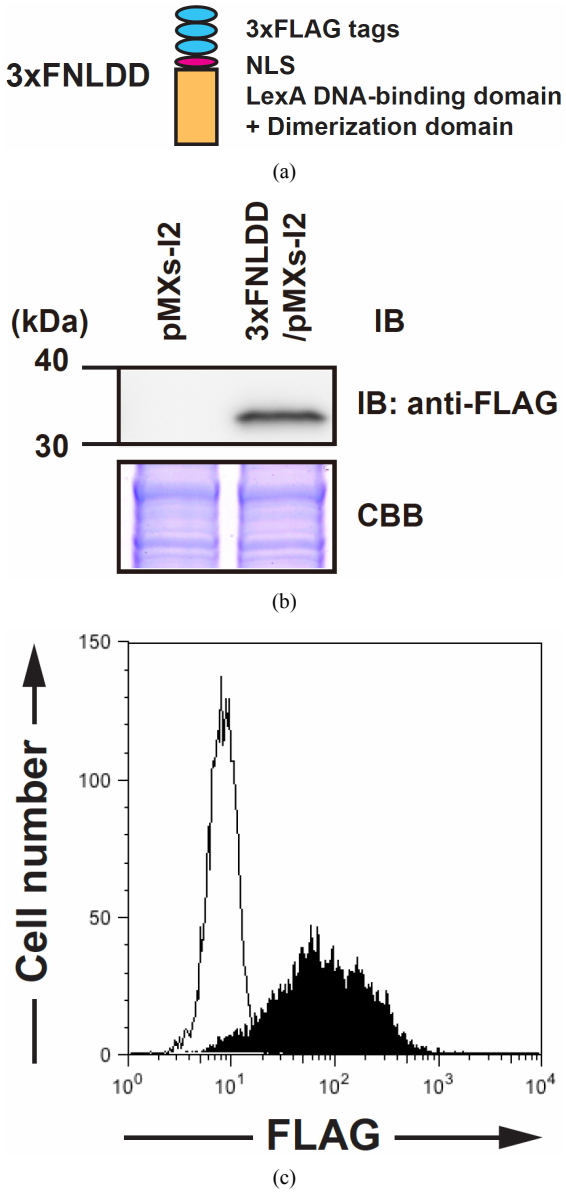

Figure 1. Scheme of 3×FNLDD. (a) 3×FNLDD consists of 3 × FLAG tags, a nuclear localization signal (NLS), the DNA-binding domain (DB) and the dimerization domain of the LexA protein; (b) Expression of 3×FNLDD in 293T cells. Nuclear extracts were subjected to immunoblot analysis with anti-FLAG Ab. The Coomassie Brilliant Blue staining (CBB) is shown as a protein loading control; (c) Flowcytometric detection of 3×FNLDD. pMXs-I2 or 3×FNLDD/pMXs-I2 was transfected into 293T cells with Lipofectamine 2000. Two days after transfection, cells were harvested and stained with PE-conjugated anti-hCD2 Ab. Subsequently, cells were intracellularly stained with FITC-conjugated anti-FLAG M2. hCD2 (+) cells were gated to quantify expression levels of 3×FNLDD. Open: pMXs-I2-trans-fected cells; solid: 3×FNLDD/pMXs-I2-transfected cells. Mean fluorescent intensity of FLAG: pMXs-I2, 8.48; 3×FNLDD/pMXs-IG, 122. Mean fluorescent intensity of hCD2: pMXs-IG, 623; 3×FNLDD/pMXs-IG, 636.

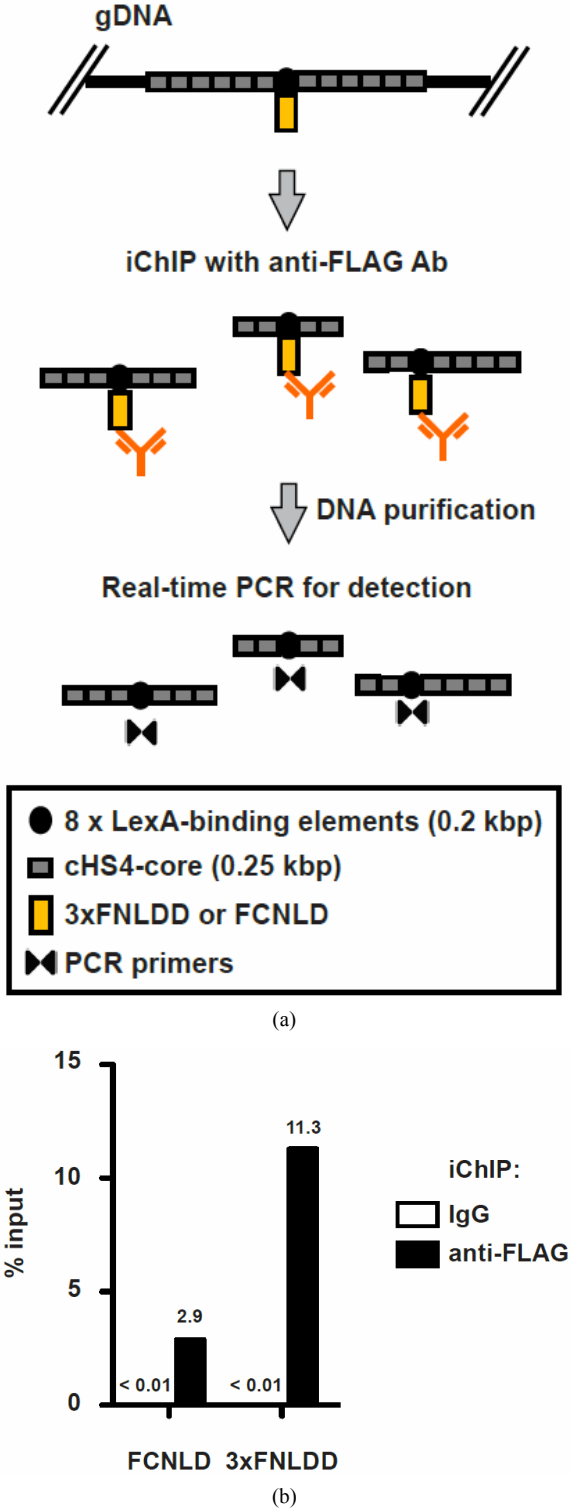

Figure 2. Efficient isolation of specific genomic regions by iChIP with 3×FNLDD. (a) Scheme of iChIP in this study. The cHS4c × 12-LexA regions integrated into the genome of Ba/F3 cell line were isolated by iChIP with anti-FLAG Ab and analyzed by real-time PCR with LexA primers; (b) Quantification of the amounts of the immunoprecipitated regions by real-time PCR.

LexA, 3×FNLDD, for more efficient iChIP analysis. By using 3×FNLDD, we were able to isolate target genomic regions much more efficiently than by using FCNLD. % input by using 3×FNLDD reached more than 10%, which would enable biochemical analysis of specific genomic regions much easier.

In addition, we showed that expression of 3×FNLDD can be detected and quantified by flowcytometry. Flowcytometric detection of 3×FNLDD would be useful in easy quantification of its expression levels in individual cells. Taken together, 3×FNLDD would be a useful tool for iChIP analysis of specific genomic regions.

5. ACKNOWLEDGEMENTS

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