Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–4, 2016

www.elsevier.com/locate/jbiosc

Enhanced transgene expression by plasmid-specific recruitment of histone acetyltransferase

Mika Nishihara,[1,2,‡] Genki N. Kanda,[2,3,‡] Tetsuya Suzuki,[1,‡] Shin'ichiro Yamakado,[2] Hideyoshi Harashima,[3] and Hiroyuki Kamiya[1,2,3,*]

Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan,[1] Graduate School of Science and Engineering, Ehime University, 2-5 Bunkyo-cho, Matsuyama 790-8577, Japan,[2] and Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan[3]

Received 22 June 2016; accepted 14 September 2016
Available online xxx

Histone acetylation is associated with the activation of genes on chromosomes. Transgene expression from plasmid DNA might be increased by the acetylation of histones bound to plasmid DNA. To examine this hypothesis, we employed a positive feedback system, using a fusion protein of the sequence-specific DNA binding domain of yeast GAL4 and the histone acetyltransferase (HAT) domain of mouse CREB-binding protein (GAL4-HAT), in which GAL4-HAT promotes its own expression as well as that of a reporter gene product (luciferase). The activator plasmid DNA carrying the gene encoding GAL4-HAT was introduced into mouse Hepa1-6 cells, together with the reporter plasmid DNA, by lipofection. Significantly increased luciferase expression was observed by the co-introduction of the activator plasmid DNA. Moreover, the acetylation of histones bound to the reporter plasmid DNA was enriched by the activator plasmid DNA. These results indicated that the GAL4-HAT system is useful for enhanced transgene expression.

[**Key words:** Transgene expression; Artificial histone acetyltransferase; Plasmid DNA; Chromatin immunoprecipitation; Histone deacetylase inhibitor]

Mammalian chromosomal DNA binds to various proteins and the DNA-protein complexes form DNA packaging units, called nucleosomes. Histones are the chief protein components of nucleosomes and perform pivotal functions in chromosomal gene regulation. Moreover, many types of chemical modifications of histones, such as acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation, are believed to play important roles in the modulation of chromatin function (histone code hypothesis) (1). Among them, acetylation is a well-characterized modification. Hyperacetylated and hypoacetylated histones are regarded as hallmarks of nucleosomes at active and inactive genes, respectively (2–5). Acetylation may affect gene expression by modifying the chromatin conformation and/or the recruitment of regulatory factors.

Nucleosomes are also formed on non-integrated plasmid DNAs delivered by nonviral vectors, indicating that plasmid DNAs bind histones in the nuclei (6,7). In agreement with these findings, transgene expression was influenced by the introduction of DNA sequences that modulate histone positioning into plasmid DNAs (8–12). Thus, the binding of histones to plasmid DNA is a key factor for the intranuclear disposition of exogenous DNA and efficient transgene expression (13). Based on the dynamics of histones that bind to chromosomal DNA, those bound to plasmid DNA could also be chemically modified. This hypothesis suggests that transgene expression might be regulated by altering the histone modification patterns. For instance, the acetylation of histones that bind to plasmid DNA might upregulate transgene expression.

Histone acetylation is controlled by two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (14–16). Inhibitors of HDACs are used for the enrichment of acetylated histones in cells. Treatments with HDAC inhibitors increased transgene expression from episomal/integrated plasmid DNAs and adenoviral DNA, as well as that in cell lines transduced by a lentivirus (17–20). These reports strongly support the idea that the acetylation of histones that bind to plasmid DNA enhances transgene expression. However, the treatments with HDAC inhibitors result in the acetylation of histones bound to chromosomal DNA and affect genome-wide gene expression. Thus, plasmid DNA-specific histone acetylation is required.

We previously described efficient transgene expression from plasmid DNA, using artificial transcription factors that bind to their recognition sites within the plasmid DNA (21–23). In this system, a fusion protein of the sequence-specific DNA binding domain of yeast (*Saccharomyces cerevisiae*) GAL4 and the transcription activation domains of viral and mammalian transcription factors were used. The reporter plasmid DNA contains five tandem copies of the 17-bp GAL4 DNA binding site (G5) in both the upstream and downstream regions of the luciferase gene expression cassette. The G5 sequences are also present in both the upstream and downstream regions of the GAL4-transcription factor expression cassette in the activator plasmid DNA. As a result, the expression of both the reporter and activator genes was strongly promoted by the plasmid-specific transcription factors. We noticed that the

* Corresponding author at: Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan. Tel.: +81 82 257 5300; fax: +81 82 257 5334.
E-mail address: hirokam@hiroshima-u.ac.jp (H. Kamiya).
‡ The first three authors contributed equally to this work.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.09.008

replacement of the transcription factor with HAT achieved plasmid DNA-specific histone acetylation by the artificial HAT, and consequently increased transgene expression.

In this study, we constructed an activator plasmid DNA containing the gene encoding a fusion protein of GAL4 and the HAT domain of mouse CREB-binding protein (CREBBP) (amino acid residues 1092–1764) (24). We introduced the activator GAL4-HAT and the reporter luciferase plasmid DNAs into mouse Hepa1-6 cells by lipofection. The expression of the activator plasmid DNA enriched the acetylated histones bound to the reporter plasmid and efficiently acted as an activator. These results indicated that this strategy is useful to promote transgene expression.

MATERIALS AND METHODS

Materials Oligodeoxyribonucleotides were obtained from Sigma Genosys Japan (Ishikari, Japan), Fasmac (Atsugi, Japan), and Eurofins Genomics (Tokyo, Japan) in purified forms. The pBluescript II SK(+) plasmid was obtained from Agilent Technologies (Santa Clara, CA, USA). The pG5-ALB-luc-G5 plasmid (21), containing the luciferase gene driven by the mouse albumin promoter and two G5 sequences, was used as the reporter plasmid (Fig. 1a).

Cell culture Hepa1-6 cells were provided by the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of the MEXT, Japan, and maintained in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B, at 37 °C in a humidified atmosphere with 5% CO_2.

Construction of plasmid DNA cDNA was prepared from total RNA isolated from BALB/c mouse kidney. The DNA fragment corresponding to mouse CREBBP (1092–1764) was amplified with high fidelity KOD FX DNA polymerase (Toyobo, Osaka, Japan). The primers used were Nd-HAT FW2 (5′-dAAGCACATCTAGACATATGCGCCAGGCACTTATGCCAA-3′) and HAT-N RV2 (5′-dTATTCAGGTACCTCGCCGGCGGCTCTTGGACTGTGGCTCA-3′). The amplified PCR fragment was ligated with the large Nde I-Not I fragment of the pG5-ALB-GAL4-p53-G5 plasmid (23), using a GeneArt Seamless Cloning and Assembly kit (Thermo Fisher Scientific, Waltham, MA, USA), to yield the pG5-ALB-HAT-G5 plasmid (Fig. 1a).

Reporter assay Hepa1-6 cells (4×10^4 cells) were plated onto 24-well dishes 24 h before transfection. The reporter and activator plasmid DNAs (30 fmol each) were mixed with an appropriate amount of the pBluescript II SK(+) plasmid DNA, to keep the total amount of DNA constant (400 ng). Transfection into Hepa1-6 cells was performed with the Lipofectamine Reagent (Thermo Fisher Scientific), according to the supplier's instructions. When necessary, trichostatin A (TSA) (final concentration of 250 nM) was added to the medium after 24 h. The luciferase activity was measured with a Luciferase Assay System (Promega, Madison, WI, USA) at 48 h after transfection.

Quantification of intranuclear plasmid DNA Extraction of DNA after isolation of the nuclei was conducted at 48 h after transfection and quantitative PCR (qPCR) was performed using the Ad5-Luc (+) and Ad5-Luc (−) primers (their base sequences are shown below), as described previously (23).

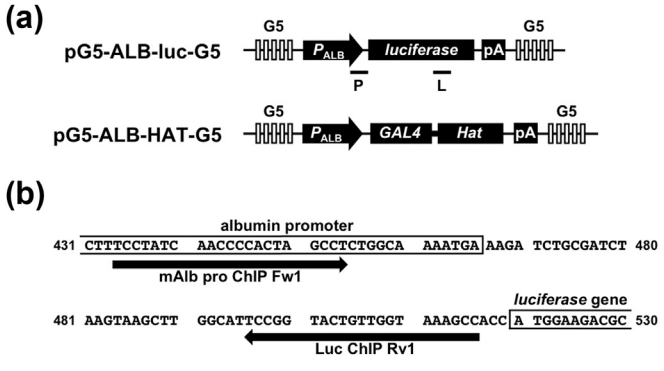

FIG. 1. (a) Schematic diagrams of the reporter and activator plasmid DNAs used in this study. The white box indicates the 17-bp GAL4 binding site, and P_{ALB} and pA refer to the mouse albumin promoter and the SV40 polyA signal, respectively. Bars under the diagram of the pG5-ALB-luc-G5 plasmid DNA correspond to the downstream region of the promoter (P) and a part of the luciferase-coding region (L), amplified in the PCR experiments. (b) Base sequence of the downstream region of the promoter in the pG5-ALB-luc-G5 plasmid, including the region amplified in the ChIP experiments. The two primers used in the amplification are indicated by arrows.

Chromatin immunoprecipitation assay Hepa1-6 cells (2×10^5 cells) were plated onto 6-well dishes 24 h before transfection. Transfection was performed as described above, using five-fold amounts of DNAs and other reagents. After 48 h, the transfected cells were treated with 1% formaldehyde for 5 min at room temperature. The chromatin immunoprecipitation (ChIP) assay was performed using an EpiScope ChIP kit (anti-mouse IgG) (Takara, Otsu, Japan), according to the supplier's instructions. An anti-acetyl histone H3(Lys9/27) mouse monoclonal antibody (catalog number: MABI0310, Medical and Biological Laboratories, Nagoya, Japan) was used for the immunoprecipitation.

The amounts of immunoprecipitated DNA were quantified by qPCR. Primer sets, mAlb pro ChIP Fw1 (5′-dTCCTATCAACCCCACTAGCCT-3′) plus Luc ChIP Rv1 (5′-dGGCTTTACCAACAGTACCGGA-3′) (Fig. 1b) and Ad5-Luc (+) (5′-dGGTCCTATGATTATGTCCGGTTATG-3′) plus Ad5-Luc (−) (5′-dATGTAGCCATCCATCCTTGTCAAT-3′) were used for amplification of the promoter region and the luciferase coding region, respectively (Fig. 1a). The precipitation ratio (pull-down/input) was calculated by determining the amounts of precipitated DNA relative to the input DNA.

Statistical analysis Statistical significance was examined by the Student's *t*-test. Levels of $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Enhanced luciferase expression by the activator plasmid DNA carrying the gene encoding GAL4-HAT We used the plasmid DNA carrying the gene encoding a fusion protein of the sequence-specific DNA binding domain of yeast GAL4 and the HAT domain of mouse CREBBP as the activator (pG5-ALB-HAT-G5, Fig. 1a). The activator and reporter (pG5-ALB-luc-G5) plasmids contain the 17-bp GAL4 binding sequences. The GAL4-HAT protein produced in the cells would specifically bind to the plasmid DNAs and acetylate the nearby histones. This would result in the specific acetylation of the histones bound to the plasmid DNAs.

The reporter pG5-ALB-luc-G5 plasmid DNA was transfected into Hepa1-6 cells, along with the activator pG5-ALB-HAT-G5 plasmid DNA. As a control, the pBluescript II SK(+) plasmid DNA, which lacks DNA elements functioning in mammalian cells, was co-introduced with the reporter plasmid DNA.

As shown in Fig. 2a, the co-transfection of the pG5-ALB-luc-G5 and pG5-ALB-HAT-G5 plasmid DNAs enhanced the transgene expression (open and closed columns). The production of luciferase was increased approximately 50-fold, relative to the pG5-ALB-luc-G5 plasmid alone. Thus, the pG5-ALB-HAT-G5 plasmid actually activated the transgene expression from the reporter plasmid.

Amounts of reporter plasmid DNA We expected that the enhanced luciferase expression would be due to the recruitment of the GAL4-HAT protein to the G5 sites in the luciferase plasmid, resulting in the acetylation of histones bound to the plasmid. However, the expression might be increased by the higher nuclear delivery and/or the enhanced stability of the reporter plasmid DNA (25) in the presence of the activator plasmid DNA. To exclude these possibilities, the amounts of the reporter plasmid DNA in the cells were measured by qPCR after the isolation of the nuclei. As shown in Fig. 2b, the amounts were almost the same in the experiments with and without the activator plasmid DNA. Thus, the upregulated luciferase expression induced by the activator plasmid DNA was due to the increased expression efficiency per single molecule of the reporter plasmid DNA.

Effects of histone deacetylase inhibitor Expression from transgenes on integrated lentiviral and integrated/episomal plasmid DNAs is promoted upon treatment with HDAC inhibitors, which enrich acetylated histones (17,18). We surmised that the effects of an HDAC inhibitor would be weak for the co-introduction of the activator and reporter plasmids, when the histones were sufficiently acetylated by the artificial HAT. Meanwhile, the effects would be evident for the introduction of the luciferase plasmid alone.

We treated the transfected cells with an HDAC inhibitor, trichostatin A (TSA), to examine this possibility (26). As shown in Fig. 2a, the TSA treatment enhanced the luciferase expression by 11-fold for the transfection without the activator plasmid. This

Please cite this article in press as: Nishihara, M., et al., Enhanced transgene expression by plasmid-specific recruitment of histone acetyltransferase, J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.09.008