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Abstract

We studied acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 within chromatin surrounding ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes in four clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of chicken HDAC2(-/-) DT40 mutants at the early, middle and late cultivation stages. Acetylation levels of these Lys residues of the five genes were high in DT40 cells. In clone cl.2-1, acetylation levels of one or more of these Lys residues of Pax5, Aiolos and EBF1 genes were dramatically decreased at the early stage and remained unchanged until the late stage, and those of the OBF1 gene were drastically decreased until the late stage. In clones cl.2-2 and cl.2-4, acetylation levels of Pax5, Aiolos and EBF1 genes were dramatically decreased at the early stage, and those of the former two were increased until the late stage but those of the latter one remained unchanged. In clone cl.2-6, acetylation levels of Pax5, Aiolos and EBF1 genes were drastically decreased at the early stage and increased until the late stage. These results could explain the previous-mentioned manners for varied gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual HDAC2(-/-) clones during cultivation. We propose a hypothesis concerning distinct manners for gain of new cell function to eliminate accumulated IgM H- and L-chains in individual HDAC2(-/-) clones during cultivation. They acquire an ability to adapt themselves to new environment, through irreversible creation of chromatin structure plasticity caused by successive structural changes between tight and loose forms based on hypo- and hyper-acetylation levels of particular Lys residues of histone H3 within proximal ~2.0 kb 5'-upstream chromatin regions of corresponding genes via numerous generations, indicating that DT40 cells are pluri-potential, elastic and flexible to gain new cell function attributed to alterations in chromatin structure.

Keywords

Irreversible creation of chromatin structure plasticity; Epigenetic modifications; Gain of new cell function; Continuous cultivation; Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay; Changes in acetylation levels of Lys residues of histone H3 during cultivation; Proximal 5'-upstream chromatin regions of particular transcription factor genes

Introduction

In 1964, it was first proposed that chemical modifications of histones with acetyl and methyl groups should be of fundamental importance as to the regulation of RNA synthesis in eukaryotes [1]. Since then, the modulation of chromatin topology has been thought to be one of the most fundamental and important manners for expression of cell functions in eukaryotes. Mechanisms to modulate chromatin structure with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and so on, have been intensively studied in a variety of life science fields. Of such

epigenetic modifications of chromatin structure, acetylation and deacetylation of particular Lys residues of core histones (H2A, H2B, H3 and H4) catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) as chromatin modifying enzymes are undoubtedly major ones. For the last several decades, countless numbers of researches on the acetylation and deacetylation (and other epigenetic modifications) have been accumulated in more diverse life science fields; transcription/gene expression, DNA replication, differentiation, development, memory, pluri-potency, clinical medicine and so on [2-30].

By gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [31, 32], we have also systematically studied in vivo roles of numerous members of histones, histone chaperones, HATs, HDACs and transcription factors [33-66]. Analyses of various DT40 mutants, lacking individual members of HATs, HDACs and transcription factors, revealed that HDAC2 indirectly regulates gene expressions of IgM H- and L-chains through opposite controlling gene expressions of Pax5, EBF1, Aiolos, E2A and also OBF1 in wild type DT40 cells [41, 46, 52, 53, 55]. The majority of artificially accumulated IgM H- and L-chains exist as a native soluble form of large molecule complex with each other in endoplasmic reticulum of HDAC2-deficient DT40 mutants [46, 53].

Very recently, we reported following interesting and important phenomena by analyzing initially generated HDAC2-deficient DT40 mutants [53, 67] and Pax5-deficient DT40 mutants [68], all of which were continuously cultivated for varying long periods. Our results obtained from these initially generated HDAC2(-/-) mutants [67] and Pax5(-) mutants [68] revealed that IgM H- and L-chains artificially accumulated at the early stage of cultivation are diminished based on their decreased gene expressions attributed to altered gene expressions of various transcription factors and chromatin modifying enzymes during continuous cultivation. In addition, interestingly, our qualitative chromatin immuno-precipitation (ChIP) assay done on the initially generated HDAC2(-/-) mutants suggested that acetylation levels of Lys-9 residues of histone H3 (K9/H3) within some regional segments of chromatin surrounding proximal ~2.0 kb 5'-upstream region of the Pax5 gene are slightly decreased at the early stage and thereafter certainly increased at the late stage [67]. To further explore mechanisms to diminish the accumulated IgM H- and L-chains based on their increased gene expressions, we newly generated and analyzed HDAC2-deficient DT40 mutants HDAC2(-/-) [69]. As expected, in six tested individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants, IgM H- and L-chains are dramatically and considerably accumulated at the early cultivation stage. In addition, HDAC2(-/-) mutant cells at the early stage exist as a morphologically aggregative form, the reason for which is still unknown. Anyway, both of the accumulated immunoglobulin proteins and aggregative form should be uncomfortable (or painful) for the mutant cells, themselves. Remarkably, the artificially accumulated IgM H- and L-chains at the early stage are gradually reduced in almost similar pattern in all of the six HDAC2(-/-) mutant clones during cultivation and thereafter at the late stage reached to

comparable levels in DT40 cells [69]. In parallel or agreed with these changes, the aggregative form of all HDAC2(-/-) mutant clones at the early stage is altered at the late stage to the dispersive form, which must be comfortable (or peaceful) for them, similarly for DT40 cells [69].

Interestingly, in these six individual clones of HDAC2(-/-) mutants, mRNA levels (i.e., gene expression/transcription levels) of PCAF, HDAC7, HDAC9, Pax5, Aiolos, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1 and others change dramatically or considerably in distinct patterns during cultivation, though all of these mutant clones show almost the same changing pattern in protein and mRNA levels of IgM H- and L-chains and also in cell morphology [69]. Of these altered chromatin modifying enzymes and transcription factors, Pax5, Aiolos, EBF1 and OBF1 should be worthy of special mention as follows. In clone cl.2-1, mRNA levels of Pax5, Aiolos and EBF1, all of which down-regulate IgM H- and L-chain gene expressions [46, 58, 67, 69], are dramatically reduced at the early stage and remain unchanged during cultivation. By contrast, the mRNA level of OBF1, which probably up-regulates these two immunoglobulin gene expressions [41, 46, 69], is gradually and drastically reduced during cultivation until the late stage. In clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5, mRNA levels of Pax5, Aiolos and EBF1 are dramatically reduced at the early stage, and thereafter those of the former two are gradually elevated until the late stage but that of the latter one remains unchanged as undetectable level during cultivation. On the other hand, the mRNA level of OBF1 does not change so much in these four mutant clones at any cultivation stages. In clone cl.2-6, mRNA levels of Pax5, Aiolos and EBF1 are drastically reduced at the early stage and thereafter gradually elevated until the late stage, but that of OBF1 changes slightly in a somewhat complicated pattern during cultivation.

These findings, together, led the following interesting inference on manners for gene expressions of IgM H- and L-chains at the late stage in individual HDAC2(-/-) mutant clones [69]. The manner of clone cl.2-1 seems to be dependent on OBF1 and distinct from that of DT40 cells. The manner of clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seems to be dependent on Pax5 and Aiolos, and slightly similar to that of DT40 cells in appearance. Moreover, these four clones should be major type, since four initially generated HDAC2(-/-) mutant clones resembled them in several cellular properties [46, 67]. The manner of clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 and most similar to that of DT40 cells in appearance.

In this study, we clarified molecular mechanisms to alter gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF in four individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of HDAC2(-/-) mutants during cultivation. We developed and performed neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP; this abbreviation also means IP on notch of chromatin) assay on proximal 5'-upstream chromatin region (named as notch of chromatin) of each of the five remarkable genes in these four mutant clones. Surprisingly, acetylation levels of one or more of particular Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) separately

changed within chromatin surrounding proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes during cultivation. In addition, remarkably, changing patterns in acetylation levels of the above-mentioned four genes were distinct in the four individual HDAC2(-/-) mutant clones, although changing patterns in protein and mRNA levels of IgM H- and L-chains were almost similar in all of them. We assumed that hyper- (high) or hypo- (low or no) acetylation levels of one or more of these particular Lys residues should induce no binding or full binding ability of histone H3 to DNA, resulting in loose (open) or tight (closed) form of chromatin structure, leading to high or low (or no) transcription levels of corresponding gene(s). Based on these results and assumptions, we propose a hypothesis on mechanisms to eliminate artificially accumulated immunoglobulin proteins through irreversible creation of diverse chromatin structure plasticity surrounding proximal 5'-upstream regions of particular transcription factor genes with epigenetic modifications in distinct manners in individual clones of HDAC2(-/-) mutants via a lot of generations during continuous cultivation.

Methods

Cell cultures

HDAC2(-/-) DT40 mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6), which had been picked up at ~8 days after knockout of two HDAC2 alleles, cultivated for another some days to be ~10^6 cells/ml and stocked at -80℃ until use [69], were continuously cultivated and collected at the early (E; 3 days), middle (M; 33 days) and late (L; 58 days) stages of cultivation as described in our previous papers [67-69]. These three cultivation stages were practically counted from the first day of cultivation from the stock at -80℃. DT40 cells (W) were used as control.

Nucleotide sequence confirmation and primer preparation

For primers in the NotchIP assay, we had determined nucleotide sequences of ~4.9 kb 5'-upstream region containing proximal ~2.0 kb 5'-upstream and some distal 5'-upstream regions, and some open reading frames (coding regions or cDNA) of the Pax5 gene (GenBank accession number: LC060666) [67]. We confirmed nucleotide sequences of proximal ~2.0 kb 5'-upstream regions, some distal 5'-upstream regions and some open reading frames (coding regions or cDNA) of Aiolos, EBF1, OBF1 and PCAF genes based on database [67, 69]. A part of nucleotide sequences of the 5'-upstream region of the EBF1 gene was newly determined. To obtain PCR products of approximately 150 ~ 250 bp in length as mentioned below, we prepared numerous appropriate primers, which were designed based on the above-mentioned nucleotide sequences of Pax5, Aiolos, EBF1, OBF1 and PCAF genes, and listed them in Tables I ~ V.

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP) assay was done with ChIP assay kit (Millipore) according to the manufacturer's instruction. Briefly, HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at the early (E), middle (M) and late (L) cultivation stages and DT40 cells (W) (1 x 10^6) were cross-linked by the addition of 37% formaldehyde to a final concentration of 1% and incubated at 37℃ for 10 min, and the cross-linking reaction was stopped with one-tenth volume of 1.25 M glycine to a final concentration of 0.125 M at 37℃ for 5 min. After removing medium, cells were washed with ice-cold phosphate buffered saline (PBS) twice and resuspended in 200 μl of Lysis Buffer freshly supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Wako, Osaka, Japan) and 1 μg/ml approtinin (Sigma, St Louis, MO, USA). Successively, we used a Bioruptor UCD-250 (Cosmo Bio) at power L for 30-s pulses (30-s pause between pulses) at 4℃ 10 times to solubilize and to shear cross-linked chromatin DNA into fragments of approximately 200 ~ 1000 bp in length. The samples were centrifuged at 13,000 rpm at 4℃ for 10 min to remove cell debris from the crude chromatin lysate. Sonicated cell supernatant was diluted to 1/10-fold in Chip Dilution Buffer. For input, a portion (40 μl: 2%) of the diluted cell supernatant was kept to quantify the amount of DNA present in each of different samples at PCR step. Each of antibodies (2 μl) was added to 2 ml of the supernatant fraction and incubated at 4℃ for overnight. Incubation with 60 μl of Protein Agarose/Salmon Sperm DNA was continued for 1 hr, followed by centrifugation at 1,000 rpm at 4℃ for 1 min. The immuno-precipitated complexes were sequentially washed with each of low-salt solution, high-salt solution, LiCl solution once and TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) twice. The antibody/histone/DNA complexes were eluted from Protein Agarose beads by adding 250 μl of elution buffer (1% SDS, 0.1% $NaHCO_3$) twice, and the cross-linking was reversed by heating at 65℃ for 6.5 hr after adding 5 M NaCl to a final concentration of 0.2 M. Samples were treated with proteinase K at 37℃ for 1 hr to remove proteins. DNA was recovered by phenol/chloroform extraction and ethanol precipitation using 40 μg glycogen (NAKARAI TESQUE, INC.) as inner carrier. Antibodies used for pulldown were: anti-AcK9H3 (#07-352), anti-AcK14H3 (#07-353), anti-AcK18H3 (#07-354), anti-AcK23H3 (#07-355) and anti-K27H3 (#07-360) from Millipore. Recovered chromatin DNA fragments were subjected to PCR for 25 ~ 35 cycles, using the above-mentioned appropriate primers specific for 150 ~ 250 bp segments, corresponding to proximal plus distal 5'-upstream regions and open reading frames (coding regions or cDNA) of Pax5, Aiolos, EBF1, OBF1 and PCAF genes, respectively. The open reading frame of the chicken β-actin was used as control. The samples were simultaneously amplified using the same master reaction mixture in 25-μl scale. Experiments for immuno-precipitated and input DNA were performed in duplicate or once. PCR products were analyzed by 1.5% agarose gel electrophoresis. Immuno-precipitated chromatins treated with non-immune rabbit serum (Vector Laboratories) produced no PCR products and were used as negative control (data not shown).

Results

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay: A new method for clarifying manners to alter chromatin structure for varied alterations in gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF in individual clones of HDAC2(-/-) mutants during continuous cultivation

Among transcription factors Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1 and OBF1, whose gene expressions were altered in HDAC2(-/-) mutants during cultivation, Pax5, Aiolos, EBF1 and OBF1 should be influential candidates participating in decreases in gene expressions of IgM H- and L-chains in individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) [69]. The validity of this inference was supported by the findings that changing patterns of these factor gene expressions were anti-parallel or parallel with those of the immunoglobulin gene expressions in one or more of these six individual mutant clones. Additionally, Pax5, Aiolos and EBF1 were already reported to down-regulate gene expressions of IgM H- and L-chains in chicken DT40 cells by gene targeting techniques [46, 58, 69], and OBF1 was also suggested to up-regulate these two immunoglobulin gene expressions, since it functionally activates the chicken L-chain promoter in NIH 3T3 cells [41].

We studied how individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) differentially gain distinct manners for positive or negative gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF via a lot of generations during cultivation. To execute the project, we carried out minutely chromatin immuno-precipitation (ChIP) assay on chromatin surrounding their proximal ~2.0 kb 5'-upstream regions, besides distal 5'-upstream regions and open reading frame regions (coding regions). Because chromatin structure of the proximal 5'-upstream region should be directly and closely related to transcriptional activity of corresponding gene, regardless of the presence or absence of transcriptional elements within the region. Moreover, our previous unpublished data obtained by the dual-luciferase assay suggested that at least ~1.6 kb 5'-upstream region of the Pax5 gene was necessary for its gene expression, whereas those were still uncertain [67]. We designed appropriate primers based on nucleotide sequences of the proximal 5'-upstream, distal 5'-upstream and open reading frame regions of these five particular genes, which were cloned and determined by us or obtained from database and conformed by us (Tables I ~ V). We named this ChIP assay as neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP; this abbreviation also means IP on notch of chromatin) assay. Because all of DNA fragments amplified by PCR using appropriate primers, which were designed based on nucleotide sequences of the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of the above-mentioned genes, coincide with corresponding segments of the region and are laid overlapping to neighboring ones each other.

We systematically carried out the NotchIP assay on chromatin prepared from four individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E; 3 days), middle (M; 33 days) and

late (L; 58 days) stages of cultivation, together with wild-type DT40 cells (W). Throughout the NotchIP assay, we used five site-specific antibodies for acetylated Lys-9 (K9/H3), Lys-14 (K14/H3), Lys-18 (K18/H3), Lys-23 (K23/H3) and Lys-27 (K27/H3) residues of histone H3 as primary antibodies, since bulk acetylation levels of these five Lys residues of histone H3 were obviously altered in initially generated HDAC2(-/-) mutants during cultivation [67]. However, regarding the Pax5 gene in clone cl.2-2, we used only four site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3 and K27/H3 at the early (E) and late (L) stages of cultivation, because this case was the first attempt as the NotchIP assay, which was developed to clarify manners of the above-mentioned interesting phenomena, i.e., gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF changed in different manners in individual HDAC2(-/-) mutant clones during cultivation.

Throughout this study, we tentatively and qualitatively deduced the binding ability (capacity) of histone H3 to DNA based on acetylation levels of particular Lys residues of its N-terminal tail (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) obtained by the NotchIP assay, though which Lys residue(s) is really and/or mainly involved in the binding is still unclear. That is, hyper- (high), considerable hyper-, somewhat hyper- and hypo- (low or no) acetylation levels of one or more of these five Lys residues should qualitatively induce no, weak, less and full binding ability of histone H3 (or corresponding Lys residue(s)) to DNA, resulting in loose (open), considerable loose, somewhat loose and tight (closed) forms of chromatin structure, leading to high, considerable high, somewhat high and low (or no) mRNA (i.e., transcription/gene expression) levels of corresponding gene(s), respectively.

In this study, the cultivation stages were practically counted from the first day of cultivation from the stock at -80℃. The open reading frame of the chicken β-actin gene was used as control, since levels of its RT-PCR-amplified product (i.e., its mRNA) at any cultivation stages in all examined mutant clones were kept constant to be approximately 85 ~ 110% of the average value for DT40 cells (see Figs. 1 ~ 20). In addition, to simplify description of this article, we used “region(s)” for “chromatin region(s)”, “stage(s)” for “stage(s) of cultivation” and also “ORF region(s)” for “open reading frame(s) or coding region(s)” as abbreviations in most cases.

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on chromatin surrounding proximal 5’-upstream, distal 5’-upstream and open reading frame regions of the Pax5 gene in individual clones of HDAC2(-/-) mutants at early, middle and late stages of continuous cultivation

Because changing patterns in the gene expression of Pax5 during cultivation were different in individual clones of HDAC2(-/-) mutants [69], we carried out the NotchIP assay on chromatin surrounding proximal 5’-upstream, distal 5’-upstream and open reading frame regions of the Pax5 gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 (Figs. 1 ~ 4). Throughout the NotchIP assay, we used site-specific

antibodies for five acetylated Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) and appropriate primers designed based on nucleotide sequences of the ~4.9 kb 5'-upstream region of the Pax5 gene that was cloned from DT40 genomic DNA by us [67] and those of its open reading frame region obtained from database and confirmed by us (Table I). Regarding the proximal 5'-upstream region of positions -1923 ~ +30, we used primers for segments 1 ~ 12, all of which are laid overlapping to neighboring ones each other. In addition, we used primers corresponding to positions -4390 ~ -4235 (segment a) of the distal 5'-upstream region and positions +55 ~ +201, +223 ~ +391 and +490 ~ +588 (segments b, c and d) of the open reading frame region (cDNA). Since primers for segments b ~ d were designed based on nucleotide sequences from database, the nucleotide numbers were discontinuous from those of the distal and proximal 5'-upstream regions. PCR in the NotchIP assay was carried out twice for all of K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3.

Changing patterns in acetylation levels of these five Lys residues of histone H3 for the Pax5 gene in clone cl.2-1 during cultivation are represented in Figure 1. Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream chromatin regions (segments a and 1 ~ 12) of the Pax5 gene were high in DT40 cells (W). Surprisingly, in spite of the HDAC2-deficiency, those were almost completely decreased at the early (E) stage in clone cl.2-1 and thereafter remained unchanged via the middle (M) to late (L) stages. On the other hand, acetylation levels of K9/H3 within three ORF regions of the gene (segments b ~ d) were very low in DT40 cells (W) (see many PCR cycle numbers) and further decreased at any cultivation stages in clone cl.2-1, except insignificant change for the region of positions +223 ~ +391 (segment c). The reason for this insignificant change is still unknown. Acetylation levels of K14/H3 within the entire 5'-upstream regions of the Pax5 gene were high in DT40 cells. However, those were dramatically decreased at the early (E) stage in clone cl.2-1 and thereafter barely increased via the middle (M) to late (L) stages, except insignificant change for the region of positions -958 ~ -679 (segment 8). The reason for this insignificant change is also unknown. On the other hand, acetylation levels of K14/H3 in three ORF regions of the gene were very low in DT40 cells (see many PCR cycle numbers) and remained unchanged at any cultivation stages in clone cl.2-1. Acetylation levels of K18/H3 within the entire 5'-upstream regions of the Pax5 gene were high in DT40 cells. However, those were dramatically decreased at the early (E) stage in clone cl.2-1 and thereafter slightly increased via the middle (M) to late (L) stages, except insignificant change for the region of positions -958 ~ -679 (segment 8). On the other hand, acetylation levels of K18/H3 in three ORF regions of the gene were very low in DT40 cells (see many PCR cycle numbers) and remained unchanged (or were slightly decreased) at any cultivation stages in clone cl.2-1. Acetylation levels of K23/H3 within the entire 5'-upstream regions of the Pax5 gene were high in DT40 cells. However, those were very slightly decreased at the early (E) stage in clone cl.2-1 and thereafter very slightly increased via the middle (M) to late (L) stages, except insignificant change for the region of positions -958 ~ -679 (segment 8). On the

other hand, in DT40 cells acetylation levels of K23/H3 in three ORF regions of the gene resembled to those within the entire 5'-upstream regions, but in clone cl.2-1 those were decreased at the early (E) stage and remained unchanged during cultivation. Acetylation levels of K27/H3 within the entire 5'-upstream regions of the Pax5 gene were high in DT40 cells. Highly similar to changing patterns for K9/H3, those were almost completely decreased at the early (E) stage in clone cl.2-1 and thereafter remained unchanged via the middle (M) to late (L) stages, except insignificant change for the region of positions -958 ~ -679 (segment 8). On the other hand, acetylation levels of K27/H3 within three ORF regions of the gene were very low in DT40 cells (see many PCR cycle numbers) and remained unchanged in clone cl.2-1 during cultivation.

These results indicated not only that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 possessed no binding ability of histone H3 to DNA based on their hyper-acetylation levels within chromatin surrounding the proximal 5'-upstream region of positions -1923 ~ +30 of the Pax5 gene in DT40 cells, but also that predominantly K9/H3, K14/H3, K18/H3 and K27/H3 exhibited full binding ability of histone H3 to DNA based on their hypo-acetylation levels at any cultivation stages in clone cl.2-1. Therefore, we speculated that the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells, but change to tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the late (L) stage. As a result, the gene expression of Pax5, which is high level in DT40 cells, is dramatically decreased at the early (E) stage in clone cl.2-1 and thereafter remains unchanged during cultivation [69].

As mentioned above, since the NotchIP assay for the Pax5 gene in clone cl.2-2 was truly the first employment in a series of these investigations, we carried out this assay, using only four site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3 and K27/H3, on chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and open reading frame regions of the Pax5 gene only at the early (E) and late (L) stages of cultivation and DT40 cells (W) (Fig. 2). Acetylation levels of K9/H3 within the entire 5'-upstream regions of the gene were almost completely reduced at the early (E) stage in clone cl.2-2. Very surprisingly, the reduced acetylation levels were dramatically elevated at the late (L) stage to comparable levels in DT40 cells. On the other hand, acetylation levels of K9/H3 within two ORF regions (segments b and d) of the gene were further reduced at the early (E) stage in clone cl.2-2 and thereafter elevated at the late (L) stage to almost the same levels in DT40 cells, but insignificantly changed within the residual ORF region of positions +223 ~ +391 (segment c). Acetylation levels of K14/H3 within the entire 5'-upstream regions of the gene were dramatically reduced at the early (E) stage in clone cl.2-2, except two proximal and distal 5'-upstream regions of positions -958 ~ -679 (segment 8) and positions -4390 ~ -4235 (segment a), which showed no change and insignificant reduction during cultivation, respectively. The reduced acetylation levels were dramatically elevated at the late (L) stage to almost similar levels in DT40 cells. However, acetylation levels of K14/H3 within three ORF regions

of the gene remained unchanged in clone cl.2-2 during cultivation. Acetylation levels of K18/H3 within the entire 5'-upstream regions of the gene were considerably reduced at the early (E) stage in clone cl.2-2 as a whole, except no change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels of K18/H3 were elevated at the late (L) stage to almost the same levels in DT40 cells. On the other hand, acetylation levels of K18/H3 within three ORF regions of the gene remained unchanged in clone cl.2-2 during cultivation. Acetylation levels of K27/H3 within the entire 5'-upstream regions of the gene were almost completely reduced at the early (E) stage in clone cl.2-2. The reduced acetylation levels were dramatically elevated at the late (L) stage to almost similar levels in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged in clone cl.2-2 during cultivation.

These results indicated that K9/H3, K14/H3, K18/H3 and K27/H3 within the proximal 5'-upstream chromatin region of the Pax5 gene exhibited full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early (E) stage in clone cl.2-2. Surprisingly, thereafter, these four Lys residues should gradually lose the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels until the late (L) stage via a lot of generations during cultivation. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the Pax5 gene should change to tight form at the early (E) stage in clone cl.2-2, and thereafter, remarkably, change to loose form until the late (L) stage during cultivation. As a result, the gene expression of Pax5 is dramatically decreased at the early (E) stage in clone cl.2-2 and thereafter gradually and certainly increased until the late (L) stage to comparable levels in DT40 cells [69].

Next, we carried out the NotchIP assay, using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and open reading frame regions of the Pax5 gene in clone cl.2-4 at the early (E), middle (M) and late (L) stages of cultivation and DT40 cells (W) (Fig. 3). Acetylation levels of K9/H3 within the entire 5'-upstream regions of the gene were almost completely reduced at the early (E) stage in clone cl.2-4. Surprisingly but as expected in part, the reduced acetylation levels were gradually elevated via the middle (M) until late (L) stages to comparable levels in DT40 cells. On the other hand, in clone cl.2-4, acetylation levels of K9/H3 within the ORF region of positions +55 ~ +201 (segment b) of the gene changed in almost the same pattern with the entire 5'-upstream regions during cultivation, but those within two other ORF regions (segments c and d) remained unchanged. Acetylation levels of K14/H3 within the entire 5'-upstream regions of the gene were dramatically reduced at the early (E) stage in clone cl.2-4, except a slight change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels were elevated via the middle (M) until late (L) stages to almost similar levels in DT40 cells. However, acetylation levels of K14/H3 within three ORF regions of the gene remained unchanged until the late (L) stage in clone cl.2-4. Acetylation levels of K18/H3 within the entire 5'-upstream

regions of the gene were drastically reduced at the early (E) stage in clone cl.2-4, except insignificant change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels of K18/H3 were dramatically elevated more quickly compared with those of K9/H3 and K14/H3 and at the middle (M) or late (L) stage reached to very close or almost similar levels in DT40 cells. On the other hand, acetylation levels of K18/H3 within three ORF regions of the gene remained unchanged until the late (L) stage in clone cl.2-4. Acetylation levels of K23/H3 within the entire 5'-upstream regions and three ORF regions of the gene were detected to be slightly lower in both DT40 and clone cl.2-4 (see many PCR cycle numbers) than those observed in clones cl.2-1 and cl.2-6 (see below) as a whole in appearance, probably because the lot of the antibody used in this assay differed in potency from that of the antibody used for other clones. Anyway, acetylation levels of K23/H3 within the proximal 5'-upstream region of the gene were certainly reduced at the early (E) stage in clone cl.2-4. The reduced acetylation levels were elevated quickly and at the middle (M) and late (L) stages reached to very close or almost similar levels in DT40 cells. On the other hand, in clone cl.2-4, acetylation levels of K23/H3 within the distal 5'-upstream region (segment a) and two ORF regions (segments b and c) of the gene remained unchanged during cultivation, but those for another ORF region (segment d) were reduced at the early (E) stage and elevated at the middle (M) and late (L) stages. Acetylation levels of K27/H3 within the proximal 5'-upstream region of the gene were drastically reduced at the early (E) stage in clone cl.2-4, but those showed a little reduction for the distal 5'-upstream region of positions -4390 ~ -4235 (segment a). The reduced acetylation levels were elevated via the middle (M) until late (L) stages to almost similar levels in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged until the late (L) stage in clone cl.2-4.

These results indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 within the proximal 5'-upstream chromatin region of the Pax5 gene possessed full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early (E) stage in clone cl.2-4. Surprisingly, thereafter, these five Lys residues should gradually lose the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels until the late (L) stage. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the Pax5 gene should change to tight form at the early (E) stage in clone cl.2-4, and thereafter, surprisingly, change to loose form via a lot of generations until the late (L) stage. As a result, the gene expression of Pax5 is dramatically decreased at the early (E) stage in clone cl.2-4 and thereafter gradually and certainly increased until the late (L) stage during cultivation [69].

Finally, we carried out the NotchIP assay, using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and open reading frame regions of the Pax5 gene in clone cl.2-6 at the early (E), middle (M) and late (L) stages of cultivation and DT40 cells (W) (Fig. 4). Acetylation levels of K9/H3 within the

entire 5'-upstream regions of the Pax5 gene were almost completely reduced at the early (E) stage in clone cl.2-6. Surprisingly but as expected in part, the reduced acetylation levels were gradually elevated via the middle (M) until late (L) stages to comparable levels in DT40 cells. On the other hand, acetylation levels of K9/H3 within three ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-6 but thereafter slightly elevated via the middle (M) until late (L) stages to almost similar levels in DT40 cells. Acetylation levels of K14/H3 within the entire 5'-upstream regions of the gene were dramatically reduced at the early (E) stage in clone cl.2-6, except no change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels were more rapidly elevated than those in clone cl.2-4 via the middle (M) until late (L) stages to almost the same levels in DT40 cells. On the other hand, acetylation levels of K14/H3 within three ORF regions of the gene remained unchanged in clone cl.2-6 until the late (L) stage. Acetylation levels of K18/H3 within the entire 5'-upstream regions of the gene were drastically reduced at the early (E) stage in clone cl.2-6, except no change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels were elevated more quickly than those in clone cl.2-4 and at the middle (M) or late (L) stage reached to very close or almost similar levels in DT40 cells. On the other hand, acetylation levels of K18/H3 within three ORF regions of the gene remained unchanged until the late (L) stage in clone cl.2-6. Changing patterns in acetylation levels of K23/H3 were remarkably distinct from those of K9/H3, K14/H3 and K18/H3 (and also K27/H3) in clone cl.2-6. That is, acetylation levels of K23/H3 were slightly reduced only for the proximal 5'-upstream region of positions -685 ~ +30 (segments 9 ~ 12) and three ORF regions of the gene at the early (E) stage in clone cl.2-6 and thereafter elevated via the middle (M) until late (L) stages to almost similar levels in DT40 cells. On the other hand, those for the remaining proximal and distal 5'-upstream regions of positions -1923 ~ -679 (segments 1 ~ 8) and -4390 ~ -4235 (segment a) of the gene remained unchanged in clone cl.2-6 during cultivation. Acetylation levels of K27/H3 within the entire 5'-upstream regions of the gene were almost completely reduced at the early (E) stage in clone cl.2-6. The reduced acetylation levels were elevated via the middle (M) until late (L) stages to almost the same levels in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged until the late (L) stage in clone cl.2-6.

These results indicated that mainly K9/H3, K14/H3, K18/H3 and K27/H3 within the proximal 5'-upstream chromatin region of the Pax5 gene exhibited full binding ability of histone H3 to DNA based on their hypo-acetylation levels at the early (E) stage in clone cl.2-6. Surprisingly, thereafter, these Lys residues should gradually lose the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels during cultivation. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the Pax5 gene should change to tight form at the early (E) stage in clone cl.2-6, and thereafter, remarkably, change to loose form via a lot of

generations until the late (L) stage. As a result, the gene expression of Pax5 is dramatically decreased at the early (E) stage in clone cl.2-6 and thereafter gradually and certainly increased during cultivation [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the Aiolos gene in individual clones of HDAC2(-/-) mutants at early, middle and late stages of continuous cultivation

Because changing patterns in the gene expression of Aiolos during cultivation were distinct in individual clones of HDAC2(-/-) mutants [69], we carried out the NotchIP assay on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the Aiolos gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 (Figs. 5 ~ 8). Throughout the NotchIP assay, we used site-specific antibodies for five acetylated Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) and appropriate primers for the proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the Aiolos gene, which were designed based on nucleotide sequences from database that were confirmed by us (Table II). Regarding the proximal 5'-upstream region of positions -2250 ~ +145, we used primers corresponding segments 1 ~ 14, all of which are laid overlapping to neighboring ones each other. In addition, we used primers corresponding to positions -3524 ~ -3367 and -2735 ~ -2528 of the distal 5'-upstream region (segments a and b) and positions +212 ~ +361 and +1265 ~ +1417 of the open reading frame (cDNA) (segments c and d). PCR in the NotchIP assay was carried out twice for K9/H3 and K27/H3 and once for K14/H3, K18/H3 and K23/H3.

Changing patterns in acetylation levels of these five Lys residues of histone H3 for the Aiolos gene in clone cl.2-1 during cultivation are represented in Figure 5. Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream regions (segments a, b and 1 ~ 14) and two ORF regions (segments c and d) of the Aiolos gene were high in DT40 cells (W). Surprisingly, those were dramatically reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged via the middle (M) until late (L) stages. Acetylation levels of K14/H3 within the entire 5'-upstream regions and two ORF regions of the gene were high in DT40 cells. However, those within the proximal 5'-upstream region (segments 1 ~ 13) and the ORF region of positions +1265 ~ +1417 (segment d) of the gene were slightly reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged via the middle (M) until late (L) stages. On the other hand, changes in acetylation levels of K14/H3 were insignificant in the residual distal and proximal 5'-upstream regions of positions -3524 ~ -3367 plus -2735 ~ -2528 (segments a and b) and positions -64 ~ +145 (segment 14) and the residual ORF region (segment c). Acetylation levels of K18/H3 within the entire 5'-upstream regions and two ORF regions of the gene were high in DT40 cells. In clone cl.2-1, those were slightly reduced at the early (E) stage, and further reduced at the middle (M) stage but remained unchanged at the late (L) stage. Acetylation levels of K23/H3 within the entire

5'-upstream regions and two ORF regions of the gene were high in DT40 cells and as a whole remained unchanged from the early (E) to late (L) stages in clone cl.2-1. Acetylation levels of K27/H3 within the entire 5'-upstream regions and two ORF regions of the gene were high in DT40 cells. On the other hand, as a whole those were slightly reduced at the early (E) stage in clone cl.2-1 and remained unchanged from the middle (M) until late (L) stages.

These results indicated that five Lys residues K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 possessed no binding ability of histone H3 to DNA based on their hyper-acetylation levels within chromatin surrounding the proximal 5'-upstream region from positions -2250 ~ +145 of the Aiolos gene in DT40 cells. However, those except K23/H3 exhibited full or less binding ability of histone H3 to DNA based on their hypo- or somewhat hyper-acetylation levels at any cultivation stages in clone cl.2-1. Therefore, we speculated that the structure of chromatin surrounding the proximal ~2.3 kb 5'-upstream region of the Aiolos gene, which may consist of ~11 nucleosomes, should be loose form in DT40 cells, but change to tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the late (L) stage. As a result, the gene expression of Aiolos, which is high level in DT40 cells, is drastically decreased at the early (E) stage in clone cl.2-1 and thereafter remains unchanged during cultivation [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the Aiolos gene in clone cl.2-2 during cultivation are represented in Figure 6. Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1 ~ 14) and two ORF regions (segments c and d) of the gene were obviously reduced at the early (E) stage in clone cl.2-2 and thereafter slowly and certainly elevated via the middle (M) until late (L) stages as a whole. Acetylation levels of K14/H3 within the entire 5'-upstream regions and two ORF regions of the gene changed insignificantly or remained unchanged from the early (E) via middle (M) to late (L) stages in clone cl.2-2 as a whole. Acetylation levels of K18/H3 within the entire 5'-upstream regions and two ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-2 and thereafter remained unchanged via the middle (M) to late (L) stages. Acetylation levels of K23/H3 within the entire 5'-upstream regions and two ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-2, followed by insignificant changes via the middle (M) to late (L) stages. Acetylation levels of K27/H3 within the entire 5'-upstream regions and two ORF regions of the gene were certainly reduced at the early (E) stage in clone cl.2-2 and thereafter slightly elevated or remained unchanged via the middle (M) until late (L) stages as a whole.

These results indicated that mainly the binding capacity of K9/H3 and K27/H3 (and probably K18/H3 and K23/H3) to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene was strengthened to less binding ability based on their hypo- or slight hyper-acetylation levels at the early (E) and middle (M) stages in clone cl.2-2 during cultivation. Thereafter, predominantly, the binding capacity of K9/H3 (and probably K27/H3) to DNA was obviously weakened to no binding ability based

on their hyper- or considerable hyper-acetylation levels at the late (L) stage. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the Aiolos gene should change to tight form at the early (E) and middle (M) stages in clone cl.2-2 and thereafter change to loose (or considerable loose) form at the late (L) stage. As a result, the gene expression of Aiolos is dramatically decreased at the early (E) stage in clone cl.2-2 and thereafter gradually increased until the late (L) stage [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the Aiolos gene in clone cl.2-4 during cultivation are represented in Figure 7. Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1 ~ 14) and the ORF region of positions +1265 ~ +1417 (segment d) of the Aiolos gene were obviously reduced at the early (E) stage in clone cl.2-4 and thereafter certainly elevated via the middle (M) to late (L) stages as a whole, but changed insignificantly within the remaining ORF region (segment c). Acetylation levels of K14/H3 within the entire 5'-upstream regions and two ORF regions of the gene remained unchanged from the early (E) via middle (M) to late (L) stages in clone cl.2-4. Acetylation levels of K18/H3 within the entire 5'-upstream regions and two ORF regions of the gene were very slightly reduced at the early (E) stage in clone cl.2-4 and thereafter remained unchanged via the middle (M) to late (L) stages as a whole. Acetylation levels of K23/H3 within the entire 5'-upstream regions and two ORF regions of the gene remained unchanged from the early (E) via middle (M) to late (L) stages in clone cl.2-4. Acetylation levels of K27/H3 within the entire 5'-upstream regions of the gene were very slightly reduced at the early (E) stage in clone cl.2-4 and thereafter slightly elevated or remained unchanged via the middle (M) until late (L) stages as a whole. However, those of K27/H3 within two ORF regions of the gene remained unchanged in clone cl.2-4 during cultivation.

These results indicated that predominantly the binding capacity of K9/H3 (and probably K18/H3 and K27/H3) to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene in clone cl.2-4 was strengthened to less binding ability based on the hypo- or slight hyper-acetylation levels at the early (E) and middle (M) stages. Thereafter, mainly the binding capacity of K9/H3 (and probably K27/H3) to DNA was certainly weakened to no binding ability based on the hyper- or considerable hyper-acetylation levels at the late (L) stage. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the Aiolos gene should change to tight form at the early (E) and middle (M) stages in clone cl.2-4 and thereafter change to loose (or considerable loose) form at the late (L) stage. As a result, the gene expression of Aiolos is obviously decreased at the early (E) stage in clone cl.2-4 and thereafter certainly increased at the late (L) stage [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the Aiolos gene in clone cl.2-6 during cultivation are represented in Figure 8. Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1 ~ 14) and two ORF regions (segments c and d) of the gene were apparently decreased at the early (E) stage in clone cl.2-6 and thereafter gradually increased via the

middle (M) to late (L) stages to be clearly higher than those in DT40 cells. Acetylation levels of K14/H3 within the entire 5'-upstream regions and two ORF regions of the gene remained unchanged at the early (E) stage in clone cl.2-6 but thereafter were gradually increased via the middle (M) to late (L) stages to be considerably higher than those in DT40 cells. Acetylation levels of K18/H3 within the entire 5'-upstream regions of the gene were very slightly decreased at the early (E) stage in clone cl.2-6 and thereafter gradually increased via the middle (M) to late (L) stages to be considerably higher than those in DT40 cells. On the other hand, those of K18/H3 within two ORF regions of the gene remained unchanged in clone cl.2-6 during cultivation. Acetylation levels of K23/H3 within the entire 5'-upstream regions of the gene remained unchanged at the early (E) and middle (M) stages in clone cl.2-6 as a whole but thereafter were slightly increased at the late (L) stage to be certainly higher than those in DT40 cells. However, those of K23/H3 within two ORF regions of the gene insignificantly changed in clone cl.2-6 during cultivation. Acetylation levels of K27/H3 within the 5'-upstream region upper from position -1230 (segments a, b and 1 ~ 6) of the gene remained unchanged at the early (E) stage in clone cl.2-6 and thereafter were gradually increased via the middle (M) to late (L) stages to be higher than those in DT40 cells. On the other hand, those of K27/H3 within the 5'-upstream region down from position -1232 (segments 7 ~ 14) were slightly decreased at the early (E) stage in clone cl.2-6 and thereafter rapidly increased via the middle (M) to late (L) stages to almost the same levels in DT40 cells as a whole. Acetylation levels of K27/H3 within two ORF regions of the gene remained unchanged or insignificantly changed during cultivation.

These results indicated that predominantly the binding capacity of K9/H3 and K27/H3 to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene in clone cl.2-6 was certainly strengthened to less binding ability based on their hypo- or slight hyper-acetylation levels at the early (E) stage. Thereafter, K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) gradually lost the binding capacity to DNA to no binding ability based on their hyper-acetylation levels via the middle (M) until late (L) stages. Naturally, it was possible that the extent of no binding ability at the middle (M) and late (L) stages should be less than that in DT40 cells, because acetylation levels at both cultivation stages in clone cl.2-6 were higher than those in DT40 cells. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the Aiolos gene should change to tight form at the early (E) stage in clone cl.2-6 and thereafter change to loose form at the late (L) stage. As a result, the gene expression of Aiolos is obviously decreased at the early (E) stage in clone cl.2-6 and thereafter increased at the late (L) stage [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the EBF1 gene in individual clones of HDAC2(-/-) mutants at early, middle and

late stages of continuous cultivation

Because changing patterns in the gene expression of EBF1 during cultivation were distinct in individual clones of HDAC2(-/-) mutants [69], we carried out the NotchIP assay on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the EBF1 gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 (Figs. 9 ~ 12). Throughout the NotchIP assay, we used site-specific antibodies for five acetylated Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) and appropriate primers for the proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the EBF1 gene, which were designed based on nucleotide sequences from database that were confirmed or determined by us (Table III). Regarding the proximal 5'-upstream region of positions -2031 ~ +200, we used primers recognizing respective segments 1 ~ 14, all of which are laid overlapping to neighboring ones each other. In addition, we used primers, corresponding to positions -3996 ~ -3770 and -2888 ~ -2730 of the distal 5'-upstream region (segments a and b) and positions +179 ~ +291, +649 ~ +768 and +787 ~ +900 of the open reading frame (cDNA) (segments c, d and e). PCR was carried out twice for these five acetylated Lys residues in the NotchIP assay.

Changing patterns in acetylation levels of these five Lys residues of histone H3 for the EBF1 gene in clone cl.2-1 during cultivation are represented in Figure 9. Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream regions (segments a, b and 1 ~ 14) and the ORF region of positions +179 ~ +291 (segment c) of the EBF1 gene were high in DT40 cells. As a whole, those were dramatically reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged via the middle (M) to late (L) stages. On the other hand, acetylation levels of K9/H3 within two residual ORF regions (segments d and e) of the gene were very low in DT40 cells (see many PCR cycle numbers), and slightly reduced at the early (E) stage in clone cl.2-1 but thereafter slightly elevated via the middle (M) to late (L) stages. Acetylation levels of K14/H3 within the entire 5'-upstream regions and three ORF regions of the gene were high (or slightly high) in DT40 cells. In clone cl.2-1 acetylation levels of K14/H3 within the proximal 5'-upstream and ORF regions of positions -1898 ~ -1582 (segments 2 and 3), -872 ~ +200 (segments 8 ~ 14) and +179 ~ +291 (segment c) of the gene were slightly reduced at the early (E) stage and thereafter changed insignificantly or remained unchanged via the middle (M) to late (L) stages as a whole. However, those did not change so much for the residual distal 5'-upstream (segments a and b), proximal 5'-upstream (segments 1 and 4 ~ 7) and ORF (segment e) regions of the gene. Acetylation levels of K18/H3 within the entire 5'-upstream regions and the ORF region of positions +179 ~ +291 (segment c) of the gene were high in DT40 cells. In clone cl.2-1 those were certainly reduced at the early (E) stage, except a slight decrease within the distal and proximal 5'-upstream regions of positions -3996 ~ -3770 (segment a) and -1299 ~ -598 (segments 6 ~ 9). The reduced acetylation levels remained unchanged via the middle (M) to late (L) stages as a whole. On the other hand, acetylation levels of K18/H3 within two residual ORF regions (segments d and e) of the gene

were slightly low in DT40 cells (see many PCR cycle numbers) and remained unchanged at any cultivation stages in clone cl.2-1. Acetylation levels of K23/H3 within the entire 5'-upstream regions and the ORF region (segment c) of the gene were high in DT40 cells, but those were relatively low within two residual ORF regions of positions +649 ~ +768 and +787 ~ +900 (segments d and e) (see many PCR cycle numbers). As a whole, acetylation levels of K23/H3 remained unchanged from the early (E) to late (L) stages in clone cl.2-1, except a slight decrease within the proximal 5'-upstream region of positions -2031 ~ -1283 (segments 1 ~ 5). Acetylation levels of K27/H3 were considerably high within the entire 5'-upstream regions and the ORF region of positions +179 ~ +291 (segment c) of the gene, but those were relatively low in two residual ORF regions of positions +649 ~ +768 and +787 ~ +900 (segments d and e) (see many PCR cycle numbers) in DT40 cells. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -1037 ~ +200 (segments 7 ~ 14) and +179 ~ +291 (segment c) were certainly reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged via the middle (M) to late (L) stages. On the other hand, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change so much during cultivation.

These results indicated not only that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 exhibited no binding ability of histone H3 to DNA based on their hyper-acetylation levels within chromatin surrounding the proximal 5'-upstream region from positions -2031 ~ +200 of the EBF1 gene in DT40 cells, but also that especially K9/H3, K18/H3 and K27/H3 possessed full binding ability based on their hypo- or slight hyper-acetylation levels at any cultivation stages in clone cl.2-1. Therefore, we speculated that the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells, but change to tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the late (L) stage during cultivation. As a result, the gene expression of EBF1, which is high level in DT40 cells, is almost completely suppressed at the early (E) stage in clone cl.2-1 and thereafter remains unchanged via the middle (M) to late (L) stages during cultivation [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the EBF1 gene in clone cl.2-2 during cultivation are represented in Figure 10. Acetylation levels of K9/H3 within the entire 5'-upstream regions and the ORF region of positions +179 ~ +291 (segment c) of the EBF1 gene were dramatically reduced at the early (E) stage in clone cl.2-2 and thereafter remained unchanged via the middle (M) to late (L) stages. On the other hand, acetylation levels of K9/H3 within two residual ORF regions (segments d and e) of the gene were slightly reduced at the early (E) stage in clone cl.2-2 and thereafter slightly elevated via the middle (M) to late (L) stages. Acetylation levels of K14/H3 within the proximal 5'-upstream and ORF regions of positions -872 ~ +200 (segments 8 ~ 14) and +179 ~ +291 (segment c) of the gene were certainly reduced at the early (E) stage in clone cl.2-2 and thereafter remained unchanged via the middle (M) to late (L) stages as a whole. However, acetylation levels of

K14/H3 within the proximal 5'-upstream regions of positions -1599 ~ -1363 and -1037 ~ -847 (segments 4 and 7) were reduced via the middle (M) to late (L) stages, but those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change so much. Acetylation levels of K18/H3 within the entire 5'-upstream and three ORF regions of the gene were certainly reduced at the early (E) stage in clone cl.2-2, except insignificant change within the proximal 5'-upstream region of positions -1299 ~ -598 (segments 6 ~ 9). The reduced acetylation levels were slightly elevated at the middle (M) stage and remained unchanged at the late (L) stage. The recovered acetylation levels at the middle (M) and late (L) stages were lower than those in DT40 cells as a whole, except those within two ORF regions of positions +649 ~ +768 and +787 ~ +900 (segments d and e). Acetylation levels of K23/H3 within the entire 5'-upstream and three ORF regions of the gene in clone cl.2-2 remained unchanged as a whole during cultivation at almost the same levels in DT40 cells. Acetylation levels of K27/H3 within the 5'-upstream regions upper from position -762 (segments a, b and 1 ~ 8) of the gene were considerably low in DT40 cells. In clone cl.2-2, acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -1826 ~ -1363 (segments 3 and 4), -1037 ~ +200 (segments 7 ~ 14) and +179 ~ +291 (segment c) were dramatically or almost completely reduced at the early (E) stage and thereafter remained unchanged via the middle (M) to late (L) stages. However, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions insignificantly changed during cultivation.

These results indicated that predominantly K9/H3, K14/H3, K18/H3 and K27/H3 exhibited full binding ability of histone H3 to DNA based on their hypo- or slight hyper-acetylation levels within the proximal 5'-upstream chromatin region of the EBF1 gene at any cultivation stages in clone cl.2-2. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the EBF1 gene should change to tight form at the early (E) stage in clone cl.2-2 and remain unchanged via the middle (M) until late (L) stages. As a result, the gene expression of EBF1 is almost completely suppressed at the early (E) stage in clone cl.2-2 and thereafter remains unchanged during cultivation [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the EBF1 gene in clone cl.2-4 during cultivation are represented in Figure 11. Acetylation levels of K9/H3 within the entire 5'-upstream regions and the ORF region of positions +179 ~ +291 (segment c) of the EBF1 gene were dramatically or almost completely decreased at the early (E) stage in clone cl.2-4. Thereafter, those remained unchanged via the middle (M) until late (L) stages, except the gradual increase within two distal 5'-upstream regions of positions -3996 ~ -3770 and -2888 ~ -2730 (segments a and b). On the other hand, acetylation levels of K9/H3 within two residual ORF regions (segments d and e) of the gene remained unchanged in clone cl.2-4 during cultivation. Acetylation levels of K14/H3 within the proximal 5'-upstream and ORF regions of positions -1898 ~ -1744 (segment 2), -621 ~ +200 (segments 10 ~ 14) and +179 ~ +291 (segment c) of the gene were slightly decreased at the early (E) stage in clone

cl.2-4 and thereafter remained unchanged via the middle (M) to late (L) stages. However, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change so much during cultivation. Acetylation levels of K18/H3 within the entire 5'-upstream and ORF regions of the gene were certainly decreased at the early (E) stage in clone cl.2-4, except insignificant change within the proximal 5'-upstream and ORF regions of positions -1299 ~ -598 (segments 6 ~ 9), +649 ~ +768 and +787 ~ +900 (segments d and e). The decreased acetylation levels were slightly increased via the middle (M) to late (L) stages to somewhat lower than those in DT40 cells as a whole. Acetylation levels of K23/H3 within the entire 5'-upstream and ORF regions of the gene remained unchanged in clone cl.2-4 as a whole during cultivation. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -1037 ~ +200 (segments 7 ~ 14) and +179 ~ +291 (segment c) were dramatically or considerably decreased at the early (E) stage in clone cl.2-4 and thereafter remained unchanged via the middle (M) to late (L) stages. On the other hand, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions insignificantly changed or slightly decreased during cultivation.

These results indicated that especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibited full binding ability of histone H3 to DNA based on their hypo- or slight hyper-acetylation levels within the proximal 5'-upstream chromatin region of the EBF1 gene at any cultivation stages in clone cl.2-4. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the EBF1 gene should change to tight form at the early (E) stage in clone cl.2-4 and remain unchanged until the late (L) stage. As a result, the gene expression of EBF1 is almost completely diminished at the early (E) stage in clone cl.2-4 and thereafter remains unchanged during cultivation [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the EBF1 gene in clone cl.2-6 during cultivation are represented in Figure 12. Acetylation levels of K9/H3 within the distal 5'-upstream and proximal 5'-upstream regions of positions -2888 ~ -2730 and -2031 ~ +200 (segments b and 1 ~ 14) and the ORF region of positions +179 ~ +291 (segment c) of the EBF1 gene were almost completely or dramatically reduced at the early (E) stage in clone cl.2-6. Interestingly, the reduced acetylation levels were gradually and dramatically elevated via the middle (M) until late (L) stages to almost similar levels in DT40 cells. On the other hand, acetylation levels of K9/H3 within the residual distal 5'-upstream and ORF regions of positions -3996 ~ -3770 (segment a), +649 ~ +768 and +787 ~ +900 (segments d and e) of the gene remained unchanged at any cultivation stages in clone cl.2-6. Acetylation levels of K14/H3 within the proximal 5'-upstream and ORF regions of positions -621 ~ +200 (segments 10 ~ 14) and +179 ~ +291 (segment c) of the gene were slightly reduced at the early (E) stage in clone cl.2-6 and thereafter gradually elevated via the middle (M) until late (L) stages to almost the same levels in DT40 cells. On the other hand, acetylation levels of K14/H3 within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions remained unchanged at any cultivation stages in

clone cl.2-6. Acetylation levels of K18/H3 within the proximal 5'-upstream and ORF regions of positions -621 ~ +200 (segments 10 ~ 14) and +179 ~ +291 (segment c) of the gene were slightly reduced at the early (E) stage in clone cl.2-6 and thereafter rapidly elevated via the middle (M) until late (L) stages to almost similar levels in DT40 cells. However, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change so much at any cultivation stages in clone cl.2-6. Acetylation levels of K23/H3 within the entire 5'-upstream and ORF regions of the gene in clone cl.2-6 did not change so much during cultivation. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -872 ~ +200 (segments 8 ~ 14) and +179 ~ +291 (segment c) of the gene were dramatically reduced at the early (E) stage in clone cl.2-6 and thereafter elevated gradually and dramatically via the middle (M) to late (L) stages to almost the same levels in DT40 cells. On the other hand, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions insignificantly changed at any cultivation stages in clone cl.2-6.

These results indicated that especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibited full binding ability of histone H3 to DNA based on their hypo- or slight hyper-acetylation levels within the proximal 5'-upstream chromatin region of the EBF1 gene at the early (E) stage in clone cl.2-6, but thereafter, interestingly, lost the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels via the middle (M) until late (L) stages. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the EBF1 gene should change to tight form at the early (E) stage in clone cl.2-6 and thereafter change to loose form via the middle (M) until late (L) stages. As a result, the gene expression of EBF1 is almost completely suppressed at the early (E) stage in clone cl.2-6 and thereafter gradually increased until the late (L) stage to comparable levels in DT40 cells [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on chromatin surrounding proximal 5'-upstream and open reading frame regions of the OBF1 gene in individual clones of HDAC2(-/-) mutants at early, middle and late stages of continuous cultivation

Because changing patterns in the gene expression of OBF1 during cultivation were distinct in individual clones of HDAC2(-/-) mutants [69], we carried out the NotchIP assay on chromatin surrounding proximal 5'-upstream and open reading frame regions of the OBF1 gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 (Figs. 13 ~ 16). Throughout the NotchIP assay, we used site-specific antibodies for five acetylated Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) and appropriate primers for the proximal 5'-upstream and ORF regions of the OBF1 gene, which were designed based on nucleotide sequences from database that were confirmed or determined by us (Table IV). Regarding the proximal 5'-upstream region of positions -2138 ~ +164, we used primers for

respective segments 1 ~ 14, all of which are laid overlapping to neighboring ones each other. In addition, we used primers, corresponding to positions +17 ~ +131 and +776 ~ +937 (segments a and b) of the open reading frame (cDNA). PCR was carried out twice for K9/H3 and K27/H3 and once for K14/H3, K18/H3 and K23/H3 in the NotchIP assay.

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the OBF1 gene in clone cl.2-1 during cultivation are represented in Figure 13. Acetylation levels of K9/H3 within the proximal 5'-upstream region (segments 1 ~ 14) and two ORF regions (segments a and b) of the OBF1 gene were high in DT40 cells but those were certainly reduced at the early (E) stage in clone cl.2-1 as a whole. Interestingly, the reduced acetylation levels were further reduced to undetectable levels at the middle (M) stage and thereafter remained unchanged until the late (L) stage. Acetylation levels of K14/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged from the early (E) to late (L) stages in clone cl.2-1. Acetylation levels of K18/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged at the early (E) stage in clone cl.2-1, but were certainly reduced at the middle (M) stage and thereafter slightly elevated or remained unchanged at the late (L) stage as a whole. Acetylation levels of K23/H3 within the proximal 5'-upstream and two ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-1, except insignificant changes within the proximal 5'-upstream region of positions -1493 ~ -1068 (segments 5 and 6). Further, the reduced acetylation levels were very slightly reduced at the middle (M) stage and thereafter remained unchanged at late (L) stage as a whole. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-1, except insignificant reduction within the proximal 5'-upstream region of positions -1493 ~ -1068 (segments 5 and 6). The reduced acetylation levels were further and clearly reduced at the middle (M) stage and thereafter remained unchanged at the late (L) stage as a whole.

These results indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 exhibited no binding ability of histone H3 to DNA based on their hyper-acetylation levels within chromatin surrounding the proximal 5'-upstream region from positions -2138 ~ +164 of the OBF1 gene in DT40 cells. On the other hand, especially K9/H3 and K27/H3 (and probably K23/H3) certainly possessed weak binding ability to DNA based on their considerable hyper-acetylation levels at the early (E) stage in clone cl.2-1. Further, predominantly, the weak binding ability of K9/H3 and K27/H3 was dramatically increased to full binding ability based on their hypo-acetylation levels at the middle (M) stage and remained unchanged until the late (L) stage. Therefore, we speculated that the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells. On the other hand, in clone cl.2-1 the chromatin structure should change to considerable loose form at the early (E) stage and thereafter change to tight form at the middle (M) and late (L) stages. As a result, the gene expression of OBF1, which is high level in DT40 cells, is certainly

decreased at the early (E) stage in clone cl.2-1 and thereafter dramatically decreased via the middle (M) to late (L) stages to very low levels [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the OBF1 gene in clone cl.2-2 during cultivation are represented in Figure 14. Acetylation levels of K9/H3 within the proximal 5'-upstream and two ORF regions of the OBF1 gene in clone cl.2-2 were dramatically decreased at the early (E) stage as a whole. Interestingly, the decreased acetylation levels remained unchanged at the middle (M) stage but thereafter were obviously increased at the late (L) stage. The increased acetylation levels at the late (L) stage were less than those in DT40 cells as a whole. Acetylation levels of K14/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged as a whole in clone cl.2-2 during cultivation. Acetylation levels of K18/H3 within the proximal 5'-upstream and two ORF regions of the gene showed insignificant change in clone cl.2-2 during cultivation as a whole, except a slight decrease at the middle (M) stage. Acetylation levels of K23/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged as a whole in clone cl.2-2 during cultivation. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were considerably decreased at the early (E) stage in clone cl.2-2, and further decreased at the middle (M) stage but thereafter obviously increased at the late (L) stage.

These results indicated that especially K9/H3 and K27/H3 within the proximal 5'-upstream chromatin region of the OBF1 gene obviously possessed full or less binding ability to DNA based on their hypo- or slight hyper-acetylation levels at the early (E) stage in clone cl.2-2. At the middle (M) stage, the full binding ability of K9/H3 remained unchanged, and the less binding ability of K27/H3 was further increased to full binding ability based on the hypo-acetylation levels. Thereafter, the full binding ability of K9/H3 and K27/H3 was obviously decreased to no binding ability based on their hyper-acetylation levels at the late (L) stage. The extent of the binding capacity at the late (L) stage should be higher than that in DT40 cells, because the acetylation levels at the late (L) stage in clone cl.2-2 were lower than those in DT40 cells. Therefore, we speculated that in clone cl.2-2 the structure of chromatin surrounding the proximal 5'-upstream region of the OBF1 gene should change to tight or somewhat loose form at the early (E) or middle (M) stage of cultivation. Thereafter, the tightened chromatin structure should become loose form at the late (L) stage, which may be slightly tighter than that in DT40 cells. As a result, the gene expression of OBF1 is certainly decreased at the early (E) stage in clone cl.2-2 and thereafter obviously increased via the middle (M) to late (L) stages [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the OBF1 gene in clone cl.2-4 during cultivation are represented in Figure 15. Acetylation levels of K9/H3 within the proximal 5'-upstream and two ORF regions of the OBF1 gene were obviously reduced at the early (E) stage in clone cl.2-4. The reduced acetylation levels remained unchanged at the middle (M) stage but thereafter were certainly elevated at the late (L) stage; the extent of the acetylation levels at the late (L)

stage being less than those in DT40 cells. Acetylation levels of K14/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged as a whole in clone cl.2-4 during cultivation. Acetylation levels of K18/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged as a whole in clone cl.2-4 during cultivation. Acetylation levels of K23/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged in clone cl.2-4 during cultivation. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-4, and remained unchanged at the middle (M) stage as a whole but thereafter slightly elevated at the late (L) stage, except no change within some segments of the proximal 5'-upstream region.

These results indicated that especially the binding capacity of K9/H3 (and probably K27/H3) to DNA within the proximal 5'-upstream chromatin region of the OBF1 gene was certainly but slightly increased to weak or less binding ability based on their considerable or slight hyper-acetylation levels at the early (E) stage in clone cl.2-4. The weak or less binding ability remained unchanged at the middle (M) stage but thereafter was obviously decreased to no binding ability based on their hyper-acetylation levels at the late (L) stage; the extent of the binding capacity at the late (L) stage being probably slightly higher than that in DT40 cells. Therefore, we speculated that in clone cl.2-4 the structure of chromatin surrounding the proximal 5'-upstream region of the OBF1 gene should change to somewhat loose form at the early (E) and middle (M) stages and thereafter at the late (L) stage change to loose form, which should be almost similar to that in DT40 cells. As a result, the gene expression of OBF1 is certainly decreased at the early (E) and middle (M) stages in clone cl.2-4 and thereafter obviously increased at the late (L) stage [69].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the OBF1 gene in clone cl.2-6 during cultivation are represented in Figure 16. Acetylation levels of K9/H3 within the proximal 5'-upstream and two ORF regions of the OBF1 gene were obviously decreased at the early (E) stage in clone cl.2-6. The decreased acetylation levels were gradually increased via the middle (M) until late (L) stages to almost the same levels in DT40 cells. Acetylation levels of K14/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged as a whole in clone cl.2-6 during cultivation. Acetylation levels of K18/H3 within the proximal 5'-upstream and two ORF regions of the gene remained unchanged as a whole in clone cl.2-6 during cultivation. Acetylation levels of K23/H3 within the proximal 5'-upstream region of positions -2138 ~ -1068 (segments 1 ~ 6) of the gene remained unchanged as a whole in clone cl.2-6 during cultivation. On the other hand, those within the proximal 5'-upstream region of positions -1071 ~ +164 (segments 7 ~ 14) and two ORF regions of positions +17 ~ +131 and +776 ~ +937 (segments a and b) were slightly decreased at the early (E) and middle (M) stages and thereafter certainly increased at the late (L) stage to almost the same levels in DT40 cells. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were certainly decreased at the early (E) stage in clone cl.2-6 and remained unchanged at the middle

(M) stage. Thereafter, those were obviously increased at the late (L) stage to almost similar or higher levels in DT40 cells.

These results indicated that in clone cl.2-6 K9/H3 and K27/H3 (and probably K23/H3) within the proximal 5'-upstream chromatin region of the OBF1 gene obviously possessed full or less binding ability to DNA based on their hypo- or slight hyper-acetylation levels at the early (E) stage. The full or less binding ability was slightly decreased at the middle (M) stage and thereafter further and obviously decreased to no binding ability based on their hyper-acetylation levels at the late (L) stage. Therefore, we speculated that in clone cl.2-6 the structure of chromatin surrounding the proximal 5'-upstream region of the OBF1 gene should change to tight form at the early (E) stage and thereafter change to loose form, like that in DT40 cells, via the middle (M) until late (L) stages. As a result, the gene expression of OBF1 is certainly decreased at the early (E) stage in clone cl.2-6 and thereafter obviously increased via the middle (M) to late (L) stages [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the PCAF gene in individual clones of HDAC2(-/-) mutants at early, middle and late stages of continuous cultivation

Because changing patterns in the gene expression of PCAF during cultivation were distinct in individual clones of HDAC2(-/-) mutants [69], we carried out the NotchIP assay on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and open reading frame regions of the PCAF gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 (Figs. 17 ~ 20). Throughout the NotchIP assay, we used site-specific antibodies for five acetylated Lys residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) and appropriate primers for the proximal 5'-upstream, distal 5'-upstream and ORF regions of the PCAF gene, which were designed based on nucleotide sequences from database that were confirmed or determined by us (Table V). Regarding the proximal 5'-upstream region of positions -2005 ~ +231, we used primers recognizing respective segments 1 ~ 14, which are laid overlapping to neighboring ones each other. In addition, we used primers, corresponding to positions -3681 ~ -3527 and -2712 ~ -2523 of the distal 5'-upstream region (segments a and b) and positions +209 ~ +320 and +628 ~ +803 of ORF region (cDNA) (segments c and d). PCR was carried out once for all of the five Lys residues in the NotchIP assay.

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the PCAF gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation are represented in Figures 17 ~ 20. In DT40 cells (W), acetylation levels of K9/H3 and K27/H3 within the entire (proximal and distal) 5'-upstream and two ORF regions of the PCAF gene were considerably low (see many PCR cycle numbers), but those of K14/H3, K18/H3 and K23/H3 were relatively high. In clones cl.2-1 and cl.2-2, acetylation levels of

K9/H3 within the entire 5'-upstream and two ORF regions of the gene were slightly decreased at the early (E) stage and thereafter slightly increased via the middle (M) until late (L) stages, but in clone cl.2-4 those remained unchanged as a whole during cultivation. In addition, in clones cl.2-1, cl.2-2 and cl.2-4, acetylation levels of K18/H3 within the entire 5'-upstream and two ORF regions of the gene were slightly decreased at the early (E) stage and thereafter slightly increased at the middle (M) and late (L) stages, but those of K14/H3, K23/H3 and K27/H3 remained unchanged as a whole during cultivation. In clone cl.2-6, acetylation levels of K9/H3 and K18/H3 within the entire 5'-upstream and two ORF regions of the gene were slightly decreased (or remained unchanged) at the early (E) stage, and thereafter increased at the middle (M) stage but again decreased at the late (L) stage. On the other hand, those of K14/H3, K23/H3 and K27/H3 remained unchanged as a whole during cultivation.

These results, together with previous findings [69], led to tentative conclusions on correlation among acetylation levels of the five Lys residues, binding ability (capacity) of histone H3 to DNA, chromatin structure and gene expression levels regarding the PCAF gene as follows. In DT40 cells, the five Lys residues (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) exhibited no binding ability of histone H3 to DNA based on their hyper-acetylation levels within chromatin surrounding the proximal 5'-upstream region of positions -2005 ~ +231 and two distal 5'-upstream regions of the PCAF gene. In three mutant clones cl.2-1, cl.2-2 and cl.2-4, K9/H3 and K18/H3 possibly exhibited weak binding ability of histone H3 to DNA based on their considerable hyper-acetylation levels at the early (E) stage and thereafter exhibited no binding ability based on their hyper-acetylation levels at the middle (M) and late (L) stages. Therefore, we speculated that the structure of chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the PCAF gene, which may consist of ~10 nucleosomes, should be loose form in DT40 cells, but change to considerable loose form at the early (E) stage in clones cl.2-1, cl.2-2 and cl.2-4 and thereafter change to loose form at the middle (M) and late (L) stages. On the other hand, in these three clones, the gene expression of PCAF is really very low at the early (E) stage, like that in DT40 cells, but dramatically increased via the middle (M) to late (L) stages during cultivation [69]. In clone cl.2-6, K9/H3 and K18/H3 possessed less binding ability of histone H3 to DNA based on their somewhat hyper-acetylation levels within chromatin surrounding the proximal 5'-upstream region of positions -2005 ~ +231 and two distal 5'-upstream regions of the PCAF gene at the early (E) stage. Interestingly, these Lys residues lost their less binding ability to no binding ability based on their hyper-acetylation levels at the middle (M) stage and thereafter again gained less binding ability based on their slight hyper-acetylation levels at the late (L) stage. Therefore, we speculated that in clone cl.2-6 the structure of chromatin surrounding the proximal 5'-upstream region of the PCAF gene should be loose form at the middle (M) stage, like in DT40 cells, but somewhat loose form at the early (E) and late (L) stages. On the other hand, as described previously [69], the gene expression of PCAF, which is really very low in DT40 cells, is gradually increased from the early (E) stage to the stage nearly prior the middle (M) stage

in clone cl.2-6 but thereafter dramatically decreased via the middle (M) to late (L) stages to almost undetectable level.

These results, therefore, indicated that the examined distal and proximal 5'-upstream regions are not directly correlated so much to the gene expression of PCAF in DT40 cells and also HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) at any cultivation stages, regardless of loose (or considerable loose) form of chromatin structure. In consequence, we assumed that undefined manners, including other distal 5'-upstream regions, should be preferentially involved in the gene expression of PCAF in HDAC2(-/-) mutants during cultivation.

Discussion

As mentioned previously [67, 69], mRNA and protein levels of IgM H- and L-chains, which are very low in DT40 cells [46, 53], are dramatically and certainly increased at the early stage of cultivation in all of HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6 (and also cl.33-12, cl.33-28, cl.33-30 and cl.45-28) examined, and thereafter gradually and obviously decreased in almost similar changing pattern via the middle (M) to late (L) stages to nearly equal levels in DT40 cells. Since alterations in gene expressions of PCAF, HDAC7 and HDAC9 are neither parallel nor anti-parallel with those of IgM H- and L-chains in one or more of the examined HDAC2(-/-) mutant clones during cultivation [69], they should not directly and/or mainly participate in decreases in gene expressions of the two immunoglobulin proteins. These results were roughly supported by the findings that the PCAF-deficient or HDAC7-deficient mutant shows a slight or no influence on gene expressions of IgM H- and L-chains [46]. Apart from that, the HDAC9-deficient one is not available yet. By contrast, Pax5, Aiolos and EBF1 or OBF1 have been verified or strongly suggested to be involved in down- or up-regulation of gene expressions of IgM H- and L-chains [41, 46, 58, 69]. Noticeably, individual gene expressions of the four transcription factors change in anti-parallel or parallel with those of the two immunoglobulin proteins in one or more of HDAC2(-/-) mutant clones during cultivation [67, 69]. Therefore, these four transcription factors should be influential candidates participating in decreased gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation.

To explore the basis for mechanisms to vary gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF in each of HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation, we performed the NotchIP assay, using appropriate primers and five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on chromatin surrounding proximal ~2.0 kb 5'-upstream regions, distal 5'-upstream regions and open reading frames (coding regions) of these transcription factor and chromatin modifying enzyme genes (Figs. 1 ~ 20). Based on the results obtained, we assumed that loose (open) or tight (closed) form of chromatin structure surrounding the proximal 5'-upstream region of certain gene, which surely causes its high or low (or no) gene expression level, should be tentatively and

qualitatively deduced from no or full binding ability of histone H3 to DNA based on hyper- or hypo- (or no) acetylation levels of one or more of the five particular Lys residues; probably K9/H3 and K27/H3 are dominant.

In DT40 cells, as a whole, chromatin structure of the proximal ~2.0 kb 5'-upstream region of each of Pax5, Aiolos, EBF1 and OBF1 genes was loose form due to no binding ability of histone H3 to DNA based on hyper-acetylation levels of one or more of the five particular Lys residues. Consequently, these results could explain the facts that their gene expressions are obviously high levels in DT40 cells [46, 67, 69].

In mutant clone cl.2-1, chromatin structure of the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes was tight form due to the full binding ability of histone H3 to DNA based on hypo-acetylation levels of one or more of the particular Lys residues at the early stage and remained nearly unchanged until the late stage. By contrast, chromatin structure of the proximal 5'-upstream region of the OBF1 gene was considerable loose form due to the weak binding ability of histone H3 to DNA based on hyper-acetylation levels at the early stage but thereafter gradually and dramatically changed to tight form due to the full binding ability of histone H3 to DNA based on hypo-acetylation levels until the late stage. These results could explain the observations that in mutant clone cl.2-1 gene expressions of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage and remain unchanged until the late stage, and remarkably, that of the OBF1 gene is gradually and drastically decreased from the early to late stages [69].

In mutant clones cl.2-2 and cl.2-4, chromatin structure of the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes was tight form based on hypo-acetylation levels of one or more of the particular Lys residues at the early stage. Thereafter, chromatin structure of the former two changed to loose form based on hyper-acetylation levels until the late stage but that of the latter one remained unchanged based on hypo-acetylation levels during cultivation. On the other hand, chromatin structure of the proximal 5'-upstream region of the OBF1 gene was somewhat loose form based on slight hyper-acetylation levels at the early stage but changed to loose form based on hyper-acetylation levels at the late stage. These results could roughly explain the observations that gene expressions of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage in mutant clones cl.2-2 and cl.2-4, and thereafter those of the former two are drastically increased until the late stage but that of the latter one remains unchanged during cultivation [69]. In addition, the gene expression of OBF1 is slightly decreased at the early stage and thereafter slightly increased at the late stage.

In mutant clone cl.2-6, chromatin structure of the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes was tight form based on hypo-acetylation levels of one or more of the particular Lys residues at the early stage. Thereafter, chromatin structure of these three genes changed to loose form based on hyper-acetylation levels until the late stage. On the other hand, chromatin structure of the

proximal 5'-upstream region of the OBF1 gene was tight form based on hypo-acetylation levels at the early stage but changed to loose form based on hyper-acetylation levels at the late stage. These results could roughly explain the observations that gene expressions of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage and thereafter drastically increased until the late stage [69]. In addition, the gene expression of OBF1 is certainly decreased at the early stage and thereafter clearly increased at the late stage.

Results on alterations in acetylation levels (hyper or hypo) of one or more of these particular Lys residues of histone H3, form (loose or tight) of chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA levels (gene expression/transcription levels) (high or low) in four individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) are roughly and schematically represented in Figure 21.

On the other hand, chromatin structure of the proximal and distal 5'-upstream regions of the PCAF gene was loose form as a whole based on hyper-acetylation levels of the particular Lys residues in four mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 at any cultivation stages as well as in DT40 cells. By contrast, the gene expression of PCAF, which is very low in DT40 cells [46], dramatically and distinctly changes in these mutant clones during cultivation [67, 69]. Therefore, other than the tested proximal and distal 5'-upstream regions, unknown mechanisms including more distal 5'-upstream regions should be involved in the gene expression of PCAF. In addition, remarkably, acetylation levels of the particular Lys residues of histone H3 within the examined open reading frames (coding regions) of Pax5, Aiolos, EBF1, OBF1 and PCAF genes were very low in DT40 cells and remained unchanged or very slightly changed in mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation. These findings suggested that the manner and the machinery for epigenetic modifications with acetyl group of the particular Lys residues of histone H3 should be clearly different between the proximal 5'-upstream regions and open reading frames of these five genes.

Taken together, these results supported our previous results that gene expressions of IgM H- and L-chains are decreased in almost similar pattern but in distinct manners attributed to alterations in gene expression levels of Pax5, Aiolos, EBF1 and OBF1 in individual HDAC2(-/-) mutant clones during cultivation [69]. That is, concerning gene expressions of these two immunoglobulin proteins at the late cultivation stage, individual clones of HDAC2(-/-) mutants could be classified into following three distinct types [69]. Namely, the manner of mutant clone cl.2-1 seems to be dependent upon OBF1 and distinct from that of wild-type DT40 cells. The manner of mutant clones cl.2-2 and cl.2-4 seems to be dependent upon Pax5 and Aiolos and slightly similar to that of DT40 cells in appearance. Besides, mutant clones cl.2-2 and cl.2-4 seem to be major type, because they resembled two other mutant clones cl.2-3 plus cl.2-5 [69] and also four initially generated HDAC2(-/-) mutant clones cl.33-12, cl.33-28, cl.33-30 plus cl.45-28 [53] in several cellular properties [46, 67]. The manner of mutant clone cl.2-6

seems to be dependent upon Pax5, Aiolos and EBF1 and most similar to that of DT40 cells in appearance.

As mentioned above, acetylation levels of each of the five particular Lys residues of histone H3 within chromatin surrounding the proximal 5'-upstream regions are separately and complicatedly altered in obviously different patterns not only for the above-mentioned five particular genes but also in the four individual HDAC2(-/-) mutant clones. In addition, as mentioned previously [69], gene expressions of PCAF, HDAC7, HDAC9, Blimp1, E2A, Ikaros, PU.1 and XBP-1 also dramatically or moderately change in different patterns in each of these mutant clones during cultivation. These findings, combined, suggested that besides gene expressions of IgM H- and L-chains (and also Pax5, Aiolos, EBF1 and OBF1) and cell morphology [69], some other undefined cellular characteristics should be undoubtedly altered in individual clones of HDAC2(-/-) mutants during cultivation. Moreover, such presumable altered characteristics may be complicated and diverse.

In conclusion, based on these results and previous findings [41, 46, 53, 55, 58, 67-69], we propose a hypothesis on distinct manners to diminish artificially accumulated IgM H- and L-chains caused by the HDAC2-deficiency through irreversible creation of varied chromatin structure plasticity surrounding proximal 5'-upstream regions of particular transcription factor genes in individual clones of HDAC2(-/-) mutants via a lot of generations during continuous cultivation as follows. First of all, the accumulation of IgM H- and L-chains is recognized as uncomfortable environment change, and subsequently putative signal(s) concerning the accumulation is genome-widely transmitted to chromatin within nucleus. The environment change should induce alterations in chromatin structure of numerous genes encoding chromatin modifying enzymes and transcription factors (such as PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1, Oct2 and so on), resulting in a slight alteration in their gene expression levels. Next, the signal transduction and response for the environment change should be successively converged to chromatin structure surrounding the proximal 5'-upstream regions of various genes encoding particular factors and enzymes, such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC7, HDAC9 and so on. As a result, remarkably, chromatin structure surrounding the proximal 5'-upstream region (notch of chromatin) of each of Pax5, Aiolos, EBF1, OBF1 and other genes should dramatically and complicatedly change in individual clones of HDAC2(-/-) mutants via a lot of generations, resulting in drastic alterations in their gene expressions. The basis for these events is that such successive response to the environment change causes varied epigenetic modifications of chromatin structure. Of these epigenetic modifications, acetylation and deacetylation of particular Lys residues of core histones may be major ones, and participating positions of the particular Lys residues and/or kinds of core histones should be diverse. In the case mentioned above, acetylation and deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. Consequently, the epigenetic modifications of one or more of these five Lys residues of histone H3 with acetyl group should be separately altered within restricted chromatin structure surrounding the proximal 5'-upstream regions of

above-mentioned particular genes via a lot of generations during cultivation. The binding ability of the N-terminal tail of histone H3 to DNA is tentatively and qualitatively deduced based on acetylation levels of one or more of these particular Lys residues, though which Lys residue(s) is really and/or mainly involved in the binding is still unclear. Namely, hyper- (high) or hypo- (low or no) acetylation levels should induce no binding or full binding ability, resulting in loose (open) or tight (closed) form of chromatin structure. Thus, chromatin structure plasticity should be irreversibly created through successive structural changes due to binding ability of histone H3 to DNA based on changes in acetylation levels of these particular Lys residues. As a result, the loose or tight form of chromatin structure surrounding the proximal 5'-upstream regions of corresponding genes should cause their high or low (or no) gene expression levels. Notably, in the concrete, manners to create chromatin structure plasticity are distinct in individual HDAC2(-/-) mutant clones, though the accumulation of IgM H- and L-chains as the environment change is the same for all of them. That is, to exclude artificially accumulated immunoglobulin proteins, individual clones of HDAC2(-/-) mutants should differently alter chromatin structure surrounding the proximal 5'-upstream regions of particular genes, such as Pax5, Aiolos, EBF1 and OBF1 genes. Detailed manner and machinery for irreversible creation of chromatin structure plasticity, including recognition of the accumulation of IgM H- and L-chains, signal transduction pathway and chromatin conformation change, remain to be elucidated.

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Figure legends

Figure 1. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during cultivation

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Pax5 gene. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) at the early (E), middle (M) and late (L) cultivation stages and DT40 (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified twice by PCR using appropriate primers for segments 1 ~ 12 and a ~ d of the Pax5 gene (Table I). Chicken β-actin was used as control. A portion of cell supernatants as input was amplified once by PCR using the same primers. PCR products were analyzed by 1.5% agarose gel electrophoresis. Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 2. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Pax5 gene for clone cl.2-2 of HDAC2(-/-) as in Figure 1, except the middle (M) cultivation stage and antiserum specific for acetylated K23 residue of histone H3.

Figure 3. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Pax5 gene for clone cl.2-4 of HDAC2(-/-) as in Figure 1.

Figure 4. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Pax5 gene for clone cl.2-6 of HDAC2(-/-) as in Figure 1.

Figure 5. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during

cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Aiolos gene. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) at the early (E), middle (M) and late (L) cultivation stages and DT40 (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified twice (for K9 and K27) or once (for K14, K18 and K23) by PCR using appropriate primers for segments 1 ~ 14 and a ~ d of the Aiolos gene (Table II). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 6. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Aiolos gene for clone cl.2-2 of HDAC2(-/-) as in Figure 5.

Figure 7. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Aiolos gene for clone cl.2-4 of HDAC2(-/-) as in Figure 5.

Figure 8. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Aiolos gene for clone cl.2-6 of HDAC2(-/-) as in Figure 5.

Figure 9. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the EBF1 gene. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) at the early (E), middle (M) and late (L) cultivation stages and DT40 (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3.

After de-crosslinking, co-precipitated chromatins were amplified twice by PCR using appropriate primers for segments 1 ~ 14 and a ~ e of the EBF1 gene (Table III). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 10. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the EBF1 gene for clone cl.2-2 of HDAC2(-/-) as in Figure 9.

Figure 11. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the EBF1 gene for clone cl.2-4 of HDAC2(-/-) as in Figure 9.

Figure 12. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the EBF1 gene for clone cl.2-6 of HDAC2(-/-) as in Figure 9.

Figure 13. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream and ORF regions of the OBF1 gene. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) at the early (E), middle (M) and late (L) cultivation stages and DT40 (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified twice (for K9 and K27) or once (for K14, K18 and K23) by PCR using appropriate primers of segments 1 ~ 14 and a plus b of the OBF1 gene (Table IV). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 14. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants

during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream and ORF regions of the OBF1 gene for clone cl.2-2 of HDAC2(-/-) as in Figure 13.

Figure 15. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream and ORF regions of the OBF1 gene for clone cl.2-4 of HDAC2(-/-) as in Figure 13.

Figure 16. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream and ORF regions of the OBF1 gene for clone cl.2-6 of HDAC2(-/-) as in Figure 13.

Figure 17. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the PCAF gene. Cross-linked chromatins were prepared from clone cl.2-1 of HDAC2(-/-) at the early (E), middle (M) and late (L) cultivation stages and DT40 (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After de-crosslinking, co-precipitated chromatins were amplified once by PCR using appropriate primers for segments 1 ~ 14 and a ~ d of the PCAF gene (Table V). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 18. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the PCAF gene for clone cl.2-2 of HDAC2(-/-) as in Figure 17.

Figure 19. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants

during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the PCAF gene for clone cl.2-4 of HDAC2(-/-) as in Figure 17.

Figure 20. Alterations in acetylation levels of particular Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation

The NotchIP assay was carried out on chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the PCAF gene for clone cl.2-6 of HDAC2(-/-) as in Figure 17.

Figure 21. Summary on alterations in gene expression (mRNA) levels (high or low), acetylation levels (hyper or hypo) of particular Lys residues of histone H3 and chromatin structure (loose or tight) of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) DT40 mutants during cultivation