Fundamental and distinct ways for irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for gain of new cell function to exclude accumulated IgM H- and L-chains in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation[**]

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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 surrounding proximal ~2.0 kb 5'-upstream chromatin regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes in four individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of chicken histone deacetylase-2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) at the early, middle and later cultivation stages. Acetylation levels of the five specific Lys residues of these transcription factor and chromatin-modifying enzyme genes were high in DT40 cells. In clone cl.2-1, acetylation levels of one or more of the Lys residues of Pax5, Aiolos and EBF1 genes were dramatically decreased at the early stage and remained unchanged until the later stage, and those of the OBF1 gene were drastically decreased until the later 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 first two were increased until the later stage but those of the last one remained unchanged. In clone cl.2-6, acetylation levels of Pax5, Aiolos and EBF1 genes were drastically decreased at the early stage and thereafter increased until the later stage. These results could explain the previously mentioned ways for varied gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual HDAC2(-/-) clones during cultivation. We propose a hypothesis concerning distinct ways for gain of new cell function to eliminate accumulated IgM H- and L-chains in individual HDAC2(-/-) clones during continuous cultivation. They have an ability to adapt themselves to new environments by means of irreversible creation of the chromatin structure plasticity caused by successive conformational (structural) changes between the tight and loose forms depending on hypo- and hyper-acetylation levels of specific Lys residues of histone H3 surrounding proximal ~2.0 kb 5'-upstream chromatin regions of corresponding genes through various generations, indicating that DT40 cells are pluri-potent, elastic and flexible for gaining new cell function, attributed to alterations in the 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 specific Lys residues of histone H3 during cultivation, proximal 5'-upstream chromatin regions of specific 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 for 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 ways for expression of cell functions in eukaryotes. Mechanisms to modulate the chromatin structure with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination,

sumoylation, ADP ribosylation, etc., have been intensively studied in a variety of life science fields. Of such epigenetic modifications of the chromatin structure, acetylation and deacetylation of specific 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 acetylation and deacetylation (and other epigenetic modifications) are accumulating in more diverse life science fields, e.g. transcription/gene expression, DNA replication, differentiation, development, memory, pluri-potency, clinical medicine and so on [2-30].

Using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [31, 32], we have 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 as a supervisor indirectly regulates gene expressions of IgM H- and L-chains in wild-type DT40 cells through opposite controlling gene expressions of Pax5, EBF1, Aiolos, E2A and also OBF1 [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 depending on their decreased gene expressions, attributed to alterations in 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 chromatin segments surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene are slightly decreased at the early stage and thereafter certainly increased at the later stage [67].

To explore the mechanisms to diminish the accumulated IgM H- and L-chains depending on their increased gene expressions in further detail, 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 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, the accumulated immunoglobulin proteins and the aggregative form should be abnormal and uncomfortable (or painful) environments for

the mutant cells, themselves. Remarkably, the artificially accumulated IgM H- and L-chains at the early stage are dramatically reduced in almost similar pattern in all of the six HDAC2(-/-) mutant clones during cultivation and thereafter at the later stage reach comparable levels as in DT40 cells [69]. In parallel or agreed with these changes, the aggregative form of all HDAC2(-/-) mutant clones at the early stage changes at the later stage to the dispersive form, which must be normal and comfortable (or peaceful) for them, similar to those for DT40 cells [69].

Interestingly, in the six individual clones of HDAC2(-/-) mutants, mRNA (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 in cell morphology [69]. Of these changed 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 drastically reduced during cultivation until the later 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 first two are gradually elevated until the later stage but that of the last one remains unchanged as a very low level during cultivation. On the other hand, the mRNA level of OBF1 does not change by 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 later stage, but that of OBF1 slightly changes in a somewhat complicated pattern during cultivation.

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

In this study, we clarified molecular mechanisms to change 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 continuous 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 the 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 specific Lys (K) residues of histone H3 (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) separately changed within the 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 specific Lys residues induce no binding or full binding ability of histone H3 to DNA, resulting in the loose (open) or tight (closed) form of the chromatin structure, leading to high or low (or no) transcription levels of corresponding gene(s). Based on these results and assumptions, we proposed a hypothesis on the mechanisms to irreversibly create the diverse chromatin structure plasticity surrounding the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications in distinct ways to exclude IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various 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 later (L; 58 days) stages of cultivation as described in our previous studies [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 a control.

Nucleotide sequence confirmation and primer preparation

Prior to the design of the primers for the NotchIP assay, we had determined nucleotide sequences of the ~4.9 kb 5'-upstream region containing proximal ~2.0 kb 5'-upstream and some distal 5'-upstream regions and some open reading frame (ORF) regions (coding regions or cDNA) of the Pax5 gene (GenBank accession number: LC060666) [67]. We also confirmed nucleotide sequences of the proximal ~2.0 kb 5'-upstream, some distal 5'-upstream and some ORF regions of Aiolos, EBF1, OBF1 and PCAF genes, which are available from a database [67, 69]. A part of nucleotide sequences of the proximal 5'-upstream region of the EBF1 gene was newly determined. To obtain polymerase chain reaction (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 using the 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 later (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 then 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 re-suspended in 200 μl of Lysis Buffer freshly supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Wako, Osaka, Japan) and 1 μg/ml aprotinin (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 an 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 the 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, and 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 hrs 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 an 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 5'-upstream, distal 5'-upstream and ORF regions (coding regions or cDNA) of Pax5, Aiolos, EBF1, OBF1 and PCAF genes, respectively. The ORF region of the chicken β-actin was used as a control. The samples were simultaneously amplified using the same master reaction mixture in 25-μl scale. Experiments for immuno-precipitated or 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 studying ways to change chromatin structure for varied alterations in gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

Among transcription factors Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1 and OBF1, whose gene expressions changed 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 four 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 gene expressions of these two immunoglobulin proteins, since it functionally activates the chicken L-chain promoter in NIH 3T3 cells [41].

We studied how four individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) differentially gain distinct ways for gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF through various generations during cultivation. To execute the project, we closely carried out chromatin immuno-precipitation (ChIP) assay on the chromatin surrounding their proximal ~2.0 kb 5'-upstream regions, distal 5'-upstream regions and ORF regions (coding regions). Because the chromatin structure of the proximal 5'-upstream region is 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 the inference is still uncertain [67]. We designed appropriate primers based on nucleotide sequences of the proximal 5'-upstream, distal 5'-upstream and ORF regions of these five specific genes, which were cloned and determined by us or obtained from a database and confirmed 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 five genes, coincide with corresponding segments of the region and are laid overlapping to each other with the neighboring ones.

We systematically carried out the NotchIP assay on the 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 later (L; 58 days) stages of cultivation and from 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 obviously changed 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 later (L) stages of cultivation, because this case was the first attempt as the NotchIP assay, which was developed to clarify ways for the above-mentioned interesting phenomena, i.e., gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF change in different ways 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 specific Lys residues of its N-terminal tail (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) obtained by the NotchIP assay, though it is still unclear which Lys residue(s) is really and/or mainly involved in the binding. 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 the loose (open), considerable loose, somewhat loose and tight (closed) forms of the chromatin structure surrounding the proximal 5'-upstream region(s). These four forms cause 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 ORF region of the chicken β-actin gene was used as a control, since levels of its reverse transcription (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 the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF

regions of the Pax5 gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later 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 the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF 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 on those of its ORF region obtained from a 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 each other with the neighboring ones. 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 ORF region (cDNA). Since primers for segments b ~ d were designed based on nucleotide sequences from a 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 presented 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 through the middle (M) to later (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 through the middle (M) to later (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 within 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 through the middle (M) to later (L) stages, except insignificant change for the region of

positions -958 ~ -679 (segment 8). On the other hand, acetylation levels of K18/H3 within 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 through the middle (M) to later (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 within 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 through the middle (M) to later (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 possess no binding ability of histone H3 to DNA based on their hyper-acetylation levels within the 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 exhibit 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 chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the Pax5 gene, which may consist of ~10 nucleosomes, should be the loose form in DT40 cells, but change to the tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the later (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 the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-2 only at the early (E) and later (L) stages of cultivation and in 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 later (L) stage and reached comparable levels as 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 later (L) stage and reached almost the same levels as 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 later (L) stage and reached almost similar levels as 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 later (L) stage and reached almost the same levels as 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 later (L) stage and reached almost similar levels as 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 exhibit 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 later (L) stage through various generations during cultivation. Therefore, we speculated that the chromatin structure surrounding the proximal 5'-upstream region of the Pax5 gene should change to the tight form at the early (E) stage in clone cl.2-2, and thereafter, remarkably, change to the loose form until the later (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 later (L) stage and reach comparable levels as 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 the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-4 at the early (E), middle (M) and later (L) stages of cultivation and in 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 through the middle (M) until later (L) stages to comparable levels as 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 those within the entire 5'-upstream regions during

cultivation, but the acetylation levels 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 through the middle (M) until later (L) stages to almost similar levels as in DT40 cells. However, acetylation levels of K14/H3 within three ORF regions of the gene remained unchanged until the later (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 at the middle (M) or later (L) stage and reached very close or almost similar levels as in DT40 cells. On the other hand, acetylation levels of K18/H3 within three ORF regions of the gene remained unchanged until the later (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 in 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 at the middle (M) and later (L) stages and reached very close or almost similar levels as 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 later (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 through the middle (M) until later (L) stages and reached almost similar levels as in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged until the later (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 possess 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 later (L) stage. Therefore, we speculated that the chromatin structure surrounding the proximal 5'-upstream region of the Pax5 gene should change to the tight form at the early (E) stage in clone cl.2-4, and thereafter, surprisingly, change to the loose form through various

generations until the later (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 later (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 the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-6 at the early (E), middle (M) and later (L) stages of cultivation and in 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 through the middle (M) until later (L) stages and reached comparable levels as 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 through the middle (M) until later (L) stages and reached almost similar levels as 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 through the middle (M) until later (L) stages and reached almost the same levels as 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 later (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 later (L) stage reached very close or almost similar levels as in DT40 cells. On the other hand, acetylation levels of K18/H3 within three ORF regions of the gene remained unchanged until the later (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 through the middle (M) until later (L) stages and reached almost similar levels as 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 through the middle (M) until later (L) stages and reached almost the same levels as in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged until the later (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 exhibit 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 chromatin structure surrounding the proximal 5'-upstream region of the Pax5 gene should change to the tight form at the early (E) stage in clone cl.2-6, and thereafter, remarkably, change to the loose form through various generations until the later (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 the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Aiolos gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later 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 the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF 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 ORF regions of the Aiolos gene, which were designed based on nucleotide sequences from a 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 each other with the neighboring ones. 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 ORF regions (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 presented 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 through the middle (M) until later (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 through the middle (M) until later (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 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 later (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 later (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 through the middle (M) until later (L) stages.

These results indicated that five Lys residues K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 possess no binding ability of histone H3 to DNA based on their hyper-acetylation levels within the chromatin surrounding the proximal 5'-upstream region from positions -2250 ~ +145 of the Aiolos gene in DT40 cells. However, those except K23/H3 exhibit 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 chromatin structure surrounding the proximal ~2.3 kb 5'-upstream region of the Aiolos gene, which may consist of ~11 nucleosomes, should be the loose form in DT40 cells, but change to the tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the later (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 presented 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 through the middle (M) until later (L) stages as a whole. Acetylation levels of K14/H3 within the entire 5'-upstream regions and two ORF regions of the gene insignificantly changed or remained unchanged from the early (E) through middle (M) to later (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 through the middle (M) to later (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 through the middle (M) to later (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 through the middle (M) to later (L) stages as a whole.

These results indicated that the binding ability of mainly 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 becomes less 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 ability of K9/H3 (and probably K27/H3) to DNA disappears almost completely based on their hyper- or considerable hyper-acetylation levels at the later (L) stage. Therefore, we speculated that the chromatin structure surrounding the proximal 5'-upstream region of the Aiolos gene should change to the tight form at the early (E) and middle (M) stages in clone cl.2-2 and thereafter change to the loose (or considerable loose) form at the later (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 later (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 presented 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 through the middle (M) to later (L) stages as a whole, but insignificantly changed 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) through middle (M) to later (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 through the middle (M) to later (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) through middle (M) to later (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 through the middle (M) to later (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 ability 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 becomes less based on the hypo- or slight hyper-acetylation levels at the early (E) and middle (M) stages. Thereafter, mainly the binding ability of K9/H3 (and probably K27/H3) to DNA disappears almost completely based on the hyper- or considerable hyper-acetylation levels at the later (L) stage. Therefore, we speculated that the chromatin structure surrounding the proximal 5'-upstream region of the Aiolos gene should change to the tight form at the early (E) and middle (M) stages in clone cl.2-4 and thereafter change to the loose or considerably loose form at the later (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 later (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 presented 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 through the middle (M) to later (L) stages and were 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 through the middle (M) to later (L) stages and were 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 through the middle (M) to later (L) stages and were 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 later (L) stage and were 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 through the middle (M) to later (L) stages and were 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 through the middle (M) to later (L) stages and were almost the same levels as 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 ability of K9/H3 and K27/H3 to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene in clone cl.2-6 certainly becomes less 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 lose the binding ability to DNA and finally reach the state of almost no binding ability based on their hyper-acetylation levels from the middle (M) until later (L) stages. Naturally, it was possible that the extent of no binding ability at the middle (M) and later (L) stages should be less than that as in DT40 cells, because acetylation levels at both cultivation stages in clone cl.2-6 are higher than those in DT40 cells. Therefore, we speculated that the chromatin structure surrounding the proximal 5'-upstream region of the Aiolos gene should change to the tight form at the early (E) stage in clone cl.2-6 and thereafter change to the loose form at the later (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 later (L) stage [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the EBF1 gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later 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 the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF 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 ORF regions of the EBF1 gene, which were designed based on nucleotide sequences from a 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 each other with the neighboring ones. 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 ORF region (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 presented 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 through the middle (M) to later (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 through the middle (M) to later (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 insignificantly changed or remained unchanged through the middle (M) to later (L) stages as a whole. However, those did not change by 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 through the middle (M) to later (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 later (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 corresponding to positions +179 ~ +291 (segment c) of the gene, but those were relatively low in two residual ORF regions corresponding to 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 corresponding to 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 through the middle (M) to later (L) stages. On the other hand, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change by much during cultivation.

These results indicated not only that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 exhibit no binding ability of histone H3 to DNA based on their hyper-acetylation levels within the 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 possess 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 chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene, which may consist of ~10 nucleosomes, should be the loose form in DT40 cells, but change to the tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the later (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 through the middle (M) to later (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 presented 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 through the middle (M) to later (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 through the middle (M) to later (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 through the middle (M) to later (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 through the middle (M) to later (L) stages, but those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change by 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 an 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 later (L) stage. The recovered acetylation levels at the middle (M) and later (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 as 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 through the middle (M) to later (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 exhibit 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 chromatin structure surrounding the proximal 5'-upstream region of the EBF1 gene should change to the tight form at the early (E) stage in clone cl.2-2 and remain unchanged through the middle (M) to later (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 presented 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, they remained unchanged through the middle (M) until later (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 through the middle (M) to later (L) stages. However, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change by 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 an 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 through the middle (M) to later (L) stages and were 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 through the middle (M) to later (L) stages. On the other hand, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions insignificantly changed or were slightly decreased during cultivation.

These results indicated that especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) exhibit 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 chromatin structure surrounding the proximal 5'-upstream region of the EBF1 gene should change to the tight form at the early (E) stage in clone cl.2-4 and remain unchanged until the later (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 presented 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 dramatically elevated through the middle (M) to later (L) stages and reached almost similar levels as 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 through the middle (M) to later (L) stages and reached almost the same levels as 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 through the middle (M) to later (L) stages and reached almost similar levels as in DT40 cells. However, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions did not change by 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 by 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 dramatically elevated through the middle (M) to later (L) stages and reached almost the same levels as 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) exhibit 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, lose the binding capacity of histone H3 to DNA and finally reach the state of almost no binding ability based on their hyper-acetylation levels through the middle (M) to later (L) stages. Therefore, we speculated that the chromatin structure surrounding the proximal 5'-upstream region of the EBF1 gene should change to the tight form at the early (E) stage in clone cl.2-6 and thereafter change to the loose form through the middle (M) to later (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 during cultivation and at the later (L) stage reaches comparable levels as in DT40 cells [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on the chromatin surrounding proximal 5'-upstream and ORF regions of the OBF1 gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later 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 the chromatin surrounding proximal 5'-upstream and ORF 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 a 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 each other with the neighboring ones. In addition, we used primers, corresponding to positions +17 ~ +131 and +776 ~ +937 (segments a and b) of the ORF region (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 presented 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 they 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 later (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 later (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 later (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 an insignificant change 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 later (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 an 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 later (L) stage as a whole.

These results indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 exhibit no binding ability of histone H3 to DNA based on their hyper-acetylation levels within the 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 possess 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 later (L) stage. Therefore, we speculated that the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene, which may consist of ~10 nucleosomes, should be the loose form in DT40 cells. On the other hand, in clone cl.2-1 the chromatin structure should change to the considerable loose form at the early (E) stage and thereafter change to the tight form at the middle (M) and later (L) stages. As a result, the gene expression of OBF1, which is at high level in DT40 cells, is certainly decreased at the early (E) stage in clone cl.2-1 and thereafter dramatically decreased through the middle (M) to later (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 presented 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 later (L) stage. The increased acetylation levels at the later (L) stage were less than those as 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 an 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 later (L) stage.

These results indicated that especially K9/H3 and K27/H3 within the proximal 5'-upstream chromatin region of the OBF1 gene obviously possess 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 remains unchanged, and the less binding ability of K27/H3 is further increased to full binding ability based on the hypo-acetylation levels. Thereafter, the full binding ability of K9/H3 and K27/H3 is obviously decreased to no binding ability based on their hyper-acetylation levels at the later (L) stage. The extent of the binding capacity at the later (L) stage should be higher than that in DT40 cells, because the acetylation levels at the later (L) stage in clone cl.2-2 are lower than those in DT40 cells. Therefore, we speculated that in clone cl.2-2 the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene should change to the tight or somewhat loose form at the early (E) or middle (M) stage of cultivation. Thereafter, the tightened chromatin structure should

become the loose form at the later (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 through the middle (M) to later (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 presented 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 later (L) stage; the extent of the acetylation levels at the later (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 later (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 is 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 remains unchanged at the middle (M) stage but thereafter is obviously decreased to no binding ability based on their hyper-acetylation levels at the later (L) stage; the extent of the binding capacity at the later (L) stage being probably slightly higher than that in DT40 cells. Therefore, we speculated that in clone cl.2-4 the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene should change to the somewhat loose form at the early (E) and middle (M) stages and thereafter at the later (L) stage change to the 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 later (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 presented 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 through the middle (M) to later (L) stages and reached almost the same levels as 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 later (L) stage and reached almost the same levels as 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, they were obviously increased at the later (L) stage and reached almost similar or higher levels as 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 possess 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 is 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 later (L) stage. Therefore, we speculated that in clone cl.2-6 the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene should change to the tight form at the early (E) stage and thereafter change to the loose form, like that in DT40 cells, through the middle (M) to later (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 through the middle (M) to later (L) stages [69].

NotchIP assay using site-specific antibodies for acetylated K9, K14, K18, K23 and K27 residues of histone H3 on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the PCAF gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later 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 the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF 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 a 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 each other with the

neighboring ones. 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 presented 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 through the middle (M) until later (L) stages, but in clone cl.2-4 they 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 later (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 later (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, the 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) exhibit no binding ability of histone H3 to DNA based on their hyper-acetylation levels within the 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 exhibit weak binding ability of histone H3 to DNA based on their considerable hyper-acetylation levels at the early (E) stage and thereafter exhibit no binding ability based on their hyper-acetylation levels at the middle (M) and later (L) stages. Therefore, we speculated that the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of the PCAF gene, which may consist of ~10 nucleosomes, should be the loose form in DT40 cells, but change to the considerable loose form at the early (E) stage in clones cl.2-1, cl.2-2 and cl.2-4 and thereafter change to the loose form at the middle (M) and later (L) stages. On the other hand, in these three clones, the gene expression of PCAF is really a low level at the early (E) stage, like that as in DT40 cells, but dramatically increased through the middle (M) to later (L) stages during cultivation [69]. In clone cl.2-6, K9/H3 and K18/H3 possess less binding ability of histone H3 to DNA based on their somewhat

hyper-acetylation levels within the 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 lose their less binding ability to no binding ability based on their hyper-acetylation levels at the middle (M) stage and thereafter again gain less binding ability based on their slight hyper-acetylation levels at the later (L) stage. Therefore, we speculated that in clone cl.2-6 the chromatin structure surrounding the proximal 5'-upstream region of the PCAF gene should be the loose form at the middle (M) stage, like in DT40 cells, but the somewhat loose form at the early (E) and later (L) stages. On the other hand, as described previously [69], the gene expression of PCAF, which is really a very low level 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 through the middle (M) to later (L) stages to almost undetectable level.

These results, therefore, indicated that the examined distal and proximal 5'-upstream regions are not directly correlated by 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 the loose (or considerable loose) form of the chromatin structure. In consequence, we assumed that undefined ways, including other distal 5'-upstream regions, should be preferentially involved in the gene expression of PCAF in HDAC2(-/-) mutants during cultivation.

Discussion

As reported previously [67, 69], mRNA and protein levels of IgM H- and L-chains, which are very low in DT40 cells [46, 53], are dramatically increased at the early stage of cultivation in all of the examined 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), and thereafter obviously decreased in almost similar changing pattern through the middle (M) to later (L) stages and finally reach nearly equal levels as 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 these 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 should be roughly supported by the findings that the PCAF-deficient mutant 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, the respective 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, Pax5, Aiolos, EBF1 and OBF1 should be influential candidates that participate in

decreased gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation.

To explore the fundamental mechanisms that 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 the chromatin surrounding proximal ~2.0 kb 5'-upstream, distal 5'-upstream, and ORF regions of these transcription factor and chromatin-modifying enzyme genes (Figs. 1 ~ 20). Based on the results obtained, we assumed that the loose (open) or tight (closed) form of the chromatin structure surrounding the proximal 5'-upstream region of a certain gene, which surely causes its high or low (or no) gene expression level, should be 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 specific Lys residues; probably K9/H3 and K27/H3 are dominant.

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

In mutant clone cl.2-1, the chromatin structure surrounding the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is in the tight form due to the full binding ability of histone H3 to DNA based on hypo-acetylation levels of one or more of the five specific Lys residues at the early stage and thereafter remains nearly unchanged until the later stage. By contrast, the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene is in the 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 dramatically changes to the tight form due to the full binding ability of histone H3 to DNA based on hypo-acetylation levels until the later 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 thereafter remain unchanged until the later stage, but remarkably, that of the OBF1 gene is drastically decreased from the early to later stages [69].

In mutant clones cl.2-2 and cl.2-4, the chromatin structure surrounding the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is in the tight form based on hypo-acetylation levels of one or more of the five specific Lys residues at the early stage. Thereafter, the chromatin structure of the first two changes to the loose form based on hyper-acetylation levels until the later stage but that of the last one remains unchanged based on hypo-acetylation levels during cultivation. On the other hand, the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene is in the somewhat loose form based on slight hyper-acetylation levels at the early stage but thereafter changes to the loose form based on hyper-acetylation levels at the later 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 first two are drastically increased until the later stage but that of the last 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 later stage.

In mutant clone cl.2-6, the chromatin structure surrounding the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is in the tight form based on hypo-acetylation levels of one or more of the five specific Lys residues at the early stage. Thereafter, the chromatin structure of these three genes changes to the loose form based on hyper-acetylation levels until the later stage. On the other hand, the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene is in the tight form based on hypo-acetylation levels at the early stage but thereafter changes to the loose form based on hyper-acetylation levels at the later 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 later stage [69]. In addition, the gene expression of OBF1 is certainly decreased at the early stage and thereafter clearly increased at the later stage.

The results on alterations in acetylation levels (hyper or hypo) of one or more of the five specific Lys residues of histone H3, the form (loose or tight) of the chromatin structure surrounding the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (as 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 presented in Figure 21.

On the other hand, the results concerning the PCAF gene and the ORF regions of the examined five genes are as follows. The chromatin structure surrounding the proximal and distal 5'-upstream regions of the PCAF gene is in the loose form as a whole based on hyper-acetylation levels of the five specific 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], changes dramatically and distinctly in these four 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 five specific Lys residues of histone H3 within the examined ORF regions of Pax5, Aiolos, EBF1, OBF1 and PCAF genes are very low in DT40 cells and remain unchanged or very slightly change in four mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation. These findings suggested that the way and the machinery for epigenetic modifications with acetyl group of the five specific Lys residues of histone H3 should be clearly different between the proximal 5'-upstream regions and ORF regions of these five specific genes.

Taken together, these results obtained by the NotchIP assay support our previous results that gene

expressions of IgM H- and L-chains are decreased in almost similar changing pattern but in distinct ways attributed to alterations in gene expressions 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 later cultivation stage, individual clones of HDAC2(-/-) mutants could be classified into following three distinct types [69]. Namely, the way of mutant clone cl.2-1 seems to be dependent upon OBF1 and distinct from that of wild-type DT40 cells. The ways of mutant clones cl.2-2 and cl.2-4 seem 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 way of mutant clone cl.2-6 seems to be dependent upon Pax5, Aiolos and EBF1 and mostly similar to that of DT40 cells in appearance.

As mentioned above, acetylation levels of each of the five specific Lys residues of histone H3 within the chromatin surrounding the proximal 5'-upstream regions separately and complicatedly change in obviously different patterns not only in the Pax5, Aiolos, EBF1 and OBF1 genes but also in the four individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6). These results should be fundamentals of the above-mentioned inferences on gene expressions of IgM H- and L-chains. 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 four mutant clones during cultivation. These findings, combined, suggest that besides alterations in gene expressions of IgM H- and L-chains (and also Pax5, Aiolos, EBF1 and OBF1) and in cell morphology [69], some other undefined cellular characteristics might be undoubtedly and separately changing among the four individual clones of HDAC2(-/-) mutants during cultivation. Moreover, such presumable changed 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 ways to diminish artificially accumulated IgM H- and L-chains caused by the HDAC2-deficiency by means of irreversible creation of the varied chromatin structure plasticity surrounding proximal 5'-upstream regions of the specific transcription factor genes in individual clones of HDAC2(-/-) mutants through various generations during continuous cultivation as follows. First of all, the accumulation of IgM H- and L-chains is recognized as an abnormal and uncomfortable environment change, and subsequently putative signal(s) concerning the accumulation is genome-widely transmitted to the chromatin within the nucleus. The abnormal environment change should induce slight alterations in the chromatin structure of the numerous genes encoding the chromatin-modifying enzymes and transcription factors (such as PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1, Oct2, etc.), resulting in a slight alteration in their gene expression levels.

Subsequently, the signal transduction and response to the environment change should be successively converged to the chromatin structure surrounding the proximal 5'-upstream regions of the various genes encoding specific transcription factors and chromatin-modifying enzymes, such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC7, HDAC9 and so on. As a result, remarkably, the 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 through various generations, resulting in drastic alterations in their gene expressions. That is, the basis of these remarkable events is that the successive response to the environment change causes varied epigenetic modifications of the chromatin structure. Of these epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones may be major ones, and then the participating positions of the specific 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 the restricted chromatin structure surrounding the proximal 5'-upstream regions of the above-mentioned specific genes through various generations during cultivation. The binding ability of the N-terminal tail of histone H3 to DNA is qualitatively deduced based on acetylation and/or deacetylation levels of one or more of these specific Lys residues, though it is still unclear which Lys residue(s) is really and/or mainly involved in the binding. Namely, hyper- (high) or hypo- (low or no) acetylation levels should induce no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure. Thus, the 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 and/or deacetylation levels of these specific Lys residues. As a result, the loose or tight form of the 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, ways to create the chromatin structure plasticity are distinct in individual HDAC2(-/-) mutant clones, though the accumulation of IgM H- and L-chains as the abnormal environment change is the same for all of them. That is, to exclude artificially accumulated immunoglobulin proteins, individual clones of HDAC2(-/-) mutants should differently change the chromatin structure surrounding the proximal 5'-upstream regions of specific genes encoding Pax5, Aiolos, EBF1 and OBF1. Detailed way and machinery to irreversibly create the 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.

Conflict of interest statement

There are no conflicts of interest.

Postscript

The studies in Ref. 69 were reviewed in Ref. 70. The studies in the article were reviewed in Ref. 71.

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Figure legends

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

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay was carried out on the 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 later (L) cultivation stages and from DT40 (W), and co-precipitated by five 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 a control. A portion of cell supernatants as an 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal

5'-upstream chromatin region of Aiolos gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 later (L) cultivation stages and from DT40 (W), and co-precipitated by five 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 later (L) cultivation stages and from DT40 (W),

and co-precipitated by five 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 later (L) cultivation stages and from DT40 (W), and co-precipitated by five 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 specific Lys residues of histone H3 within proximal

5'-upstream chromatin region of OBF1 gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-1 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 later (L) cultivation stages and from DT40 (W), and co-precipitated by five 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-2 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal

5'-upstream chromatin region of PCAF gene in clone cl.2-4 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of PCAF gene in clone cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation.

The NotchIP assay was carried out on the 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 of alterations in gene expression (mRNA) levels (high or low), acetylation levels (hyper or hypo) of specific Lys residues of histone H3 and the chromatin structure (loose or tight) surrounding 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 continuous cultivation.