Chapter 5

A fundamental way for irreversible creation of chromatin structure plasticity with epigenetic modifications for gaining new cell function to exclude IgM H- and L-chains accumulated in HDAC2(-/-) DT40 mutants through various generations during continuous cultivation

by Masami Nakayama and Tatsuo Nakayama

SUMMARY

We studied acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 within the chromatin surrounding proximal ~2.0 kb 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) during continuous cultivation by neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay. Acetylation levels of these five Lys residues of the four genes were high in DT40 cells. In clone cl.2-1, acetylation levels of one or more of these Lys residues of Pax5, Aiolos and EBF1 genes were almost completely suppressed at the early stage of cultivation and remained unchanged until the later stage, and those of the OBF1 gene were dramatically decreased until the later stage. In clones cl.2-2 and cl.2-4, acetylation levels of Pax5, Aiolos and EBF1 genes were almost completely suppressed at the early stage, and thereafter those of the first two were obviously elevated 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 almost completely suppressed at the early stage and thereafter obviously elevated until the later stage. These results could fully explain the previous-mentioned ways for varied gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual HDAC2(-/-) mutant clones during cultivation [Chap. 4], indicating that the chicken DT40 cell line is pluri-potent, elastic and flexible for gaining new cell function attributed to alterations in the chromatin structure.

We proposed a hypothesis concerning distinct ways for gaining new cell function to eliminate IgM H- and L-chain proteins artificially accumulated in individual HDAC2(-/-) mutant clones during cultivation. They acquire an ability to adapt themselves to newly encountered abnormal environment change, by means of irreversible creation of the chromatin structure plasticity caused by successive structural alterations between the tight and loose forms depending on hypo- and hyper-acetylation levels of specific Lys residues of histone H3 within proximal ~2.0 kb 5'-upstream chromatin regions of corresponding transcription factor genes through various generations.

INTRODUCTION

Our previous results obtained from both initially generated HDAC2- and Pax5-deficient DT40 mutants, HDAC2(-/-) [1, Chap. 2] and Pax5(-) [Chap. 3], revealed that IgM H- and L-chains artificially accumulated at the early stage of cultivation in these mutants are dramatically diminished depending on their decreased gene expressions, attributed to changes in gene expressions of various transcription factors and chromatin-modifying enzymes during continuous cultivation. In addition, interestingly, our qualitative chromatin immuno-precipitation (ChIP) assay on the initially generated HDAC2(-/-) mutant clones suggested that acetylation levels of Lys-9 residues of histone H3 (K9/H3) within some regional segments of the chromatin 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 [Chap. 2].

To further explore mechanisms to diminish the accumulated IgM H- and L-chain proteins depending on their increased gene expressions, we again generated and analyzed HDAC2-deficient DT40 mutants, HDAC2(-/-) [Chap. 4]. As expected, in six tested HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6, IgM H- and L-chains are dramatically and considerably accumulated at the early cultivation stage, because HDAC2 regulates gene expressions of these two immunoglobulin proteins through controlling gene expressions of Pax5, EBF1, Aiolos, E2A and also OBF1 in wild-type DT40 cells [2-4, Chaps. 2, 3 and 4]. 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(-/-) mutants [3, Chap. 4]. In addition, HDAC2(-/-) mutant cells at the early stage exist as a morphologically aggregative form [Chap. 4], the reason for which is still unknown. Anyway, both of the accumulated immunoglobulin proteins and the aggregative form should be abnormal and uncomfortable (or painful) for the mutant cells, themselves. Remarkably, the artificially accumulated IgM H- and L-chains at the early stage are gradually reduced in almost similar pattern in all of the six HDAC2(-/-) mutant clones during cultivation and thereafter at the later stage reach comparable levels as in DT40 cells [Chap. 4]. 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 comfortable (or peaceful) for these mutant cells, similarly for DT40 cells [Chap. 4].

Interestingly, in these six individual clones of HDAC2(-/-) mutants, gene expressions of PCAF, HDAC7, HDAC9, Pax5, Aiolos, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1 and others dramatically or considerably change in distinct patterns during cultivation, though these mutant clones show almost the same changing pattern in protein and mRNA levels of IgM H- and L-chains and in cell morphology as mentioned above [Chap. 4]. Of these changed chromatin-modifying enzymes and transcription factors, Pax5, Aiolos, EBF1 and OBF1 are 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 [3, 4, Chaps. 2 and 4], are dramatically reduced at the early stage and remain unchanged during cultivation. By contrast, the mRNA level of OBF1, which probably up-regulates gene expressions of the two immunoglobulin proteins [2], is gradually and drastically reduced during cultivation until the later stage. In four 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 undetectable level during cultivation. However, 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 decreased at the early stage and thereafter gradually increased until the later stage, but that of OBF1 slightly changes in a somewhat complicated pattern during cultivation.

These findings, together, led the following interesting speculation on the ways for gene expressions of

IgM H- and L-chains at the later stage in individual HDAC2(-/-) mutant clones [Chap. 4]. The way of clone cl.2-1 seems to be dependent on OBF1 and distinct from that of DT40. The ways of 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 in appearance. Moreover, these four clones should be major type, since four initially generated HDAC2(-/-) clones resembled them in several cellular properties [3, Chap. 2]. The way of clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 and mostly similar to that of DT40 in appearance.

In this study, we clarified molecular mechanisms to change gene expressions of Pax5, Aiolos, EBF1, OBF1 and 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 the four mutant clones. Surprisingly, acetylation levels of one or more of specific Lys 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 of these acetylation levels for the above-mentioned four genes were distinct in the four individual HDAC2(-/-) mutant clones with each other, 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 induces 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 others, we proposed a hypothesis on mechanisms to eliminate the artificially accumulated immunoglobulin proteins by means of irreversible creation of the chromatin structure plasticity with epigenetic modifications in distinct ways in individual clones of HDAC2(-/-) mutants through various generations during continuous cultivation.

RESULTS

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay: A new method for clarification of ways to diversely change gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF in individual clones of HDAC2(-/-) mutants during continuous cultivation

Among transcription factors Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1 and OBF1, gene expressions of which changed in HDAC2(-/-) mutants during cultivation, Pax5, Aiolos, EBF1 and OBF1 are influential candidates participating in the 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 [Chap. 4]. The validity of this inference was supported by the findings that changing patterns of these factor gene expressions are in anti-parallel or in parallel with those of the immunoglobulin gene expressions in one or

more of these six individual 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 [3, 4, Chap. 4], and OBF1 was suggested to up-regulate these two immunoglobulin gene expressions, since it functionally activates the chicken L-chain promoter in NIH 3T3 cells [2].

In this article, we studied how do individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 differentially gain distinct ways for positive or negative regulations of gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF through various generations during cultivation. To execute the project, we carried out chromatin immuno-precipitation (ChIP) assay on the chromatin surrounding their proximal ~2.0 kb 5'-upstream regions, distal 5'-upstream regions and open reading frame (ORF) regions (coding regions). Because the chromatin structure of the proximal 5'-upstream region is directly and closely related to transcription activity of the corresponding gene, regardless of the presence or absence of transcriptional elements within the region. 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 DNA fragments amplified by PCR using appropriate primers, which were designed from nucleotide sequences of the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of the above-mentioned genes, coincide with corresponding segments of the region and are laid overlapping to each other with neighboring ones.

We systematically performed the NotchIP assay on the chromatin in 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 in 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. We also 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 conformed by us (Suppl. Tables 5-SI to 5-SV). However, in the case of 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 the detailed mechanisms of the above-mentioned interesting phenomena, i.e., the gene expressions of Pax5, Aiolos, EBF1, OBF1 and PCAF changed 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 from acetylation levels of the specific Lys (K) residues of its N-terminal tail (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) obtained by the NotchIP assay, though which Lys residue(s) is really and/or mainly involved in the binding is still unknown. That is, hyper- (high), considerable hyper-, somewhat hyper- and hypo- (low or no) acetylation levels of one or more of these five Lys

residues 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, leading to high, considerable high, somewhat high and low (or no) transcription levels of corresponding gene(s), respectively.

In this study, the stages (and other periods) of cultivation were practically counted from the first day of cultivation from the stock of the mutant clones at -80 °C. The ORF region of the chicken β-actin gene was used as a control, since the levels of its PCR-amplified products for all examined mutant clones at any cultivation stages were kept constant to be approximately 85-110% of the average value for DT40 cells (see Figs. 5-1 ~ 5-20). In addition, to simplify description of this article, we preferentially used "region(s)" for "chromatin region(s)", "stage(s)" for "stage(s) of cultivation" and "ORF" for open reading frame(s) (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 at early, middle and later stages of continuous cultivation in individual clones of HDAC2(-/-) mutants

Because changing patterns in the gene expression of Pax5 during cultivation were different in individual clones of HDAC2(-/-) mutants [Chap. 4], we carried out the NotchIP assay on the chromatin surrounding the 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, and in DT40 as a control (Figs. 5-1 ~ 5-4). 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 [see Chap. 2] and on those of its ORF region obtained from a database and confirmed by us (Suppl. Table 5-SI). Regarding the proximal 5'-upstream region of positions -1923 ~ +30, we used primers for segments 1 to 12, all of which are laid overlapping to each other with 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 the primers for segments b to 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 of clone cl.2-1 during cultivation are presented in Figure 5-1. Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream chromatin regions (segments a and 1 to 12) of the Pax5 gene were high in DT40 cells (W). Surprisingly, in spite of the HDAC2-deficiency, those were almost completely

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 three ORF regions of the gene (segments b to d) were a very low in DT40 cells (W) (see many PCR cycle numbers) and further reduced at any cultivation stages in clone cl.2-1, except insignificant changing pattern for the region of positions +223 ~ +391 (segment c). The reason for the 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 reduced 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 the insignificant change is also unknown. On the other hand, acetylation levels of K14/H3 in three ORF regions of the gene were a 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 reduced 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 in three ORF regions of the gene were a 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 reduced 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 in three ORF regions of the gene resembled those within the entire 5'-upstream regions, but in clone cl.2-1 those were reduced 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. Similar to changing patterns for K9/H3, those were almost completely reduced 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 a 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 to +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 structure of the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of

the Pax5 gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells, but changes to the tight form at the early (E) stage in clone cl.2-1 and thereafter remains 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 [see Chap. 4].

As mentioned above, since the NotchIP assay for the Pax5 gene in clone cl.2-2 was the first employment in these investigations, we carried out the assay 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), using only four site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3 and K27/H3 (Fig. 5-2). 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 to 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 to almost the same levels as in DT40 cells, but insignificantly changed within the residual 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), respectively, which showed no change and insignificant reduction during cultivation. The reduced acetylation levels were dramatically elevated at the later (L) stage to 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 to 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 to 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 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 structure of the chromatin surrounding the proximal 5'-upstream region of the Pax5 gene changes to the tight form at the early (E) stage in clone cl.2-2, and thereafter, remarkably, changes 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 to comparable levels as in DT40 cells [see Chap. 4].

Next, we carried out the NotchIP assay 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), using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 (Fig. 5-3). 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 the entire 5'-upstream regions during cultivation, but those within two other ORF regions (segments c and d) remained unchanged. Acetylation levels of K14/H3 within the entire 5'-upstream regions of the gene were dramatically reduced at the early (E) stage in clone cl.2-4, except a slight change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels were elevated 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 an insignificant change for the region of positions -958 ~ -679 (segment 8). The reduced acetylation levels of K18/H3 were dramatically elevated more quickly compared with those of K9/H3 and K14/H3 and at the middle (M) or 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.2-4. Acetylation levels of K23/H3 within the entire 5'-upstream regions and three ORF regions of the Pax5 gene were slightly lower in both DT40 and clone cl.2-4 than those observed in clones cl.2-1 and cl.2-6 (see many PCR cycle numbers) as a whole in appearance, probably because the potency of the antibody used in this assay was lower than that of the antibody used for other clones. Anyway, acetylation levels of K23/H3 within the proximal 5'-upstream region of the gene were certainly reduced at the early (E) stage in clone cl.2-4. The reduced acetylation levels were quickly elevated and at the middle (M) and later (L) stages reached very close and 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 of the gene (segments b and c) remained unchanged during cultivation, but those for another ORF region (segment d) were decreased 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 to 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 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 structure of the chromatin surrounding the proximal 5'-upstream region of the Pax5 gene changes to the tight form at the early (E) stage in clone cl.2-4, and thereafter, surprisingly, changes 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 [see Chap. 4].

Finally, we carried out the NotchIP assay 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), using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 (Fig. 5-4). 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) to later (L) stages to 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 increased through the middle (M) until later (L) stages to 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 to 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 K27/H3) in clone cl.2-6. That is, acetylation levels of K23/H3 were slightly decreased 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 increased through the middle (M) until later (L) stages to 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 to 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 to 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 early (E) stage in clone cl.2-6. Surprisingly, thereafter, these Lys residues gradually lose the binding capacity of histone H3 to DNA to no binding ability based on their hyper-acetylation levels during cultivation. Therefore, we speculated that the structure of the chromatin surrounding the proximal 5'-upstream region of the Pax5 gene 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 [see Chap. 4].

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 at early, middle and later stages of continuous cultivation in individual clones of HDAC2(-/-) mutants

Because changing patterns in the gene expression of Aiolos during cultivation were distinct in individual clones of HDAC2(-/-) mutants [Chap. 4], 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, and in DT40 cells (Figs. 5-5 ~ 5-8). Throughout the NotchIP assay, we used five site-specific antibodies for 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. These primers were designed based on nucleotide sequences from a database and confirmed by us (Suppl. Table 5-SII). Regarding the proximal 5'-upstream region of positions -2250 ~ +145, we used primers corresponding segments 1 to 14, all of which are laid

overlapping to each other with 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 region (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 5-5. Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream regions (segments a, b and 1 to 14) and two ORF regions (segments c and d) of the Aiolos gene were high in DT40 cells. 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 to 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 the residual ORF region (segment c). Acetylation levels of K18/H3 within the entire 5'-upstream regions and two ORF regions of the gene were high in DT40 cells. In clone cl.2-1, those were slightly reduced at the early (E) stage, and further reduced at the middle (M) stage but remained unchanged at the 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 from the middle (M) to 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 to +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 structure of the chromatin surrounding the proximal ~2.3 kb 5'-upstream region of the Aiolos gene, which may consist of ~11 nucleosomes, is the loose form in DT40 cells, but changes to the tight form at the early (E) stage in clone cl.2-1 and thereafter remains 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 [see Chap. 4].

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 5-6. Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1 to 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 increased 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 increased or remained unchanged through the middle (M) until later (L) stages as a whole.

These results indicated that mainly the binding capacity of K9/H3 and K27/H3 (and probably K18/H3 and K23/H3) to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene is strengthened to less binding ability based on their hypo- or slight hyper-acetylation levels at the early (E) and middle (M) stages in clone cl.2-2 during cultivation. Thereafter, predominantly, the binding capacity of K9/H3 (and probably K27/H3) to DNA is obviously weakened to no binding ability based on their hyper- or considerable hyper-acetylation levels at the later (L) stage. Therefore, we speculated that the structure of the chromatin surrounding the proximal 5'-upstream region of the Aiolos gene changes to the tight form at the early (E) and middle (M) stages in clone cl.2-2 and thereafter changes 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 [see Chap. 4].

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 5-7. Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1 to 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 increased through the middle (M) to later (L) stages as a whole, but insignificantly changed within the 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 increased or remained unchanged through the middle (M) until 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 capacity of K9/H3 (and probably K18/H3 and K27/H3) to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene in clone cl.2-4 is strengthened to less binding ability based on the hypo- or slight hyper-acetylation levels at the early (E) and middle (M) stages. Thereafter, mainly the binding capacity of K9/H3 (and probably K27/H3) to DNA is certainly weakened to no binding ability based on the hyper- or considerable hyper-acetylation levels at the later (L) stage. Therefore, we speculated that the structure of the chromatin surrounding the proximal 5'-upstream region of the Aiolos gene changes to the tight form at the early (E) and middle (M) stages in clone cl.2-4 and thereafter changes to the loose (or considerable 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 [see Chap. 4].

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 5-8. Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1 to 14) and two ORF regions (segments c and d) of the gene were apparently reduced at the early (E) stage in clone cl.2-6 and thereafter gradually increased through the middle (M) to later (L) stages to be clearly higher than those as 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 to be considerably higher than those as in DT40 cells. Acetylation levels of K18/H3 within the entire 5'-upstream regions of the gene were very slightly reduced at the early (E) stage in clone cl.2-6 and thereafter gradually elevated through the middle (M) to later (L) stages to be considerably higher than those as 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 to be certainly higher than those as 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 to 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 to be higher than those as in DT40 cells. On the other hand, those of K27/H3 within the 5'-upstream

region down from position -1232 (segments 7 to 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 to 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 capacity (to DNA) of K9/H3 and K27/H3 to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene in clone cl.2-6 is certainly strengthened to less binding ability based on their hypo- or slight hyper-acetylation levels at the early (E) stage. Thereafter, K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) gradually lose the binding capacity to DNA to no binding ability based on their hyper-acetylation levels through the middle (M) until later (L) stages. Naturally, it is possible that the extent of no binding ability at the middle (M) and later (L) stages is less than that as in DT40 cells, because the acetylation levels at both cultivation stages in clone cl.2-6 are higher than those as in DT40 cells (W). Therefore, we speculated that the structure of the chromatin surrounding the proximal 5'-upstream region of the Aiolos gene changes to the tight form at the early (E) stage in clone cl.2-6 and thereafter changes 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 [see Chap. 4].

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 at early, middle and later stages of continuous cultivation in individual clones of HDAC2(-/-) mutants

Because changing patterns in the gene expression of EBF1 during cultivation were distinct in individual clones of HDAC2(-/-) mutants [Chap. 4], 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, and in DT40 cells (Figs. 5-9 ~ 5-12). 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. These primers were designed based on nucleotide sequences from a database and confirmed or determined by us (Suppl. Table 5-SIII). Regarding the proximal 5'-upstream region of positions -2031 ~ +200, we used primers recognizing respective segments 1 to 14, all of which are laid overlapping to each other with 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 5-9. Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream regions (segments a, b and 1 to 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 ORF regions (segments d and e) of the gene were a 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 increased 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 within 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 did not change by much. 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 to 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 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 to 5). Acetylation levels of K27/H3 were considerably high within the entire 5'-upstream regions and the ORF region of positions +179 ~ +291 (segment c) of the gene, but those were relatively low within two ORF regions of positions +649 ~ +768 and +787 ~ +900 (segments d and e) (see many PCR cycle numbers) in DT40 cells. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -1037 ~ +200 (segments 7 to 14) and +179 ~ +291 (segment c) were certainly 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, 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 to +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 structure of the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the EBF1 gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells, but changes to the tight form at the early (E) stage in clone cl.2-1 and thereafter remains 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 [see Chap. 4].

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 5-10. Acetylation levels of K9/H3 within the entire (proximal and distal) 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 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 increased 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 to 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 insignificant changes within the proximal 5'-upstream region of positions -1299 ~ -598 (segments 6 to 9). The reduced acetylation levels were slightly increased 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 as 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 to 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 to 14) and +179 ~ +291 (segment c) were dramatically or almost

completely decreased 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 structure of the chromatin surrounding the proximal 5'-upstream region of the EBF1 gene changes to the tight form at the early (E) stage in clone cl.2-2 and remains unchanged through the middle (M) until 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 [see Chap. 4].

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 5-11. Acetylation levels of K9/H3 within the entire (proximal and distal) 5'-upstream regions and the ORF region of positions +179 ~ +291 (segment c) of the EBF1 gene were dramatically or almost completely reduced at the early (E) stage in clone cl.2-4. Thereafter, those 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 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 to 14) and +179 ~ +291 (segment c) of the gene were slightly reduced 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 reduced at the early (E) stage in clone cl.2-4, except insignificant changes within the proximal 5'-upstream and ORF regions of positions -1299 ~ -598 (segments 6 to 9), +649 ~ +768 and +787 ~ +900 (segments d and e). The reduced acetylation levels were slightly increased through the middle (M) to later (L) stages to somewhat lower than those as 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 to +200 (segments 7 to 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 structure of the chromatin surrounding the proximal 5'-upstream region of the EBF1 gene changes to the tight form at the early (E) stage in clone cl.2-4 and remains 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 [see Chap. 4].

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 5-12. Acetylation levels of K9/H3 within the distal 5'-upstream and proximal 5'-upstream regions (segments b and 1 to 14) and the ORF region of positions +179 ~ +291 (segment c) of the EBF1 gene were almost completely or dramatically reduced at the early (E) stage in clone cl.2-6. Interestingly, the reduced acetylation levels were gradually and dramatically elevated through the middle (M) until later (L) stages to 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 to 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 increased through the middle (M) until later (L) stages to 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 to 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 increased through the middle (M) until later (L) stages to 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 to 14) and +179 ~ +291 (segment c) of the gene were dramatically reduced at the early (E) stage in clone cl.2-6 and thereafter increased gradually and dramatically through the middle (M) to later (L) stages to 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 to no binding ability based on their hyper-acetylation levels through the middle (M) until later (L) stages. Therefore, we speculated that the structure of the chromatin surrounding the proximal 5'-upstream region of the EBF1 gene changes to the tight form at the early (E) stage in clone cl.2-6 and thereafter changes to the loose form through the middle (M) until 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 elevated until the later (L) stage to comparable levels as in DT40 cells [see Chap. 4].

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 at early, middle and later stages of continuous cultivation in individual clones of HDAC2(-/-) mutants

Because changing patterns in the gene expression of OBF1 during cultivation were distinct in individual clones of HDAC2(-/-) mutants [Chap. 4], 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, and in DT40 cells (Figs. 5-13 ~ 5-16). 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. These primers were designed based on nucleotide sequences from a database and confirmed or determined by us (Suppl. Table 5-SIV). Regarding the proximal 5'-upstream region of positions -2138 ~ +164, we used primers for respective segments 1 to 14, all of which are laid overlapping to each other with 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 5-13. Acetylation levels of K9/H3 within the proximal 5'-upstream region (segments 1 to 14) and two ORF regions (segments a and b) of the OBF1 gene were high in DT40 cells but those were certainly reduced at the early (E) stage in clone cl.2-1 as a whole. Interestingly, the reduced acetylation levels were further reduced to undetectable levels at the middle (M) stage and thereafter remained unchanged until the 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 increased 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 insignificant changes within the proximal 5'-upstream region of positions -1493 ~ -1068 (segments 5 and 6). Further, the reduced acetylation levels were very slightly reduced at the middle (M) stage and thereafter remained unchanged at 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 to +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 is 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 structure of the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the OBF1 gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells. On the other hand, in clone cl.2-1 the chromatin structure changes to the considerable loose form at the early (E) stage and thereafter changes to the tight form at the middle (M) and later (L) stages. As a result, the gene expression of OBF1, which is high level in DT40 cells, is certainly decreased at the early (E) stage in clone cl.2-1 and thereafter further and dramatically decreased through the middle (M) to later (L) stages to a very low level [see Chap. 4].

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 5-14. 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 reduced at the early (E) stage as a whole. Interestingly, the reduced 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 reduced 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 reduced 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 is higher than that as in DT40 cells, because the acetylation levels at the later (L) stage in clone cl.2-2 are lower than those as in DT40 cells. Therefore, we speculated that in clone cl.2-2 the structure of the chromatin surrounding the proximal 5'-upstream region of the OBF1 gene changes to the tight or somewhat loose form at the early (E) or middle (M) stage of cultivation. Thereafter, the tightened chromatin structure becomes the loose form at the later (L) stage, which may be slightly tighter than that as 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 [see Chap. 4].

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 5-15. 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 increased at the later (L) stage; the extent of the acetylation levels at the later (L) stage being less than those 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.2-4 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 decreased at the early (E) stage in clone cl.2-4, and remained unchanged at the middle (M) stage as a whole but thereafter slightly increased at the later (L) stage, except no changes 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 or slightly increased to weak or less binding ability based on their considerable or slight hyper-acetylation levels at the early (E) stage in clone cl.2-4. The weak or less binding ability remained unchanged at the middle (M) stage but

thereafter 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 as in DT40 cells. Therefore, we speculated that in clone cl.2-4 the structure of the chromatin surrounding the proximal 5'-upstream region of the OBF1 gene changes to the somewhat loose form at the early (E) and middle (M) stages and thereafter at the later (L) stage changes to the loose form, which is almost similar to that as 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 [see Chap. 4].

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 ~~re~~presented in Figure 5-16. 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.2-6. The reduced acetylation levels were gradually increased through the middle (M) until later (L) stages to 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 to 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 to 14) and two ORF regions of positions +17 ~ +131 and +776 ~ +937 (segments a and b) were slightly reduced at the early (E) and middle (M) stages and thereafter certainly increased at the later (L) stage to 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, those were obviously increased at the later (L) stage to 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 reduced 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 structure of the chromatin surrounding the proximal 5'-upstream region of the OBF1 gene changes to the tight form at the early (E) stage and thereafter changes to the loose form, like that as in DT40 cells, through the middle (M) until 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 [see Chap. 4].

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 at early, middle and later stages of continuous cultivation in individual clones of HDAC2(-/-) mutants

Because changing patterns in the gene expression of PCAF during cultivation were distinct in individual clones of HDAC2(-/-) mutants [Chap. 4], we carried out the NotchIP assay on the chromatin surrounding the 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, and in DT40 cells (Figs. 5-17 ~ 5-20). 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. These primers were designed based on nucleotide sequences from a database and confirmed or determined by us (Suppl. Table 5-SV). Regarding the proximal 5'-upstream region of positions -2005 ~ +231, we used primers recognizing respective segments 1 to 14, which are laid overlapping to each other with 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 to 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 5-17 ~ 5-20. In DT40 cells, 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 those remained unchanged as a whole during cultivation. In addition, in clones cl.2-1, cl.2-2 and cl.2-4, acetylation levels of K18/H3 within the entire 5'-upstream and two ORF regions of the gene were slightly decreased at the early (E) stage and thereafter slightly increased at the middle (M) and 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 [see Chap. 4], led to tentative conclusions on correlation among the acetylation levels of the five Lys residues, the binding ability (capacity) of histone

H3 to DNA, the chromatin structure and the gene expression levels of PCAF 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 to +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 structure of the chromatin surrounding the proximal ~2.0 kb 5'-upstream region of the PCAF gene, which may consist of ~10 nucleosomes, is the loose form in DT40 cells, but changes to the considerable loose form at the early (E) stage in clones cl.2-1, cl.2-2 and cl.2-4 and thereafter changes 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 very low at the early (E) stage, like that in DT40 cells, but dramatically increased through the middle (M) to later (L) stages during cultivation [see Chap. 4]. 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 to +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 structure of the chromatin surrounding the proximal 5'-upstream region of the PCAF gene is 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 [see Chap. 4], the gene expression of the PCAF gene, which is really a very low in DT40 cells, is gradually increased from the early (E) stage to the stage nearly prior the middle (M) stage in clone cl.2-6 but thereafter dramatically decreased through the middle (M) to later (L) stages to an 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 continuous cultivation.

DISCUSSION

As mentioned previously [Chaps. 2 and 4], mRNA and protein levels of IgM H- and L-chains, which are a very low in DT40 cells [1], are dramatically and certainly increased at the early stage of cultivation

in all of 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 and drastically decreased in almost similar changing pattern through the middle (M) to later (L) stages to nearly equal levels as in DT40 cells. Since alterations in gene expressions of PCAF, HDAC7 and HDAC9 are neither in parallel nor in anti-parallel with those of IgM H- and L-chains in one or more of the examined HDAC2(-/-) mutant clones during cultivation [Chap. 4], these three chromatin-modifying enzymes are not directly and/or mainly participated in alterations in gene expressions of the two immunoglobulin proteins. These results are roughly supported by the findings that the PCAF-deficient mutant or the HDAC7-deficient mutant shows a slight or no influence on gene expressions of IgM H- and L-chains [3]. Apart from that, the HDAC9-deficient one is not available yet. By contrast, Pax5, Aiolos and EBF1 or OBF1 are verified or strongly suggested to participate in down- or up-regulation of gene expressions of IgM H- and L-chains [2-4, Chap. 4]. Noticeably, respective gene expressions of these four transcription factors change in anti-parallel or in parallel with those of the two immunoglobulin proteins in one or more of HDAC2(-/-) mutant clones during cultivation [Chaps. 2 and 4]. Therefore, these four transcription factors are influential candidates participating in decreases in gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation.

To explore the basis for mechanisms of varied gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF in HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6, we performed the NotchIP assay 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, using appropriate primers and five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 (Figs. 5-1 ~ 5-20). 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) transcription level, is tentatively and qualitatively deduced from no or full binding ability of histone H3 to DNA based on hyper- or hypo- (or no) acetylation levels of one or more of the five specific Lys residues within the region; probably K9/H3 and K27/H3 are dominant.

In DT40 cells, as a whole, the chromatin structure of the proximal ~2.0 kb 5'-upstream region of each of Pax5, Aiolos, EBF1 and OBF1 genes is the loose form due to no binding ability of histone H3 to DNA depending on the hyper-acetylation levels of any of the five specific Lys residues within the region. Consequently, these results can explain the facts that their gene expressions are obviously high levels in DT40 cells [3, Chaps. 2 and 4].

In mutant clone cl.2-1, the chromatin structure of the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is the tight form due to the full binding ability of histone H3 to DNA depending on the hypo-acetylation levels of one or more of the specific Lys residues at the early stage and remains nearly unchanged until the later stage. By contrast, the chromatin structure of the proximal 5'-upstream

region of the OBF1 gene is the considerable loose form due to the weak binding ability of histone H3 to DNA based on the hyper-acetylation levels at the early stage but thereafter gradually and dramatically changes to the tight form due to the full binding ability of histone H3 to DNA depending on the hypo-acetylation levels until the later stage. These results can explain the observations that in mutant clone cl.2-1 gene expressions of Pax5, Aiolos and EBF1 are dramatically reduced at the early stage and remains unchanged until the later stage, and remarkably, that of the OBF1 gene is gradually and drastically reduced from the early to later stages [Chap. 4].

In mutant clones cl.2-2 and cl.2-4, the chromatin structure of the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is the tight form depending on the hypo-acetylation levels of one or more of the specific Lys residues at the early stage. Thereafter, the chromatin structure of the first two changes to the loose form depending on the hyper-acetylation levels until the later stage but that of the last one remains unchanged depending on the hypo-acetylation levels during cultivation. On the other hand, the chromatin structure of the proximal 5'-upstream region of the OBF1 gene is the somewhat loose form depending on the slight hyper-acetylation levels at the early stage but changes to the loose form depending on the hyper-acetylation levels at the later stage. These results can roughly explain not only the observations that gene expressions of Pax5, Aiolos and EBF1 are dramatically reduced at the early stage in mutant clones cl.2-2 and cl.2-4, and thereafter those of the first two are drastically elevated until the later stage but that of the last one remains unchanged during cultivation, but also the observations that the gene expression of OBF1 is slightly decreased at the early stage and thereafter slightly increased at the later stage [Chap. 4].

In mutant clone cl.2-6, the chromatin structure of the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is the tight form depending on the hypo-acetylation levels of one or more of the specific Lys residues at the early stage. Thereafter, the chromatin structure of these three genes changes to the loose form depending on the hyper-acetylation levels until the later stage. On the other hand, the chromatin structure of the proximal 5'-upstream region of the OBF1 gene is the tight form depending on the hypo-acetylation levels at the early stage but changes to the loose form depending on the hyper-acetylation levels at the later stage. These results can roughly explain not only the observations that gene expressions of Pax5, Aiolos and EBF1 are dramatically reduced at the early stage and thereafter drastically elevated until the later stage, but also the observations that the gene expression of OBF1 is certainly decreased at the early stage and thereafter clearly increased at the later stage [Chap. 4].

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

On the other hand, the chromatin structure of the proximal and distal 5'-upstream region of the PCAF gene is the loose form as a whole depending on the hyper-acetylation levels of the five specific Lys residues of histone H3 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 a very low in DT40 cells [3], dramatically and distinctly changes in these four mutant clones during cultivation [Chaps. 2 and 4]. 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 within the examined ORF regions (coding regions) of Pax5, Aiolos, EBF1, OBF1 and PCAF genes are a very low in DT40 cells and remains unchanged or very slightly changes in 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 clearly differ between the proximal 5'-upstream regions and ORF regions of these tested five genes.

Taken together, these results support the previous inference that gene expressions of IgM H- and L-chains decrease in distinct ways attributed to alterations in gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual clones of HDAC2(-/-) mutants during cultivation [Chap. 4]. Namely, the way of mutant clone cl.2-1 at the later stage for gene expressions of these two immunoglobulin proteins seems to be dependent upon OBF1 and distinct from that of wild-type DT40 cells. The way of mutant clones cl.2-2 and cl.2-4 at the later stage seems to be dependent upon Pax5 and Aiolos and slightly similar to that of DT40 cells in appearance. Besides, mutant clones cl.2-2 and cl.2-4 seem to be major type, because two other mutant clones cl.2-3 plus cl.2-5 [Chap. 4] and four initially generated HDAC2(-/-) mutant clones cl.33-12, cl.33-28, cl.33-30 plus cl.45-28 [1] resemble the first two mutant clones cl.2-2 and cl.2-4 in several cellular properties [3]. The way of mutant clone cl.2-6 at the later stage seems to be dependent upon Pax5, Aiolos and EBF1 and mostly similar to that of DT40 cells in appearance.

As mentioned repeatedly, 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 ways not only for the above-mentioned five specific genes but also in the four individual HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6. In addition, as mentioned previously [Chap. 4], gene expressions of PCAF, HDAC7, HDAC9, Blimp1, E2A, Ikaros, PU.1 and XBP-1 dramatically or moderately change in different patterns in these four mutant clones during cultivation. These findings, combined, suggest that besides gene expressions of IgM H- and L-chains (and Pax5, Aiolos, EBF1 and OBF1) and also the cell morphology [see Chap. 4], some other undefined cellular characteristics should undoubtedly change in 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 [1-4, Chaps. 2, 3 and 4], we proposed a

hypothesis on distinct ways to diminish artificially accumulated IgM H- and L-chains by means of irreversible creation of the chromatin structure plasticity surrounding the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, which was caused by the HDAC2-deficiency in individual clones of HDAC2(-/-) mutants through various generations during 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 nucleus. The abnormal environment change induces alterations in the chromatin structure of various genes encoding chromatin modifying-enzymes and transcription factors (such as PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1, Oct2, etc.), resulting in changes in their gene expression levels. As a next step, the signal transduction and response to the abnormal environment change are successively converged to the chromatin structure surrounding the proximal 5'-upstream regions of several genes encoding specific enzymes and factors, such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC7, HDAC9 and others. 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 dramatically and complicatedly changes in individual clones of HDAC2(-/-) mutants through various generations, resulting in drastic alterations in their gene expressions. In general, this successive response to the abnormal environment change causes varied epigenetic modifications of the chromatin structure. Of these epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones are major ones, but positions of the specific Lys residues and/or kinds of core histones are diverse. In our 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 change 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 tentatively and qualitatively deduced depending on acetylation and deacetylation levels of one or more of the specific Lys residues, though which Lys residue(s) is really and/or mainly involved in the binding is still unknown. Namely, hyper- (high) or hypo- (low or no) acetylation levels induces no binding or binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure. Thus, the chromatin structure plasticity is irreversibly created through successive structural changes attributed to binding ability of histone H3 to DNA depending on changes in acetylation and 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 causes the high or low (or no) gene expression levels. Notably, in the concrete, the ways to create the chromatin structure plasticity are distinct in individual HDAC2(-/-) mutant clones, though the accumulation of IgM H- and L-chains as an abnormal environment change is the same for all

of them. That is, in order to exclude artificially accumulated immunoglobulin proteins, individual clones of HDAC2(-/-) mutants differently change the chromatin structure surrounding the proximal 5'-upstream regions of specific transcription factor genes, such as Pax5, Aiolos, EBF1 and OBF1 genes. Detailed way and machinery for irreversible creation of the chromatin structure plasticity, including the recognition of the accumulation of IgM H- and L-chains, the signal transduction pathway and the chromatin conformation change, remain to be elucidated.

METHODS

Cell cultures

HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 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 [1, 3, Chaps. 2, 3 and 4]. These three cultivation stages were practically counted from the first day of cultivation from the stock at -80 ℃, although all positive mutant clones were 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. DT40 cells (W) were used as a control.

Nucleotide sequence confirmation and primer preparation

For primers used in the NotchIP assay, we had determined nucleotide sequences of ~4.9 kb 5'-upstream regions, containing proximal ~2.0 kb 5'-upstream region, some distal 5'-upstream regions and some ORF regions (coding regions or cDNA) of the Pax5 gene [Chap. 2]. We confirmed nucleotide sequences of proximal ~2.0 kb 5'-upstream, distal 5'-upstream and ORF regions (coding regions or cDNA) of Aiolos, EBF1, OBF1 and PCAF genes based on a databank. A part of nucleotide sequences of 5'-upstream region of the EBF1 gene was newly determined. To obtain PCR products of approximately 150 ~ 250 bp in length (see below), we prepared various appropriate primers, which were designed based on the above-mentioned nucleotide sequences of Pax5, Aiolos, EBF1, OBF1 and PCAF genes, and listed them in Supplementary Tables 5-SI ~ 5-SV.

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP) assay was done with ChIP assay kit (Millipore) according to the manufacturer's instruction. Briefly, HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 at the early (E; 3 days), middle (M; 33 days) and later (L; 58 days) cultivation stages and DT40 cells (W) (1 x 10^6) were cross-linked by the addition of 37% formaldehyde to a final concentration of 1% and incubated at 37 ℃ for 10 min, and the cross-linking reaction was stopped with one-tenth volume of 1.25 M glycine to a final concentration of 0.125 M at 37 ℃ for 5 min. After removing medium, cells were washed with ice-cold 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 PCR step. Each of antibodies (2 μl) was added to 2 ml of the supernatant fraction and incubated at 4 ℃ for overnight. Incubation with 60 μl of Protein Agarose/Salmon Sperm DNA was continued for 1 hr, followed by centrifugation at 1,000 rpm at 4 ℃ for 1 min. The immuno-precipitated complexes were sequentially washed with each of low-salt solution, high-salt solution, LiCl solution once and TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) twice. The antibody/histone/DNA complexes were eluted from Protein Agarose beads by adding 250 μl of elution buffer (1% SDS, 0.1% $NaHCO_3$) twice, and the cross-linking was reversed by heating at 65 ℃ for 6.5 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. 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 and input DNA were performed in duplicate and once, respectively. 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 controls (data not shown).

POSTSCRIPT

The studies in Chapter 5 were reviewed in Ref. 5.

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FIGURE LEGENDS

Figure 5-1. 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 cells (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After decross-linking, co-precipitated chromatins were amplified twice by PCR using appropriate primers for segments 1~12 and a~d of the Pax5 gene (Suppl. Table 5-SI). 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 5-2. 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 in clone cl.2-2 of HDAC2(-/-) as in Fig. 5-1, except the middle (M) cultivation stage and antiserum specific for acetylated K23 residue of histone H3.

Figure 5-3. 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 in clone cl.2-4 of HDAC2(-/-) as in Fig. 5-1.

Figure 5-4. 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 in clone cl.2-6 of HDAC2(-/-) as in Fig. 5-1.

Figure 5-5. 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 cells (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After decross-linking, 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 (Suppl. Table 5-SII). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 5-6. 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 in clone cl.2-2 of HDAC2(-/-) as in Fig. 5-5.

Figure 5-7. 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 in clone cl.2-4 of HDAC2(-/-) as in Fig. 5-5.

Figure 5-8. 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 in clone cl.2-6 of HDAC2(-/-) as in Fig. 5-5.

Figure 5-9. 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 cells

(W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After decross-linking, co-precipitated chromatins were amplified twice by PCR using appropriate primers for segments 1~14 and a~e of the EBF1 gene (Suppl. Table 5-SIII). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 5-10. 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 in clone cl.2-2 of HDAC2(-/-) as in Fig. 5-9.

Figure 5-11. 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 in clone cl.2-4 of HDAC2(-/-) as in Fig. 5-9.

Figure 5-12. 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 in clone cl.2-6 of HDAC2(-/-) as in Fig. 5-9.

Figure 5-13. 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 cells (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After decross-linking, 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 (Suppl. Table 5-SIV). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 5-14. 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 in clone cl.2-2 of HDAC2(-/-) as in Fig. 5-13.

Figure 5-15. 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 in clone cl.2-4 of HDAC2(-/-) as in Fig. 5-13.

Figure 5-16. 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 in clone cl.2-6 of HDAC2(-/-) as in Fig. 5-13.

Figure 5-17. 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 cells (W) and co-precipitated by antisera specific for acetylated K9, K14, K18, K23 and K27 residues of histone H3. After decross-linking, co-precipitated chromatins were amplified once by PCR using appropriate primers for segments 1~14 and a~d of the PCAF gene (Suppl. Table 5-SV). Results are shown with locations of segments (top) and PCR cycle numbers (bottom), respectively.

Figure 5-18. 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 in clone cl.2-2 of HDAC2(-/-) as in Fig. 5-17.

Figure 5-19. 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 in clone cl.2-4 of HDAC2(-/-) as in Fig. 5-17.

Figure 5-20. 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 in clone cl.2-6 of HDAC2(-/-) as in Fig. 5-17.

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