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Review

Irreversible creation of chromatin structure plasticity of proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications to exclude IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation

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ABSTRACT

We recently studied acetylation levels of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 surrounding ~2.0 kb 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes in four clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of chicken histone deacetylase2 (HDAC2)-deficient DT40 mutants HDAC2(-/-) during continuous cultivation. In this article, we review our studies on alterations in acetylation levels of these specific Lys residues of histone H3 at the early, middle and later cultivation stages. Acetylation levels of the Lys residues of the four genes were high in DT40 cells. In clone cl.2-1, acetylation levels of one or more of the Lys residues of Pax5, Aiolos and EBF1 genes were dramatically decreased at the early stage and thereafter remained unchanged until the later stage, and those of the OBF1 gene were drastically decreased until the later stage. In clones cl.2-2 and cl.2-4, acetylation levels of Pax5, Aiolos and EBF1 genes were dramatically decreased at the early stage, and thereafter those of the first two were increased until the later stage but those of the last one remained unchanged. In clone cl.2-6, acetylation levels of Pax5, Aiolos and EBF1 genes were drastically decreased at the early stage and thereafter increased until the later stage. These results could explain the previously mentioned ways for varied gene expressions of Pax5, Aiolos, EBF1 and OBF1 in individual HDAC2(-/-) clones during cultivation. We propose a hypothesis concerning distinct ways to gain new cell function to eliminate IgM H- and L-chains accumulated in individual HDAC2(-/-) clones during cultivation. They have an ability to adapt themselves to new environments through irreversible creation of chromatin structure plasticity caused by successive structural changes between tight and loose forms depending on hypo- and hyper-acetylation levels of specific Lys residues of histone H3 surrounding proximal ~2.0 kb 5'-upstream chromatin regions of corresponding genes through various generations, indicating that DT40 cells are pluripotent, elastic and flexible to gain new cell function, attributed to alterations in the chromatin structure.

KEYWORDS: irreversible creation of chromatin structure plasticity, epigenetic modifications, gain of new cell function, continuous cultivation,

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neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay, changes in acetylation levels of Lys residues of histone H3 during cultivation, proximal 5'-upstream chromatin regions of specific transcription factor genes.

INTRODUCTION

In 1964, it was first proposed that chemical modifications of histones with acetyl and methyl groups should be of fundamental importance for the regulation of RNA synthesis in eukaryotes [1]. Since then, the modulation of chromatin topology has been thought to be one of the most fundamental and important ways for expression of cell functions in eukaryotes. Mechanisms to modulate the chromatin structure with epigenetic modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, etc., have been intensively studied in a variety of life science fields. Of such epigenetic modifications of the chromatin structure, acetylation and deacetylation of specific Lys residues of core histones (H2A, H2B, H3 and H4) catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs) as chromatin-modifying enzymes are undoubtedly major ones. For the last several decades, countless numbers of research papers on acetylation and deacetylation (and other epigenetic modifications) are accumulating in more diverse life science fields, e.g. transcription/ gene expression, DNA replication, differentiation, development, memory, pluri-potency, clinical medicine and so on [2-30].

Using gene targeting techniques in the chicken B cell line DT40 possessing homologous recombination with a very high frequency [31, 32], we have systematically studied *in vivo* roles of numerous members of histones, histone chaperones, HATs, HDACs and transcription factors [33-66]. Analyses of various DT40 mutants lacking individual members of HATs, HDACs and transcription factors revealed that HDAC2 indirectly regulates gene expressions of IgM H- and L-chains in wild-type DT40 cells through opposite controlling of gene expressions of Pax5, EBF1, Aiolos, E2A and OBF1 [41, 46, 52, 53, 55]. Furthermore, we reported following important phenomena by analyzing initially generated HDAC2-deficient DT40 mutants [53, 67, 68] and Pax5-deficient DT40 mutants [67, 69], all of which were continuously cultivated for varying long periods. In [53, 67-69], the cultivation stages and/or periods were practically counted from the first day of cultivation from the stock at -80 °C. Our results obtained from these HDAC2(-/-) and Pax5(-) mutants revealed that IgM H- and L-chains artificially accumulated at the early stage of cultivation are diminished depending on their decreased gene expressions, attributed to altered gene expressions of various transcription factors during continuous cultivation. In addition, interestingly, our qualitative chromatin immuno-precipitation (ChIP) assay done on the initially generated HDAC2(-/-) mutants suggested that acetylation levels of Lys-9 residues of histone H3 (K9/H3) within some regional chromatin segments surrounding proximal ~2.0 kb 5'-upstream region of the Pax5 gene are certainly decreased at the early stage and thereafter gradually increased at the later stage [67, 68].

To further explore mechanisms to diminish the accumulated IgM H- and L-chains depending on their increased gene expressions, we generated and analyzed HDAC2-deficient DT40 mutants HDAC2(-/-) [67, 70]. As expected, in six tested individual clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) of HDAC2(-/-) mutants, IgM H- and L-chains are dramatically accumulated at the early cultivation stage. Moreover, HDAC2(-/-) mutant cells at the early stage exist as a morphologically aggregative form. Anyway, the accumulated immunoglobulin proteins and the aggregative form should be abnormal environments for the mutant cells. Remarkably, the artificially accumulated IgM H- and L-chains at the early stage are dramatically reduced in almost similar pattern in all of the six HDAC2(-/-) mutant clones during cultivation and thereafter at the later stage reached comparable levels as in DT40 cells [67, 70]. In parallel with these changes, the aggregative form of all HDAC2(-/-) mutant clones at the early stage is altered at the later stage to the dispersive form, which must be normal environments for them, similar to those for DT40 cells [67, 70]. Interestingly, in the six individual clones of HDAC2(-/-) mutants, mRNA (i.e., gene expression/transcription) levels of PCAF, HDAC7, HDAC9, Pax5, Aiolos, EBF1,

E2A, PU.1, Blimp1, XBP-1, OBF1 and others change dramatically or considerably in distinct patterns during cultivation, though all of these mutant clones show almost the same changing pattern in protein and mRNA levels of IgM H- and L-chains and in cell morphology [67, 70]. Of these altered chromatin-modifying enzymes and transcription factors, Pax5, Aiolos, EBF1 and OBF1 should be worthy of special mention. In clone cl.2-1, mRNA levels of Pax5, Aiolos and EBF1, all of which down-regulate IgM H- and L-chain gene expressions [46, 58, 67, 68, 70], are dramatically reduced at the early stage and thereafter remain unchanged during cultivation. By contrast, the mRNA level of OBF1, which probably up-regulates these immunoglobulin gene expressions [41, 46, 67, 70], is drastically reduced during cultivation until the later stage. In clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5, mRNA levels of Pax5, Aiolos and EBF1 are dramatically reduced at the early stage, and thereafter those of the first two are gradually elevated until the later stage but that of the last one remains unchanged at a very low level during cultivation. On the other hand, the mRNA level of OBF1 does not change by much in these four mutant clones at any cultivation stages. In clone cl.2-6, mRNA levels of Pax5, Aiolos and EBF1 are drastically reduced at the early stage and thereafter gradually elevated until the later stage, but that of OBF1 changes slightly in a somewhat complicated pattern during cultivation. These findings led to the following interesting inference on the ways for gene expressions of IgM H- and L-chains at the later stage in individual HDAC2(-/-) mutant clones [67, 70]. The way in clone cl.2-1 seems to be dependent on OBF1 and distinct from that of DT40 cells. The ways in clones cl.2-2, cl.2-3, cl.2-4 and cl.2-5 seem to be dependent on Pax5 and Aiolos, and slightly similar to that of DT40 cells in appearance. These four clones should be major types, since four initially generated HDAC2(-/-) mutant clones resembled them in several cellular properties [46, 67, 68]. The way in clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1 and almost similar to that of DT40 cells in appearance.

As described above, among the transcription factors whose gene expressions were altered in HDAC2(-/-) mutants during cultivation, Pax5, Aiolos, EBF1 and OBF1 should be influential candidates participating in decreases in gene expressions of IgM H- and L-chains in individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6) [67, 70]. The validity of this inference was supported by the findings that changing patterns of these factor gene expressions were anti-parallel or parallel with those of the immunoglobulin gene expressions in one or more of the six individual mutant clones. Additionally, Pax5, Aiolos and EBF1 were reported to down-regulate gene expressions of IgM H- and L-chains in chicken DT40 cells [46, 58, 67, 70], and OBF1 was suggested to up-regulate these immunoglobulin gene expressions [41]. Therefore, to clarify molecular mechanisms to alter gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF in four individual clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) of HDAC2(-/-) mutants during cultivation [67, 71], we recently developed and performed neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP) assay (which will be explained in detail later) on the proximal 5’-upstream chromatin region of each of the five remarkable genes in these 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 change within the chromatin surrounding proximal ~2.0 kb 5’-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes during cultivation. In addition, remarkably, changing patterns in acetylation levels of the above-mentioned four genes are distinct in the four individual HDAC2(-/-) mutant clones, although changing patterns in protein and mRNA levels of IgM H- and L-chains are almost similar in all of them. Based on these results, we proposed a hypothesis on the mechanisms to irreversibly create plasticity of the chromatin structure surrounding proximal 5’-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes with epigenetic modifications for exclusion of IgM H- and L-chains accumulated in individual clones of HDAC2(-/-) DT40 mutants through various generations during continuous cultivation [67, 71]. This article is the review of the studies in ref. 71.

Neighboring overlapping tiling chromatin immuno-precipitation (NotchIP) assay: A new method to study alterations in the chromatin structure surrounding proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 in individual clones of HDAC2(-/-) DT40 mutants during continuous cultivation

We studied how individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) differentially gain distinct ways for gene expressions of Pax5, Aiolos, EBF1, OBF1 and also PCAF through various generations during cultivation. To execute the project, we carried out chromatin immuno-precipitation (ChIP) assay on the chromatin surrounding their proximal ~2.0 kb 5'-upstream, distal 5'-upstream and coding (open reading frame; ORF) regions [67, 71], because the chromatin structure surrounding the proximal 5'-upstream region should be directly and closely related to transcriptional activity of corresponding gene, regardless of the presence or absence of transcriptional elements within the region. Moreover, our previous data obtained by the dual-luciferase assay suggested that at least ~1.6 kb 5'-upstream region of the Pax5 gene was necessary for its gene expression (unpublished data). We used appropriate primers specific for the proximal 5'-upstream, distal 5'-upstream and ORF regions of these five particular genes. As mentioned above, we named the ChIP assay as neighboring overlapping tiling chromatin immuno-precipitation (NotchIP or Notch-IP; this abbreviation also means IP on notch of chromatin) assay, because all of DNA fragments amplified by polymerase chain reaction (PCR) using appropriate primers, which were designed based on nucleotide sequences of the proximal ~2.0 kb 5'-upstream chromatin region (named as notch of chromatin) of each of the above-mentioned genes, coincide with corresponding segments of the region and are laid overlapping to each other with neighboring ones.

We systematically carried out the NotchIP assay on the chromatin prepared from four individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E; 3 days), middle (M; 33 days) and later (L; 58 days) stages of cultivation and from wild-type DT40 cells (W). Throughout the NotchIP assay, we used five site-specific antibodies for acetylated Lys-9 (K9/H3), Lys-14 (K14/H3), Lys-18 (K18/H3), Lys-23 (K23/H3) and Lys-27 (K27/H3) residues of histone H3, since bulk acetylation levels of these five Lys residues of histone H3 obviously changed in initially generated HDAC2(-/-) mutants during cultivation [67, 68, 70]. However, regarding the Pax5 gene in clone cl.2-2, we used only four site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3 and K27/H3 at the early (E) and later (L) stages of cultivation, because this case was the first attempt of the NotchIP assay. We tentatively deduced the binding ability (capacity) of histone H3 to DNA based on acetylation levels of these specific Lys residues of its N-terminal tail (K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3) obtained by the NotchIP assay, though it is still unclear which Lys residue(s) is really and/or mainly involved in the binding. That is, hyper- (high), considerably hyper-, somewhat hyper- and hypo- (low or no) acetylation levels of one or more of these five Lys residues of histone H3 (or corresponding Lys residue(s)) should qualitatively induce no, weak, less and full binding ability to DNA, resulting in loose (open), considerably loose, somewhat loose and tight (closed) forms of the chromatin structure. These four forms cause high, considerably high, somewhat high and low (or no) mRNA (i.e., transcription/ gene expression) levels of corresponding gene(s), respectively. As detailed results on acetylation levels of K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 for Pax5, Aiolos, EBF1, OBF1 and also PCAF genes are available from refs. [67, 71], in order to simplify description of this review article, hereafter, we will explain only results on acetylation levels of K9/H3 and K27/H3 of the proximal 5'-upstream chromatin regions of Pax5, Aiolos, EBF1 and OBF1 genes.

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 open reading frame regions of the Pax5 gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later stages of continuous cultivation

As changing patterns in the gene expression of Pax5 during cultivation were different in

individual clones of HDAC2(-/-) mutants [67, 70], we carried out the NotchIP assay on the chromatin surrounding proximal 5'-upstream, distal 5'-upstream and ORF regions of the Pax5 gene in clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 and in DT40 cells (Fig. 1). We used five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 and appropriate primers designed based on nucleotide sequences of the ~4.9 kb 5'-upstream region of the Pax5 gene that was cloned from DT40 genomic DNA by us [67, 68] and of its ORF region obtained from database. Regarding the proximal 5'-upstream region of positions -1923 to +30, we used primers for segments 1-12, all of which are laid overlapping to each other with neighboring ones. In addition, we used primers corresponding to positions -4390 to -4235 (segment a) of the distal 5'-upstream region and positions +55 to +201, +223 to +391 and +490 to +588 (segments b-d) of the ORF region. Since primers for segments b-d were designed based on nucleotide sequences from database, the nucleotide numbers were discontinuous from those of the distal and proximal 5'-upstream regions.

First, we carried out the NotchIP assay, using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-1 at the early (E), middle (M) and later (L) stages of cultivation and DT40 cells (W). Changing patterns in acetylation levels of these five Lys residues of histone H3 in clone cl.2-1 during cultivation are presented in fig. 1 (cl.2-1). Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream chromatin regions (segments a and 1-12) of the Pax5 gene were high in DT40 cells (W). Surprisingly, in spite of the HDAC2-deficiency, they were almost completely decreased at the early (E) stage in clone cl.2-1 and thereafter remained unchanged throughout the middle (M) to later (L) stages. On the other hand, acetylation levels of K9/H3 within three ORF regions of the gene (segments b-d) were very low in DT40 cells (W) (see many PCR cycle numbers) and further decreased at all cultivation stages in clone cl.2-1, except for an insignificant change for the region of positions +223 to +391 (segment c). Acetylation levels of K27/H3 within the entire 5'-upstream regions of the Pax5 gene were high in DT40 cells. Highly similar to changing patterns for K9/H3, they were almost completely decreased at the early (E) stage in clone cl.2-1 and thereafter remained unchanged throughout the middle (M) and later (L) stages, except for an insignificant change for the region of positions -958 to -679 (segment 8). On the other hand, acetylation levels of K27/H3 within three ORF regions of the gene were very low in DT40 cells (see many PCR cycle numbers) and remained unchanged in clone cl.2-1 during cultivation. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 residues of histone H3 possess no binding ability 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. In addition, predominantly, K9/H3, K14/H3, K18/H3 and K27/H3 residues of histone H3 exhibit full binding ability to DNA based on their hypo-acetylation levels at all 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, should be the loose form in DT40 cells, but thereafter changes to the tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the later (L) stage. As a result, the gene expression of Pax5, which is high level in DT40 cells, is dramatically decreased at the early (E) stage in clone cl.2-1 and thereafter remains unchanged during cultivation [67, 70].

As mentioned above, we carried out NotchIP assay, using only four site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3 and K27/H3, on the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-2 only at the early (E) and later (L) stages of cultivation and in DT40 cells (W) (Fig. 1 (cl. 2-2)). 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

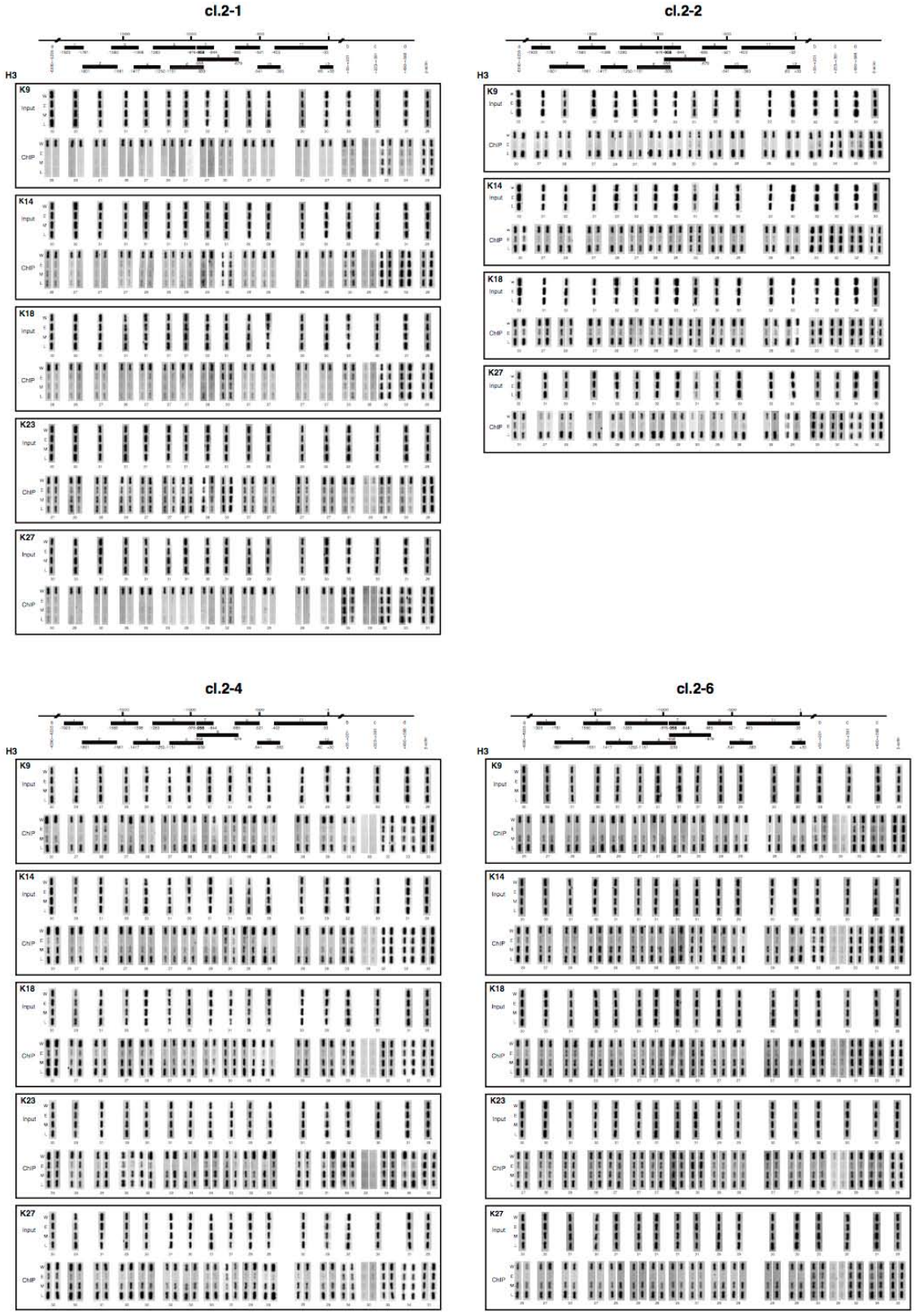

Fig. 1

were dramatically elevated at the later (L) stage and reached comparable levels as in DT40 cells. On the other hand, acetylation levels of K9/H3 within two ORF regions (segments b and d) of the gene were further reduced at the early (E) stage in clone cl.2-2 and thereafter elevated at the later (L) stage and reached almost the same levels as in DT40 cells, but insignificantly changed within the residual ORF region of positions +223 to +391 (segment c). Acetylation levels of K27/H3 within the entire 5'-upstream regions of the gene were almost completely reduced at the early (E) stage in clone cl.2-2. The reduced acetylation levels were dramatically elevated at the later (L) stage and reached almost similar levels as in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged in clone cl.2-2 during cultivation. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that K9/H3, K14/H3, K18/H3 and K27/H3 residues of histone H3 within the proximal 5'-upstream chromatin region of the Pax5 gene exhibit full binding ability to DNA based on their hypo-acetylation levels at the early (E) stage in clone cl.2-2. Thereafter, the level of binding capacity of these four Lys residues of histone H3 to DNA gradually decrease during cultivation and finally reach the state of almost no binding ability based on their 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 Pax5 gene should change to the tight form at the early (E) stage in clone cl.2-2, and thereafter, remarkably, change to 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 certainly increased during cultivation and at the later (L) stage reached comparable levels as in DT40 cells [67, 70].

Next, we carried out the NotchIP assay, using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-4 at the early (E), middle (M) and later (L) stages of cultivation and in DT40 cells (W) (Fig. 1 (cl.2-4)). 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 throughout the middle (M) until late (L) stages to comparable levels in DT40 cells. On the other hand, in clone cl.2-4, acetylation levels of K9/H3 within the ORF region of positions +55 to +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 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 to -4235 (segment a). The reduced acetylation levels were elevated throughout the middle (M) to later (L) stages and reached almost similar levels as in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged until the later (L) stage in clone cl.2-4. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 residues of histone H3 within the proximal 5'-upstream chromatin region of the Pax5 gene possess full binding

Legend to Fig. 1. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Pax5 gene in individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 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 DT40 cells (W) and individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages, and co-precipitated by five antisera specific for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers for segments 1-12 and a-d of the Pax5 gene. The figure is a set of figs. 1, 2, 3 and 4 of ref. [71].

ability to DNA based on their hypo-acetylation levels at the early (E) stage in clone cl.2-4. Thereafter, the level of binding capacity of these five Lys residues of histone H3 to DNA gradually decrease during cultivation and finally reach the state of almost no binding ability based on their 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 Pax5 gene should change to the tight form at the early (E) stage in clone cl.2-4, and thereafter, surprisingly, change to the loose form through various generations until the later (L) stage. As a result, the gene expression of Pax5 is dramatically decreased at the early (E) stage in clone cl.2-4 and thereafter gradually increased until the later (L) stage during cultivation [67, 70].

Finally, we carried out the NotchIP assay, using five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on the chromatin surrounding the distal 5'-upstream, proximal 5'-upstream and ORF regions of the Pax5 gene in clone cl.2-6 at the early (E), middle (M) and later (L) stages of cultivation and in DT40 cells (W) (Fig. 1 (cl.2-6)). 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 throughout the middle (M) to later (L) stages and reached almost similar levels as in DT40 cells. On the other hand, acetylation levels of K9/H3 within three ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-6 but thereafter slightly elevated throughout the middle (M) to later (L) stages and reached almost similar levels as in DT40 cells. 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 throughout the middle (M) to later (L) stages and reached almost similar levels as in DT40 cells. However, acetylation levels of K27/H3 within three ORF regions of the gene remained unchanged until the later (L) stage in clone cl.2-6. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that mainly K9/H3, K14/H3, K18/H3 and K27/H3 residues of histone H3 within proximal 5'-upstream chromatin region of the Pax5 gene exhibit full binding ability to DNA based on their hypo-acetylation levels at the early (E) stage in clone cl.2-6. Thereafter, the level of binding capacity of these Lys residues of histone H3 to DNA gradually decrease during cultivation and finally reach the state of almost no binding ability based on their 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 Pax5 gene should change to the tight form at the early (E) stage in clone cl.2-6, and thereafter, remarkably, change to the loose form through various generations until the later (L) stage. As a result, the gene expression of Pax5 is dramatically decreased at the early (E) stage in clone cl.2-6 and thereafter gradually increased during cultivation [67, 70].

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 open reading frame regions of the Aiolos gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later stages of continuous cultivation

As changing patterns in the gene expression of Aiolos during cultivation were distinct in individual clones of HDAC2(-/-) mutants [67, 70], 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 (Fig. 2). We used five site-specific antibodies for acetylated 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. Regarding the proximal 5'-upstream region of positions -2250 to +145, we used primers corresponding to segments 1-14, all of which are laid overlapping to each other with the neighboring ones. In addition, we used primers corresponding to positions -3524 to -3367 and -2735 to -2528 of the distal 5'-upstream region (segments a and b) and positions +212 to +361 and +1265 to +1417 of the ORF region (segments c and d). The primers were designed

based on nucleotide sequences from database and confirmed by us.

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 fig. 2 (cl.2-1). Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream regions (segments a, b and 1-14) and two ORF regions (segments c and d) of the Aiolos gene were high in DT40 cells (W). Surprisingly, they were dramatically reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged throughout the middle (M) stage until later (L) stages. 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 they were slightly reduced at the early (E) stage in clone cl.2-1 and remained unchanged throughout the middle (M) to later (L) stages. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that five Lys residues K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 of histone H3 possess no binding ability 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 residues of histone H3 except K23/H3 exhibited full or less binding ability to DNA based on their hypo-acetylation 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, should be the loose form in DT40 cells, but thereafter changes to the tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the later (L) stage. As a result, the gene expression of Aiolos, which is at high level in DT40 cells, is drastically decreased at the early (E) stage in clone cl.2-1 and thereafter remains unchanged during cultivation [67, 70].

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 fig. 2 (cl.2-2). Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1-14) and two ORF regions (segments c and d) of the gene were obviously reduced at the early (E) stage in clone cl.2-2 and thereafter slowly elevated throughout the middle (M) to later (L) stages as a whole. Acetylation levels of K27/H3 within the entire 5'-upstream regions and two ORF regions of the gene were certainly reduced at the early (E) stage in clone cl.2-2 and thereafter slightly elevated or remained unchanged throughout the middle (M) to later (L) stages as a whole. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that mainly the level of 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 becomes less based on their hypo-acetylation or slight hyper-acetylation levels at the early (E) and middle (M) cultivation stages in clone cl.2-2. Thereafter, predominantly, the binding capacity of K9/H3 (and probably K27/H3) to DNA disappears almost completely based on their hyper-acetylation 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 should change to tight form at the early (E) and middle (M) stages in clone cl.2-2 and thereafter change to loose or considerably 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 [67, 70].

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 fig. 2 (cl.2-4). Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1-14) and the ORF region of positions +1265 to +1417 (segment d) of the Aiolos gene were obviously reduced at the early (E) stage in clone cl.2-4 and thereafter certainly elevated throughout the middle (M) to later (L) stages as a whole, but changed insignificantly within the remaining ORF region (segment c). Acetylation levels of K27/H3 within the entire 5'-upstream regions of the gene were very slightly reduced at the early (E) stage in clone cl.2-4 and thereafter slightly elevated (or remained

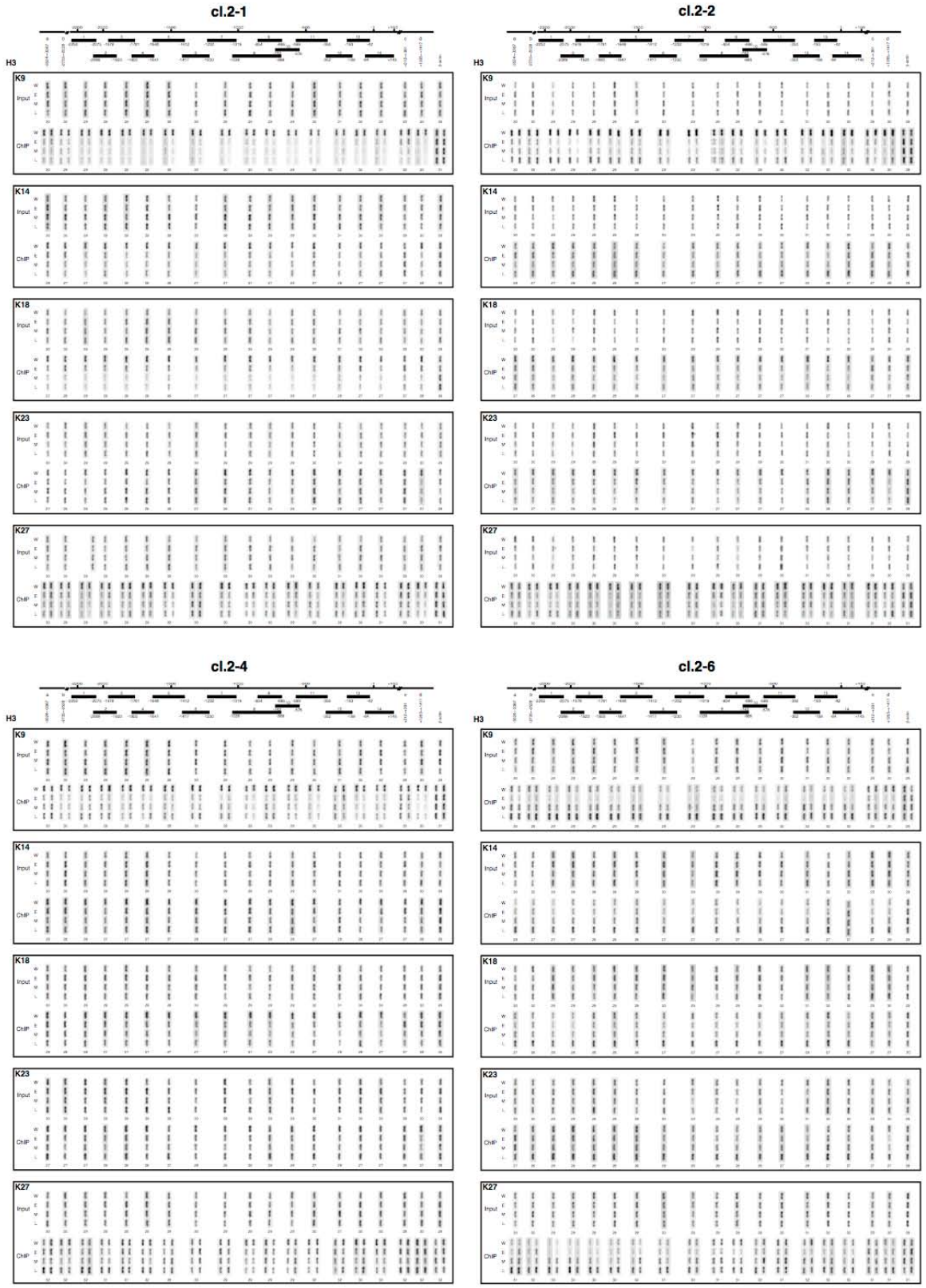

Fig. 2

unchanged) throughout the middle (M) to later (L) stages as a whole. However, those of K27/H3 within two ORF regions of the gene remained unchanged in clone cl.2-4 during cultivation. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that predominantly the level of 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 becomes less based on the hypo-acetylation or slight hyper-acetylation levels at the early (E) and middle (M) stages. Thereafter, the binding capacity of K9/H3 (and probably K27/H3) to DNA certainly disappears almost completely based on the hyper-acetylation 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 should change to the tight form at the early (E) and middle (M) stages in clone cl.2-4 and thereafter change to the loose or considerably loose form at the later (L) stage. As a result, the gene expression of Aiolos is obviously decreased at the early (E) stage in clone cl.2-4 and thereafter certainly increased at the later (L) stage [67, 70].

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 fig. 2 (cl.2-6). Acetylation levels of K9/H3 within the entire 5'-upstream regions (segments a, b and 1-14) and two ORF regions (segments c and d) of the gene were apparently decreased at the early (E) stage in clone cl.2-6 and thereafter gradually increased throughout the middle (M) to later (L) stages and were clearly higher than those in DT40 cells. Acetylation levels of K27/H3 within the 5'-upstream region upper from position -1230 (segments a, b and 1-6) of the gene remained unchanged at the early (E) stage in clone cl.2-6 and thereafter were gradually increased throughout the middle (M) to later (L) stages and were higher than those in DT40 cells. On the other hand, those of K27/H3 within the 5'-upstream region down from position -1232 (segments 7-14) were slightly decreased at the early (E) stage in clone cl.2-6 and thereafter rapidly increased throughout the middle (M) to later (L) stages and reached 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, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that predominantly the level of binding capacity of K9/H3 and K27/H3 to DNA within the proximal 5'-upstream chromatin region of the Aiolos gene in clone cl.2-6 certainly becomes less based on their hypo-acetylation 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 and finally reach the state of almost no binding ability based on their hyper-acetylation levels at the middle (M) stage to the later (L) stage. Naturally, it was possible that the extent of no binding ability at the middle (M) and later (L) stages should be less than that in DT40 cells, because acetylation levels at both cultivation stages in clone cl.2-6 were higher than those in DT40 cells. Therefore, we speculated that the structure of the chromatin surrounding the proximal 5'-upstream region of the Aiolos gene should change to the tight form at the early (E) stage in clone cl.2-6 and thereafter change to the loose form at the later (L) stage. As a result, the gene expression of Aiolos is obviously decreased at the early (E) stage in clone cl.2-6 and thereafter increased at the later (L) stage [67, 70].

Legend to Fig. 2. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of Aiolos gene in individual clones cl.2-1, cl.2-2, cl.2-4 and 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. Cross-linked chromatins were prepared from DT40 cells (W) and individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages, and co-precipitated by five antisera specific for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers for segments 1-14 and a-d of the Aiolos gene. The figure is a set of figs. 5, 6, 7 and 8 of ref. [71].

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 open reading frame regions of the EBF1 gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later stages of continuous cultivation

As changing patterns in the gene expression of EBF1 during cultivation were distinct in individual clones of HDAC2(-/-) mutants [67, 70], 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 (Fig. 3). We used five site-specific antibodies for acetylated 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. Regarding the proximal 5'-upstream region of positions -2031 to +200, we used primers recognizing respective segments 1-14, all of which are laid overlapping to each other with the neighboring ones. In addition, we used primers, corresponding to positions -3996 to -3770 and -2888 to -2730 of the distal 5'-upstream region (segments a and b) and positions +179 to +291, +649 to +768 and +787 to +900 of the ORF region (segments c-e). The primers were designed based on nucleotide sequences from database and confirmed by us.

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 fig. 3 (cl.2-1). Acetylation levels of K9/H3 within the entire (distal and proximal) 5'-upstream regions (segments a, b and 1-14) and the ORF region corresponding to positions +179 to +291 (segment c) of the EBF1 gene were high in DT40 cells. As a whole, they were dramatically reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged throughout the middle (M) to later (L) stages. On the other hand, acetylation levels of K9/H3 within two residual ORF regions (segments d and e) of the gene were very low in DT40 cells (see many PCR cycle numbers), and slightly reduced at the early (E) stage in clone cl.2-1 but thereafter slightly elevated throughout the middle (M) to later (L) stages. Acetylation levels of K27/H3 were considerably high within the entire 5'-upstream regions and the ORF region corresponding to positions +179 to +291 (segment c) of the gene, but they were relatively low in two residual ORF regions corresponding to positions +649 to +768 and +787 to +900 (segments d and e) (see many PCR cycle numbers) in DT40 cells. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions corresponding to positions -1037 to +200 (segments 7-14) and +179 to +291 (segment c) were certainly reduced at the early (E) stage in clone cl.2-1 and thereafter remained unchanged throughout 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, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 residues of histone H3 exhibit no binding ability 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. Furthermore, K9/H3, K18/H3 and K27/H3 in particular possess full binding ability based on their hypo-acetylation 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, should be the loose form in DT40 cells, but changes to the tight form at the early (E) stage in clone cl.2-1 and thereafter remain unchanged until the later (L) stage during cultivation. As a result, the gene expression of EBF1, which is at high level in DT40 cells, is almost completely suppressed at the early (E) stage in clone cl.2-1 and thereafter remains unchanged throughout the middle (M) to later (L) stages during cultivation [67, 70].

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 fig. 3 (cl.2-2). Acetylation levels of K9/H3 within the entire 5'-upstream regions and the ORF region of positions +179 to +291 (segment c) of the EBF1 gene were dramatically reduced

at the early (E) stage in clone cl.2-2 and thereafter remained unchanged throughout the middle (M) to later (L) stages. On the other hand, acetylation levels of K9/H3 within two residual ORF regions (segments d and e) of the gene were slightly reduced at the early (E) stage in clone cl.2-2 and thereafter slightly elevated throughout the middle (M) to later (L) stages. Acetylation levels of K27/H3 within the 5'-upstream regions upper from position -762 (segments a, b and 1-8) of the gene were considerably low in DT40 cells. In clone cl.2-2, acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -1826 to -1363 (segments 3 and 4), -1037 to +200 (segments 7-14) and +179 to +291 (segment c) were dramatically or almost completely reduced at the early (E) stage and thereafter remained unchanged throughout 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, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that predominantly K9/H3, K14/H3, K18/H3 and K27/H3 residues of histone H3 exhibit full binding ability to DNA based on their hypo-acetylation 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 should change to the tight form at the early (E) stage in clone cl.2-2 and remain unchanged throughout the middle (M) to later (L) stages. As a result, the gene expression of EBF1 is almost completely suppressed at the early (E) stage in clone cl.2-2 and thereafter remains unchanged during cultivation [67, 70].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the EBF1 gene in clone cl.2-4 during cultivation are represented in fig. 3 (cl.2-4). Acetylation levels of K9/H3 within the entire 5'-upstream regions and the ORF region of positions +179 to +291 (segment c) of the EBF1 gene were dramatically or almost completely decreased at the early (E) stage in clone cl.2-4. Thereafter, they remained unchanged throughout the middle (M) to later (L) stages, except for a gradual increase within two distal 5'-upstream regions of positions -3996 to -3770 and -2888 to -2730 (segments a and b). On the other hand, acetylation levels of K9/H3 within two residual ORF regions (segments d and e) of the gene remained unchanged in clone cl.2-4 during cultivation. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -1037 to +200 (segments 7-14) and +179 to +291 (segment c) were dramatically or considerably decreased at the early (E) stage in clone cl.2-4 and thereafter remained unchanged throughout 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 slightly decreased during cultivation. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) residues of histone H3 exhibit full binding ability to DNA based on their hypo-acetylation 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 should change to the tight form at the early (E) stage in clone cl.2-4 and remain unchanged until the later (L) stage. As a result, the gene expression of EBF1 is almost completely diminished at the early (E) stage in clone cl.2-4 and thereafter remains unchanged during cultivation [67, 70].

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 fig. 3 (cl.2-6). Acetylation levels of K9/H3 within the distal 5'-upstream and proximal 5'-upstream regions of positions -2888 to -2730 and -2031 to +200 (segments b and 1-14) and the ORF region of positions +179 to +291 (segment c) of the EBF1 gene were almost completely or dramatically reduced at the early (E) stage in clone cl.2-6. Interestingly, the reduced acetylation levels were dramatically elevated throughout the middle (M) to later (L) stages and reached almost similar levels as in DT40 cells. On the other hand, acetylation levels of K9/H3 within the residual

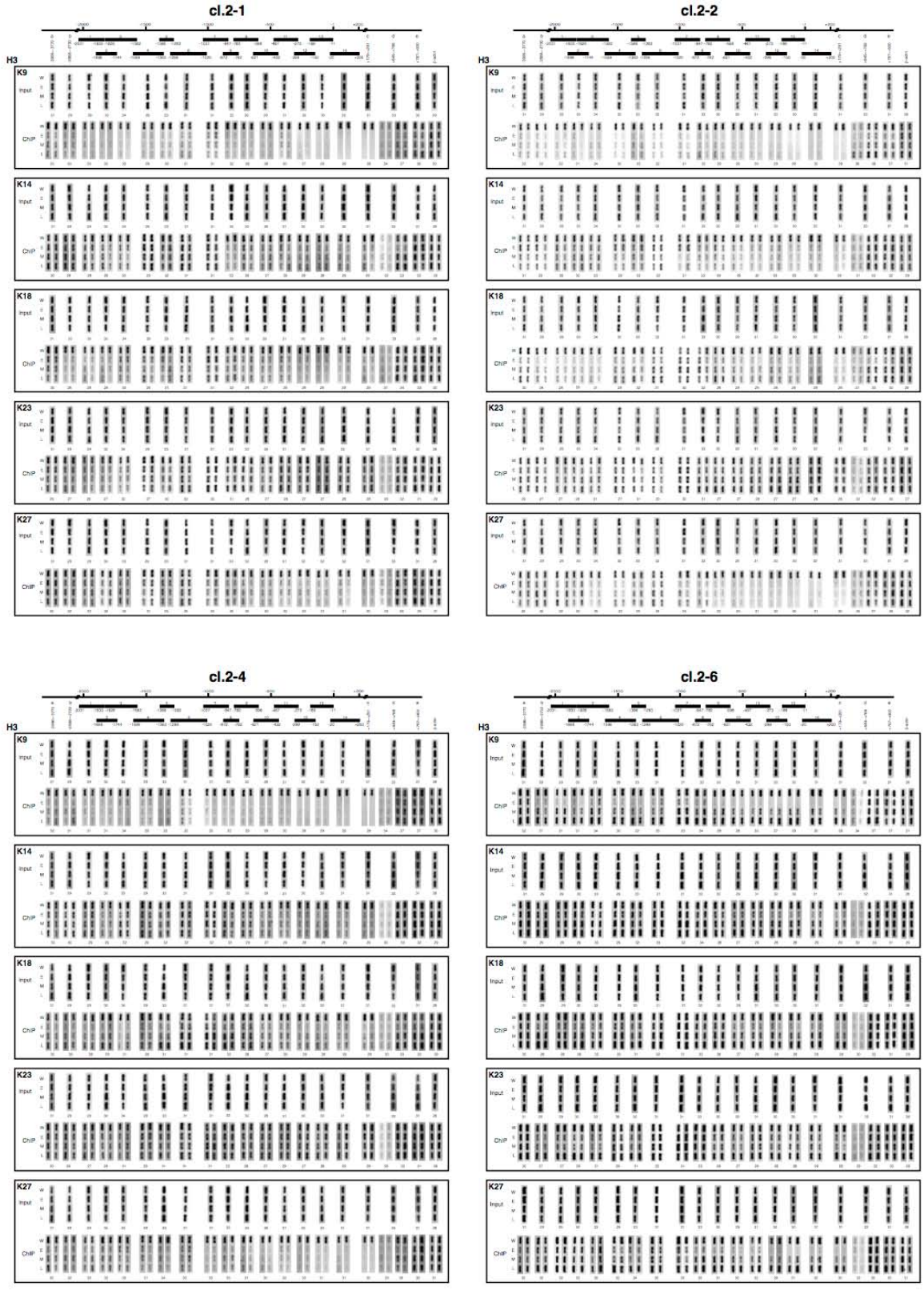

Fig. 3

distal 5'-upstream and ORF regions of positions -3996 to -3770 (segment a), +649 to +768 and +787 to +900 (segments d and e) of the gene remained unchanged at all cultivation stages in clone cl.2-6. Acetylation levels of K27/H3 within the proximal 5'-upstream and ORF regions of positions -872 to +200 (segments 8-14) and +179 to +291 (segment c) of the gene were dramatically reduced at the early (E) stage in clone cl.2-6 and thereafter elevated dramatically throughout the middle (M) to later (L) stages and reached almost the same levels as in DT40 cells. On the other hand, those within the residual distal 5'-upstream, proximal 5'-upstream and ORF regions insignificantly changed at all cultivation stages in clone cl.2-6. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that especially K9/H3 and K27/H3 (and probably K14/H3 and K18/H3) residues of histone H3 exhibit full binding ability to DNA based on their hypo-acetylation 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 to DNA and finally reach the state of almost no binding ability based on their hyper-acetylation levels throughout the middle (M) to later (L) stages. Therefore, we speculated that the structure of chromatin surrounding the proximal 5'-upstream region of the EBF1 gene should change to the tight form at the early (E) stage in clone cl.2-6 and thereafter change to the loose form throughout the middle (M) to later (L) stages. As a result, the gene expression of EBF1 is almost completely suppressed at the early (E) stage in clone cl.2-6, and thereafter gradually increased during cultivation and at the later (L) stage reached comparable levels as in DT40 cells [67, 70].

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 open reading frame regions of the OBF1 gene in individual clones of HDAC2(-/-) DT40 mutants at early, middle and later stages of continuous cultivation

As changing patterns in the gene expression of OBF1 during cultivation were distinct in individual clones of HDAC2(-/-) mutants [67, 70], 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 (Fig. 4). We used five site-specific antibodies for acetylated 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. Regarding the proximal 5'-upstream region of positions -2138 to +164, we used primers for respective segments 1-14, all of which are laid overlapping to each other with the neighboring ones. In addition, we used primers, corresponding to positions +17 to +131 and +776 to +937 (segments a and b) of the ORF region. The primers were designed based on nucleotide sequences from database and confirmed by us.

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 fig. 4 (cl.2-1). Acetylation levels of K9/H3 within the proximal 5'-upstream region (segments 1-14) and two ORF regions (segments a and b) of the OBF1 gene were high in DT40 cells but they were certainly reduced at the early (E) stage in clone cl.2-1 as a whole. Interestingly, the reduced acetylation levels were further reduced to undetectable levels at the middle (M) stage and

Legend to Fig. 3. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of EBF1 gene in individual clones cl.2-1, cl.2-2, cl.2-4 and 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. Cross-linked chromatins were prepared from DT40 cells (W) and individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages, and co-precipitated by five antisera specific for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers for segments 1-14 and a-e of the EBF1 gene. The figure is a set of figs. 9, 10, 11 and 12 of ref. [71].

thereafter remained unchanged until the later (L) stage. 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 for an insignificant reduction within the proximal 5'-upstream region of positions -1493 to -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, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3 residues of histone H3 exhibit no binding ability 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 possessed weak binding ability to DNA based on their considerable hyper-acetylation levels at the early (E) stage in clone cl.2-1. Further, predominantly, the weak binding ability of K9/H3 and K27/H3 was dramatically increased to full binding ability based on their hypo-acetylation levels at the middle (M) stage and remained unchanged until the 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, should be the loose form in DT40 cells. On the other hand, in clone cl.2-1 the chromatin structure should change to the considerably loose form at the early (E) stage and thereafter change to the tight form at the middle (M) and later (L) stages. As a result, the gene expression of OBF1, which is at high level in DT40 cells, is certainly decreased at the early (E) stage in clone cl.2-1 and thereafter dramatically decreased throughout the middle (M) to later (L) stages and reached a very low level [67, 70].

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 fig. 4 (cl.2-2). Acetylation levels of K9/H3 within the proximal 5'-upstream and two ORF regions of the OBF1 gene in clone cl.2-2 were dramatically decreased at the early (E) stage as a whole. Interestingly, the decreased acetylation levels remained unchanged at the middle (M) stage but thereafter were obviously increased at the later (L) stage. The increased acetylation levels at the later (L) stage were less than those in DT40 cells as a whole. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were considerably decreased at the early (E) stage in clone cl.2-2, and further decreased at the middle (M) stage but thereafter obviously increased at the later (L) stage. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that especially K9/H3 and K27/H3 within the proximal 5'-upstream chromatin region of the OBF1 gene obviously possessed full or less binding ability to DNA based on their hypo-acetylation or slight hyper-acetylation levels at the early (E) stage in clone cl.2-2. At the middle (M) stage, the full binding ability of K9/H3 remained unchanged, and the less binding ability of K27/H3 was further increased to full binding ability based on the hypo-acetylation levels. Thereafter, the full binding ability of K9/H3 and K27/H3 was obviously decreased to no binding ability based on their hyper-acetylation levels at the later (L) stage. The extent of the binding capacity at the later (L) stage should be higher than that in DT40 cells, because the acetylation levels at the later (L) stage in clone cl.2-2 were lower than those 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 should change to the tight or somewhat loose form at the early (E) or middle (M) stage of cultivation. Thereafter, the tightened chromatin structure should become the loose form at the later (L) stage, which may be slightly tighter than that in DT40 cells. As a result, the gene expression of OBF1 is certainly decreased at the early (E) stage in clone cl.2-2 and thereafter obviously increased throughout the middle (M) to later (L) stages [67, 70].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the OBF1 gene in clone cl.2-4 during cultivation are represented in fig. 4 (cl.2-4). Acetylation levels of K9/H3 within the proximal 5'-upstream and two ORF regions of the OBF1 gene were obviously reduced at the early (E) stage in clone cl.2-4. The reduced acetylation levels remained unchanged at the

middle (M) stage but thereafter were certainly elevated at the later (L) stage; the extent of the acetylation levels at the later (L) stage being less than those in DT40 cells. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were slightly reduced at the early (E) stage in clone cl.2-4, and remained unchanged at the middle (M) stage as a whole but thereafter slightly elevated at the later (L) stage, but did not change within some segments of the proximal 5'-upstream region. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], indicated that the binding capacity of K9/H3 (and probably K27/H3) to DNA in particular within the proximal 5'-upstream chromatin region of the OBF1 gene is certainly but slightly increased to weak or less binding ability based on their considerable acetylation or slight hyper-acetylation levels at the early (E) stage in clone cl.2-4. The weak or less binding ability remained unchanged at the middle (M) stage but thereafter was obviously decreased to no binding ability based on their hyper-acetylation levels at the later (L) stage; the extent of the binding capacity at the later (L) stage was probably slightly higher than that 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 should change to the somewhat loose form at the early (E) and middle (M) stages and thereafter at the later (L) stage change to the loose form, which should be almost similar to that in DT40 cells. As a result, the gene expression of OBF1 is certainly decreased at the early (E) and middle (M) stages in clone cl.2-4 and thereafter obviously increased at the later (L) stage [67, 70].

Changing patterns in acetylation levels of the five Lys residues of histone H3 for the OBF1 gene in clone cl.2-6 during cultivation are presented in fig. 4 (cl.2-6). Acetylation levels of K9/H3 within the proximal 5'-upstream and two ORF regions of the OBF1 gene were obviously decreased at the early (E) stage in clone cl.2-6. The decreased acetylation levels were gradually increased throughout the middle (M) to later (L) stages and reached almost the same levels as in DT40 cells. Acetylation levels of K27/H3 within the proximal 5'-upstream and two ORF regions of the gene were certainly decreased at the early (E) stage in clone cl.2-6 and remained unchanged at the middle (M) stage. Thereafter, they were obviously increased during cultivation and at the later (L) stage reached almost similar (or higher) levels as in DT40 cells. These results, together with those for K14/H3, K18/H3 and K23/H3 [67, 71], 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-acetylation or slight hyper-acetylation levels at the early (E) stage. The full or less binding ability was slightly decreased at the middle (M) stage and thereafter further and obviously decreased to no binding ability based on their hyper-acetylation levels at the 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 should change to the tight form at the early (E) stage and thereafter change to the loose form, like that in DT40 cells, throughout the middle (M) to later (L) stages. As a result, the gene expression of OBF1 is certainly decreased at the early (E) stage in clone cl.2-6 and thereafter obviously increased throughout the middle (M) to later (L) stages [67, 70].

Discussion

As reported previously [67, 68, 70], mRNA and protein levels of IgM H- and L-chains, which are very low in DT40 cells [46, 53], are dramatically increased at the early stage of cultivation in all of the examined HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-3, cl.2-4, cl.2-5 and cl.2-6 (and also cl.33-12, cl.33-28, cl.33-30 and cl.45-28), and thereafter obviously decreased in almost similar changing pattern throughout the middle to later stages and reached nearly equal levels as in T40 cells. Since alterations in gene expressions of PCAF, HDAC7 and HDAC9 are neither parallel nor anti-parallel with those of IgM H- and L-chains in one or more of the HDAC2(-/-) mutant clones during cultivation [67, 70], they should not directly and/or mainly participate in decreases in gene expressions of the two immunoglobulin proteins. These results should be roughly supported by the findings that the PCAF-deficient or HDAC7-deficient mutant shows a slight or no influence on gene expressions of

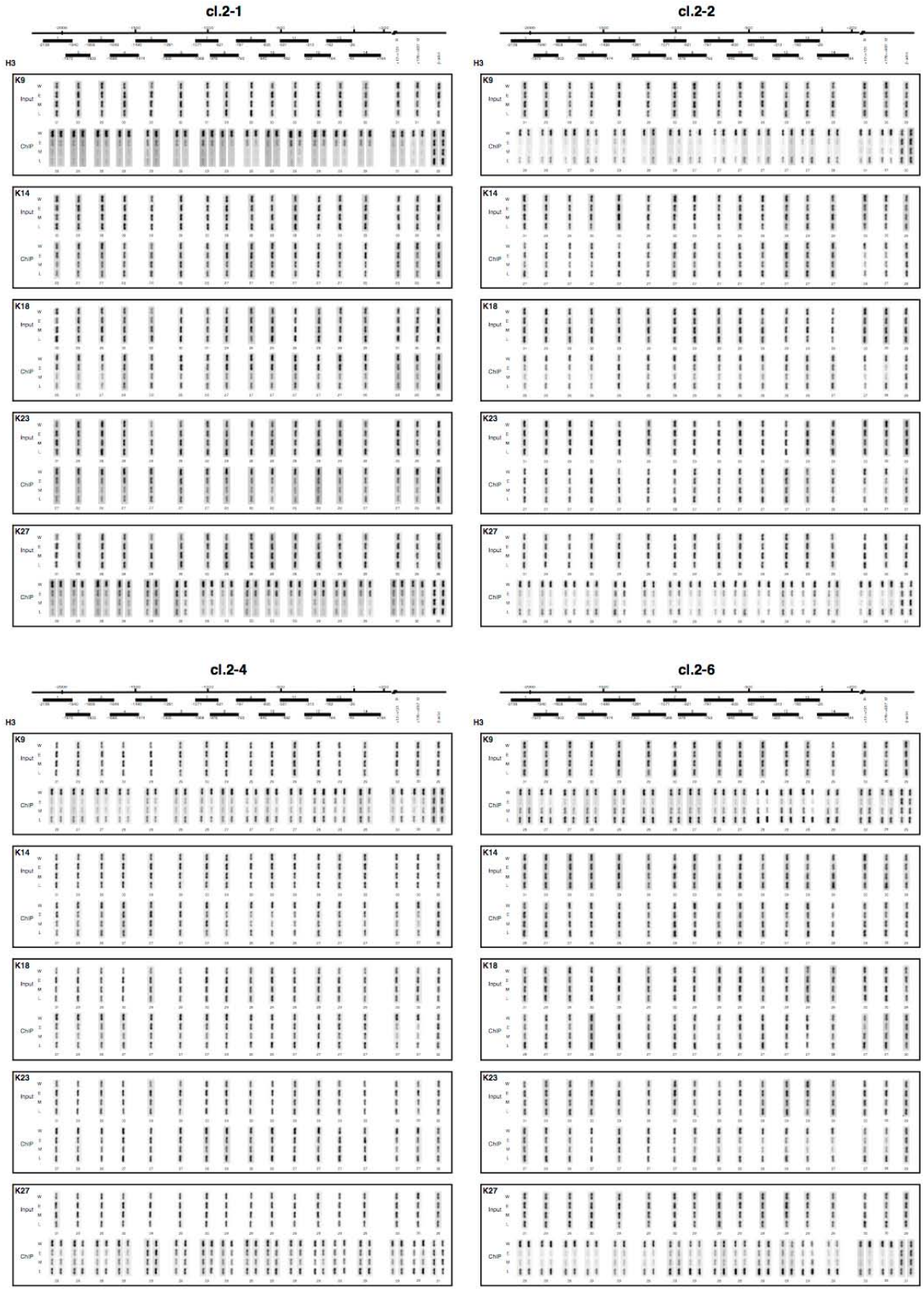

Fig. 4

IgM H- and L-chains [46]. Apart from that, the HDAC9-deficient one is not available yet. By contrast, Pax5, Aiolos and EBF1 or OBF1 have been verified or strongly suggested to be involved in down- or up-regulation of gene expressions of IgM H- and L-chains [41, 46, 58, 67, 70]. Noticeably, the respective gene expressions of the four transcription factors change in anti-parallel or parallel with those of the two immunoglobulin proteins in one or more of the HDAC2(-/-) mutant clones during cultivation [67, 68, 70]. Therefore, these four transcription factors should be influential candidates that participate in decreased gene expressions of IgM H- and L-chains in HDAC2(-/-) mutants during cultivation.

To explore the fundamental mechanisms that vary gene expressions of Pax5, Aiolos, EBF1 and OBF1 in each of the HDAC2(-/-) mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation, we performed the NotchIP assay using appropriate primers and five site-specific antibodies for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3, on the chromatin surrounding proximal ~2.0 kb 5'-upstream, distal 5'-upstream and ORF regions of these transcription factor genes (Figs. 1, 2, 3 and 4) [67, 71]. Based on the results obtained, we assume that loose (open) or tight (closed) form of the chromatin structure surrounding the proximal 5'-upstream region of a certain gene, which surely causes its high or low (or no) gene expression level, should be qualitatively deduced from no or full binding ability of histone H3 to DNA based on hyper- or hypo- (or no) acetylation levels of one or more of the five specific Lys residues; probably K9/H3 and K27/H3 are dominant. In DT40 cells, as a whole, the chromatin structure surrounding the proximal ~2.0 kb 5'-upstream region of each of Pax5, Aiolos, EBF1 and OBF1 genes is in the loose form due to no binding ability of histone H3 to DNA based on hyper-acetylation levels of one or more of the five specific Lys residues. Consequently, these results could explain the facts that their gene expressions are obviously at high levels in DT40 cells [46, 67, 68, 70]. In mutant clone cl.2-1, the chromatin structure surrounding the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is in the tight form due to the full binding ability of histone H3 to DNA based on hypo-acetylation levels of one or more of the specific Lys residues at the early stage and thereafter remains nearly unchanged until the later stage. By contrast, the chromatin structure surrounding the proximal 5'-upstream region of the OBF1 gene is in the considerable loose form due to the weak binding ability of histone H3 to DNA based on hyper-acetylation levels at the early stage but thereafter dramatically changes to the tight form due to the full binding ability of histone H3 to DNA based on hypo-acetylation levels until the later stage. These results could explain the observations that in mutant clone cl.2-1 gene expressions of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage and thereafter remain unchanged until the later stage, but remarkably, that of the OBF1 gene is drastically decreased from the early to later stages [67, 70]. In mutant clones cl.2-2 and cl.2-4, the chromatin structure surrounding the proximal 5'-upstream region of each of Pax5, Aiolos and EBF1 genes is in the tight form based on hypo-acetylation levels of one or more of the specific Lys residues at the early stage. Thereafter, the chromatin structure of the first two changes to the loose form based on hyper-acetylation levels until the later stage but that of the last one remains unchanged based on hypo-acetylation levels during cultivation. On the other hand, the chromatin structure surrounding the proximal

Legend to Fig. 4. Alterations in acetylation levels of specific Lys residues of histone H3 within proximal 5'-upstream chromatin region of OBF1 gene in individual clones cl.2-1, cl.2-2, cl.2-4 and 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. Cross-linked chromatins were prepared from DT40 cells (W) and individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) mutants at the early (E), middle (M) and later (L) cultivation stages, and co-precipitated by five antisera specific for acetylated K9/H3, K14/H3, K18/H3, K23/H3 and K27/H3. After de-crosslinking, co-precipitated chromatins were amplified by PCR using appropriate primers of segments 1-14 and a plus b of the OBF1 gene. The figure is a set of figs. 13, 14, 15 and 16 of ref. [71].

5’-upstream region of the OBF1 gene is somewhat in the loose form based on slight hyper-acetylation levels at the early stage but thereafter changes to the loose form based on hyper-acetylation levels at the later stage. These results could roughly explain the observations that gene expressions of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage in mutant clones cl.2-2 and cl.2-4, and thereafter those of the first two are drastically increased until the later stage but that of the last one remains unchanged during cultivation [67, 70]. In addition, the gene expression of OBF1 is slightly decreased at the early stage and thereafter slightly increased at the later stage. In mutant clone cl.2-6, the chromatin structure surrounding the proximal 5’-upstream region of each of Pax5, Aiolos and EBF1 genes is in the tight form based on hypo-acetylation levels of one or more of the specific Lys residues at the early stage. Thereafter, the chromatin structure of these three genes changes to the loose form based on hyper-acetylation levels until the later stage. On the other hand, the chromatin structure surrounding the proximal 5’-upstream region of the OBF1 gene is in the tight form based on hypo-acetylation levels at the early stage but thereafter changes to the loose form based on hyper-acetylation levels at the later stage. These results could roughly explain the observations that gene expressions of Pax5, Aiolos and EBF1 are dramatically decreased at the early stage and thereafter drastically increased until the later stage [67, 70]. In addition, the gene expression of OBF1 is certainly decreased at the early stage and thereafter clearly increased at the later stage.

In summary, the results for alterations in acetylation levels (hyper or hypo) of one or more of the five specific Lys residues of histone H3, the form (loose or tight) of the chromatin structure surrounding the proximal 5’-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes, and their mRNA (as gene expression/transcription) levels (high or low) in four individual HDAC2(-/-) mutant clones (cl.2-1, cl.2-2, cl.2-4 and cl.2-6) are roughly and schematically presented in fig. 5. The results obtained by the NotchIP assay reveal that 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 different patterns not only in the Pax5, Aiolos, EBF1 and OBF1genes but also in the four individual clones of HDAC2(-/-) mutants. Further, these results should be fundamentals of our previous findings that gene expressions of IgM H- and L-chains are decreased in almost similar changing pattern but in distinct ways, attributed to alterations in gene expression levels of Pax5, Aiolos, EBF1 and OBF1 in the aforesaid individual clones of HDAC2(-/-) mutants during cultivation [67, 70]. That is, concerning gene expressions of the two immunoglobulin proteins at the later cultivation stage, clone cl.2-1 seems to be dependent on OBF1, clones cl.2-2 and cl.2-4 (and clones cl.2-3 and cl.2-5) seem to be dependent on Pax5 and Aiolos, and clone cl.2-6 seems to be dependent on Pax5, Aiolos and EBF1.

In addition, in [67, 71] we reported some interesting results as follows. The chromatin structure surrounding the proximal and distal 5’-upstream regions of the PCAF gene is in the loose form based on hyper-acetylation levels of the specific Lys residues in the mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 at any cultivation stages, as well as in DT40 cells. By contrast, the gene expression of PCAF, which is very low in DT40 cells [46], changes dramatically and distinctly in these four mutant clones during cultivation [67, 68, 70]. 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. Remarkably, acetylation levels of the specific Lys residues of histone H3 within the examined ORF regions of Pax5, Aiolos, EBF1 and OBF1 (and also PCAF) genes are very low in DT40 cells and remain unchanged or very slightly change in mutant clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 during cultivation [67, 71]. These findings suggest that the way and machinery for epigenetic modifications of the specific Lys residues of histone H3 with acetyl group should be clearly different between the proximal 5’-upstream and ORF regions of these five genes. In addition, as mentioned previously [67, 70], gene expressions of PCAF, HDAC7, HDAC9, Blimp1, E2A, Ikaros, PU.1 and XBP-1 also dramatically or moderately change in different patterns in

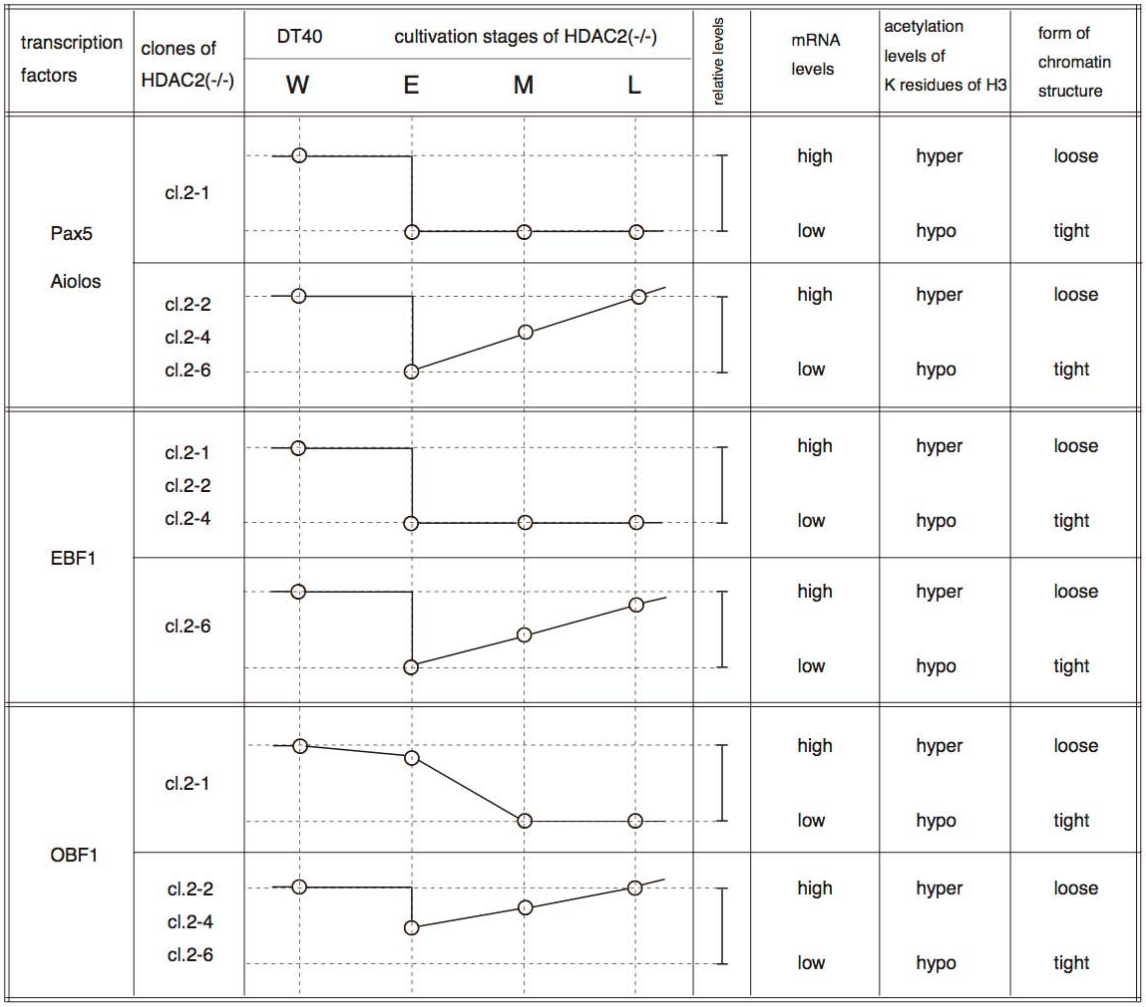

Fig. 5. Summary of alterations in mRNA (gene expression) levels (high or low), and acetylation levels (hyper or hypo) of five specific Lys residues of histone H3 and the chromatin structure (loose or tight) surrounding the proximal 5'-upstream regions of Pax5, Aiolos, EBF1 and OBF1 genes in individual clones cl.2-1, cl.2-2, cl.2-4 and cl.2-6 of HDAC2(-/-) DT40 mutants during continuous cultivation. The figure is identical with fig. 21 of ref. [71].

individual HDAC2(-/-) mutant clones during cultivation. These previous and recent results in [67, 70, 71] suggest that besides alterations in gene expressions of IgM H- and L-chains (and Pax5, Aiolos, EBF1 and OBF1) and in cell morphology, some other undefined cellular characteristics might be undoubtedly and separately changing among the four clones during cultivation, and such presumable altered characteristics may be complicated and diverse.

CONCLUSION

Based on our recent and previous results [41, 46, 53, 55, 58, 67-71], we propose a hypothesis on the distinct ways to diminish artificially accumulated IgM H- and L-chains through irreversible creation of plasticity of the varied chromatin structure surrounding proximal 5'-upstream regions of the specific transcription factor genes in individual clones of HDAC2(-/-) mutants during continuous cultivation. First of all, the accumulation of IgM H- and L-chains is recognized as an abnormal environment change, and subsequently putative signal(s) concerning the accumulation is genome-widely transmitted to the chromatin within the nucleus. The abnormal environment change should induce slight alterations in the chromatin structure of the

various genes encoding the chromatin-modifying enzymes and transcription factors (such as PCAF, HDAC7, HDAC9, Pax5, Aiolos, Ikaros, EBF1, E2A, PU.1, Blimp1, XBP-1, OBF1, Oct2, etc.), resulting in a slight alteration in their gene expression levels. Subsequently, the signal transduction and response to the environment change should be successively converged to the chromatin structure surrounding the proximal 5'-upstream regions of the various genes encoding specific factors and enzymes, such as Pax5, Aiolos, EBF1, OBF1, Blimp1, PCAF, HDAC7, HDAC9 and so on. As a result, remarkably, the chromatin structure surrounding the proximal 5'-upstream region (notch of chromatin) of each of Pax5, Aiolos, EBF1, OBF1 and other genes should dramatically and complicatedly change in individual clones of HDAC2(-/-) mutants through various generations, resulting in drastic alterations in their gene expressions. That is, the basis of these events is that the successive response to the environment change causes varied epigenetic modifications of the chromatin structure. Of these epigenetic modifications, acetylation and deacetylation of specific Lys residues of core histones may be major ones. And the positions of the specific Lys residues and/or kinds of core histones should be diverse. In the case mentioned above, acetylation and deacetylation of Lys-9, Lys-14, Lys-18, Lys-23 and Lys-27 residues of histone H3 are prominent. Consequently, the epigenetic modifications of one or more of these five Lys residues of histone H3 with acetyl group should be separately altered within the restricted chromatin structure surrounding the proximal 5'-upstream regions of the above-mentioned specific genes through various generations during cultivation. The binding ability of the N-terminal tail of histone H3 to DNA is qualitatively deduced based on acetylation levels of one or more of these specific Lys residues, though it is still unclear which Lys residue(s) is really and/or mainly involved in the binding. In particular, hyper- (high) or hypo- (low or no) acetylation levels should induce no binding or full binding ability, resulting in the loose (open) or tight (closed) form of the chromatin structure. Thus, chromatin structure plasticity should be irreversibly created through successive structural changes due to binding ability of histone H3 to DNA based on changes in acetylation levels of these specific Lys residues. As a result, the loose or tight form of the chromatin structure surrounding the proximal 5'-upstream regions of the specific genes should cause their high or low (or no) gene expression levels. Notably, the ways to create chromatin structure plasticity are distinct in individual HDAC2(-/-) mutant clones, though the accumulation of IgM H- and L-chains as the abnormal environment change is the same for all of them. That is, to exclude artificially accumulated immunoglobulin proteins, individual clones of HDAC2(-/-) mutants should differently alter the chromatin structure surrounding the proximal 5'-upstream regions of the specific genes encoding Pax5, Aiolos, EBF1, OBF1 and others. Detailed way and machinery for irreversible creation of chromatin structure plasticity, including recognition of the accumulation of IgM H- and L-chains, signal transduction pathway and chromatin conformation change, remain to be elucidated.

CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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