Histone deacetylases (HDACs) are involved in the deacetylation of core histones, which is related to transcription regulation in eukaryotes through alterations in the chromatin structure. We cloned cDNA and genomic DNA encoding a chicken HDAC, chHDAC-3, which was localized in both the nuclear and cytoplasm in DT40 cells. Although one of the two chHDAC-3 alleles could be disrupted with high efficiency, no homozygous mutants were obtained after a second round of the gene-targeting technique due to the necessity for DT40 cells. We introduced a chHDAC-3 transgene under the control of a tetracycline-responsive promoter into the heterozygous mutant and subsequently disrupted the remaining endogenous chHDAC-3 allele to generate the homozygous chHDAC-3-deficient mutant, ΔchHDAC-3/FHDAC3. Inhibition of the expression of the regulatable chHDAC-3 transgene caused apoptotic cell death of the mutant. Complementation experiments involving truncated and missense chHDAC-3 mutant proteins revealed that the 1–23 N-terminal sequence, the 389–417 C-terminal sequence, the nuclear export signal, and the deacetylation activity of chHDAC-3 were essential for the cell viability. Taken together, these results indicate that chHDAC-3 plays an essential role, probably as a scavenger in the cytoplasm, in the proliferation of DT40 cells.

The molecular mechanisms for the regulation of gene expression through alterations in the chromatin structure have been rapidly elucidated in eukaryotes. The chemical modification of core histones with acetyl groups has been thought to be of fundamental importance as to conformational changes of the chromatin (1–5). The level of acetylation is related to transcriptional activity, and the acetylation induces an open chromatin conformation that allows the transcription machinery access to promoters. The acetylation of core histones is catalyzed by histone acetyltransferases (HATs),1 which are homologs of yeast Gcn5p (general controlled nonrepressed protein) (1, 6, 7). In addition, p300/CREB-binding protein, PCAF, and TAFII230/250 were found to possess the ability to acetylate core histones (8–11).

On the other hand, the deacetylation of acetylated core histones is catalyzed by histone deacetylases (HDACs) (12), which are homologs of yeast Rpd3, which negatively regulates the global expression of genes (13). In higher eukaryotes, Mad-Max heterodimers require the interaction of Mad with mSin3, the mammalian homolog of Sin3, to repress transcription (14–16), and mSin3 exists in a complex with HDAC-1 and -2 (17). Treatment with trichostatin A (TSA), a specific inhibitor of HDACs (18), abolishes both the Mad repression and HDAC activity of the mSin3 immunocomplexes. The transcriptional corepressors SMRT and N-CoR act as silencing mediators for retinoid and thyroid hormone receptors (19–21). Transcriptional repression by nuclear receptors is mediated by a complex containing SMRT or N-CoR, mSin3, and HDAC. Mammalian HDAC-1 is recruited by the retinoblastoma protein to repress transcription (22, 23). These results therefore indicate that a complex containing HDAC deacetylates nucleosomal core histones, producing alterations in the chromatin structure that block transcription.

It has been reported that multiple forms of HDACs exist like HATs, there being at least three human HDACs (12, 24), two mouse HDACs (10, 25), and two chicken HDACs (26). These findings led us to expect that HDAC family members each play an individual, particular role and then participate in combination with one another and/or with HAT family members in the acetylation of core histones, which is related to transcriptional activity. To understand the overall picture of transcription regulation through the formation of (and/or alterations in) the chromatin structure based on the acetylation of core histones, it should be essential to determine the role of each of the multiple HDACs and HATs in vivo.

The DT40 chicken B cell line incorporates foreign DNA by means of targeted integration at frequencies similar to those for random integration at a number of different genomic loci, including the histone gene cluster (27–29). Using the conventional gene targetting technique, we generated two homozygous DT40 mutants, ΔchHDAC-1 and -2 devoid of two alleles of chHDAC-1 and -2 (26). Systematic analyses of the mutants obtained led us to some noticeable conclusions as follows. The protein patterns on two-dimensional PAGE were obviously changed for ΔchHDAC-2, but the changes were insignificant for ΔchHDAC-1. In particular, the amounts of IgM H- and L-chains increased in the former mutant. Furthermore, of the two forms of the IgM H-chain, the secreted form increased, but the membrane-bound form decreased. Interestingly, the IgM H-chain gene was transcribed more in ΔchHDAC-2 than in DT40 cells. The alternative processing of IgM H-chain pre-mRNAs...
preferentially occurred in the mutant, resulting in increases in the amounts of secreted-form mRNAs. Thus, chHDAC-2 dually controls the amounts of the secreted-form IgM H-chain at the steps of both the transcription of its gene and the switch from membrane-bound form to secreted form mRNAs, probably through alterations in the chromatin structure restricted to narrow regions surrounding the IgM H-chain gene and putative switch-related factor gene(s), which should be due to the remaining acetyl groups of particular Lys residue(s) of core histones. Most or all of the specific functions of chHDAC-2 can not be compensated for by any other chHDACs, including chHDAC-1, all of which should be different from the former in substrate specificity, i.e. in core histone subtypes, histone variants, or deacetylatable Lys residues. The difference in the functions of chHDAC-1 and -2, i.e. in the participation in the accumulation of the IgM H-chain (and IgM L-chain), should be due to the difference in their C-terminal regions of approximately 50 amino acids, where the two enzymes exhibit relatively low homology (~50%), although they show extensive homology (~95%) in the remaining regions of approximately 430 amino acids.

Our previous screening of both DT40 cDNA and genomic DNA libraries revealed not only that there was another form of chHDAC, but also that it differed remarkably in size from both chHDAC-1 and -2, suggesting that the novel chHDAC enzyme should be different in function from these two enzymes. In this study, therefore, we first cloned cDNA and genomic DNA encoding the remaining chHDAC, designated as chHDAC-3, which was localized in both the nuclei and cytoplasm. The HDAC activity in the chHDAC-3 immunoprecipitate was abolished by treatment with TSA. Using the conventional gene targeting technique, we could easily generate a heterozygous chHDAC-3-deficient mutant but not a homozygous one, indicating that chHDAC-3 is essential for DT40 cells. Next, we generated a homozygous chHDAC-3-deficient mutant, ΔchHDAC-3/ΔchHDAC3, carrying a chHDAC-3 transgene, encoding FLAG-tagged chHDAC-3 under the control of a tetracycline (tet)-responsive promoter. The inhibition of the expression of the chHDAC-3 transgene caused the slow growth of ΔchHDAC-3/ΔchHDAC3 before dying. Complementation experiments, involving truncated or missense chHDAC-3 mutant proteins, revealed that the N-terminal region, the C-terminal region, the proper nuclear export signal, and the deacetyl activity of chHDAC-3 are necessary for the specific function of the enzyme. Taken together, these results indicate that chHDAC-3 is essential for the viability of the DT40 cell line.

**EXPERIMENTAL PROCEDURES**

Cloning of cDNA and Genomic DNA Encoding chHDAC-3—To obtain full-length chHDAC-3 cDNA using the resultant chHDAC-related PCR product as a probe, we screened a DT40 λ ZAP II cDNA library, as described (26). The entire sequences of both strands of the full-length cDNA were sequenced by the dye terminator method (Applied Biosystems Division, Perkin-Elmer). Using chHDAC-3 cDNA obtained as a probe, genomic DNA specific for chHDAC-3 was isolated on the screening of a DT40 genomic DNA library in a λ phage, as described (26). The organization of the resultant genomic DNA was determined by the PCR-sequencing protocol (Amersham Pharmacia Biotech).

**Gene Constructs**—Two blunt-ended cassettes carrying neo and hisD (30), transcribed by the β-actin promoter (31, 32), were obtained as described (33, 34). Using these cassettes, we generated targeting vectors for the disruption of chHDAC-3, as follows. To obtain the pBamchHD-3neo or pBamchHD-3hisD construct, we replaced the genomic sequence between exons 5 and 6 of chHDAC-3 with a pBamHI- or hisD-carrying cassette. Namely, the 1.5-kb upstream sequence generated by PCR with the genomic clone as a template was ligated to the 5’-end of the cassette carrying neo or hisD, followed by ligation of the 1.7-kb HindIII/BglII fragment derived from downstream of exon 6 of the gene to the 3’-end of either cassette. Probe A was the 1.5-kb BamHI fragment derived from intron 2 of chHDAC-3. Probes B and C were derived from neo and hisD, as described (33, 34).

To construct the pTetFHDAC3 vector expressing FLAG-tagged chHDAC-3 under the control of the tet operator (tetO) and cytomegalovirus minimal promoter, the PCR-amplified cDNA product encoding FLAG-tagged chHDAC-3 was inserted into the pUC9 plasmid (35), and concurrently its constituent luciferase gene was replaced. To obtain the pTg4-bleo construct, a cassette of the bleomycin-resistant gene driven by the β-actin promoter was inserted into the pUHD15–1 plasmid, which contains the tet transactivator gene, controlled by the cytomegalovirus promoter (35).

In the complementation assay, we generated chHDAC-related expression vectors as follows. To obtain the HA-tagged chHDAC-1, -2, or -3 expression plasmid, each of three HA-tagged full-length chHDAC cDNAs was generated by PCR and then inserted into pAPuro (36). Various 5’- and 3’-truncated fragments of chHDAC-3 were generated by PCR and cloned into pAPuro to obtain their HA-tagged expression plasmids. Single missense mutations (H135A, H193F, and Y404F) or a double missense mutation (L29I,L31M) was generated with a Quick Change™ site-directed mutagenesis kit (Stratagene) using pHADHC3 (the HA-tagged chHDAC-3 expression plasmid obtained above) as a template. All gene constructs were verified by sequencing. The details of the plasmid constructs are available on request.

**Cell Cultures, Transfection, and Isolation of Transfectants**—DT40 cells and all subclones were cultured essentially as described (37–39) in Dulbecco’s modified medium. At the indicated times, the cells were counted to determine the growth rate.

Transfection by the electroporation method was carried out essentially as described (34, 37, 39). Drug-resistant colonies were selected on 96-well plates in medium containing appropriate concentrations of several drugs, i.e. 2 mg of G-418 (Life Technologies, Inc.; ml, 0.3 mg of phleomycin (Sigma)/ml, 0.8 mg of histidinol (Sigma)/ml, or 0.4 μg of puro (Sigma)/ml. To suppress the expression of the tet-responsive FHDC3 transgene, doxycycline (tet) (Sigma) was added at the concentration of 100 ng/ml.

**Generation of Antibodies against chHDAC-3**—To generate antiserum against chHDAC-3, the cDNA fragment encoding the C-terminal region of amino acids 362–429 of chHDAC-3 was subcloned into the pOEX-2T plasmid (CLONTECH) in-frame, and then the chHDAC-3 C-terminal peptide-GST fusion protein was synthesized in Escherichia coli, extracted, and purified to more than 95% homogeneity. According to a standard immunization protocol, New Zealand White rabbits were immunized, and then preimmune and immune sera were collected. To remove the anti-GST antibodies, the crude antiserum was passed through a column of GST bound to glutathione-Sepharose beads. To avoid the cross-reaction with chHDAC-1 and -2, the anti-chHDAC-3 anti-serum was successively passed through columns of chHDAC-1 and -2 C-terminal peptide-GST-conjugated glutathione-agarose beads (26).

**Western Blotting**—Cells (5×10⁷) were lysed in 200 μl of SDS buffer, and then aliquots (20 μl) of the resultant cell extracts were separated by SDS-PAGE, followed by electrophobting onto a nitrocellulose membrane filter as described (40). The filter was probed with a 1/1000 dilution of anti-chHDAC-3 antiserum, anti-FLAG antiserum (Sigma), or anti-HA antiserum (12CA5, Roche Molecular Biochemicals), followed by the addition of a 1/1000 dilution of secondary antibodies (horse-radish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Dako)). As a control, the preimmune serum was used at a 1/1000 dilution. The signal was detected with a Super Signal Ultra (Pierce).

**Southern Blotting**—Genomic DNAs were digested with the indicated enzymes, separated in a 0.8% agarose gel, transferred to a Hybond N membrane, and then hybridized with 32P-labeled probe A, B, or C, as described (41).

**Radioimmunoprecipitation and HDAC Activity Assay**—A radioimmunoprecipitation experiment was performed as described (42). Cells (2×10⁷) were metabolically labeled for 1 h with [35S]Met and [35S]Cys and then lysed in 1 ml of radioimmunoprecipitation buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). Aliquots (500 μl) of the cell extract obtained were incubated with anti-chHDAC-3 antisera (10 μl) in either the presence or absence of 5 μg of the chHDAC-3-related fusion protein and then incubated with protein A-Sepharose 4FF beads (Amersham Pharmacia Biotech). After washing with radioimmunoprecipitation buffer, the immunoprecipitated proteins were dissolved in SDS buffer, resolved by 10% SDS-PAGE, and then examined by autoradiography.

The in vitro HDAC activity in the immunoprecipitate obtained with anti-chHDAC-3 antisera was assayed essentially as described (12). Cells (4×10⁷) were lysed in 2 ml of phosphate-buffered saline containing 1% Nonidet P-40, and then aliquots (500 μl) of the extract were
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immunoprecipitated and washed with HD buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol). The resultant immunocomplex was incubated for 1 h at 37 °C with 100 μg (5 × 10^6 dpm) of acid-soluble histones isolated from [3H]acetate-labeled DT40 cells. The released [3H]acetic acid was extracted with ethyl acetate and then measured. Pretreatment of the immunoprecipitated sample with 100 nM TSA was performed for 2 h at 4 °C before the addition of the labeled histones.

To determine the HDAC activity of various chHDAC-3 mutant proteins, plasmids expressing them were transiently transfected into COS7 cells. After transfection for 36 h, the cells were lysed in phosphate-buffered saline containing 1% Nonidet P-40 at 4 °C for 30 min, followed by centrifugation. A portion of each of the supernatants obtained was analyzed by Western blotting using anti-HA antibody as mentioned above to determine the amounts of the chHDAC-3-related recombinant proteins. The remainder of each of the supernatants was immunoprecipitated with anti-HA antibody-conjugated agarose beads (Santa Cruz Biotechnology, CA), and then the HDAC activity in the immunoprecipitate was assayed as described (26).

**Immunofluorescence Microscopy**—An immunofluorescence microscopy involving anti-chHDAC-3 antisera was carried out according to the manufacturer’s protocol (NEN Life Science Products). DT40 subclones on slides were fixed in 4% formalin. The primary antibody (affinity-purified rabbit anti-chHDAC-3 antisera) was incubated at 1:20 dilution, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako) as a secondary antibody at 1:1000 dilution. After incubation with fluorescein isothiocyanate-conjugated thyramide (NEN Life Science Products), the cells were stained with 4’,6-diamidino-2-phenylindole. The fluorescein isothiocyanate-conjugated thyramide-labeled chHDAC-3 and nuclei were examined by fluorescence microscopy.

**Fluorescence-activated Cell Sorting Analysis**—At the indicated times, cells were labeled with 5-bromodeoxyuridine (BrdUrd) (Ameresco) and detected by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Dako) as a secondary antibody at 1:20 dilution, followed by incubation with fluorescein isothiocyanate-conjugated thyramide. The fluorescein isothiocyanate-conjugated thyramide-labeled chHDAC-3 and nuclei were examined by fluorescence microscopy.

**Complementation Assay**—To determine the ability of chHDAC-3 or its derivatives to rescue the tet-inducible lethality of Δch3ΔHDAc-3/FHDAC3 (cl 18-tet-2-5), a complementation assay was carried out essentially as described (44). The mutant cells (1 × 10^7) were transfected with pBHAHDAC3 or its derivatives for 24 h and then split into two portions. One portion was incubated in 96-well microtiter plates with medium containing 0.4 μg of puro/ml to determine the frequency of transformants and the other was incubated in 96-well microtiter plates with medium containing 0.4 μg of puro/ml plus 100 ng of tet/ml. After incubation for 10 days, the numbers of surviving colonies were determined.

**Accession Number**—The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number AF039753.

**RESULTS**

Cloning of cDNA and Genomic DNA Encoding chHDAC-3.—To identify the chicken HDACs as novel enzymes, we have cloned and sequenced their cDNAs. Forty-one clones, obtained in our previous screening using the HDAC-related PCR product of a DT40 cDNA library, were classified into three groups based on their restriction enzyme patterns. The two largest cDNA inserts of two of the three groups appeared to contain the full-length sequences of two different chicken HDAC cDNAs, chHDAC-1 and -2 (26). Sequence analysis of the largest cDNA insert of the remaining group, designated as clone 24, revealed that it contained both an initiation codon and a 3’ poly(A) tail and, thus, appeared to contain the full-length sequence of another chicken HDAC cDNA. The amino acid sequence deduced from its nucleotide sequence is shown together with those of human and mouse HDAC-3a, yeast Rpd3, and chHDAC-1 and -2 in Fig. 1. The protein encoded by clone 24 comprises 428 amino acids including a putative initiation Met and exhibits approximately 97% identity in amino acid sequence to human and mouse HDAC-3a. Thus, this protein is the chicken HDAC-3 homolog and is designated as chHDAC-3. Compared with chHDAC-1 and -2, chHDAC-3 lacks 6–7 amino acids and approximately 50–60 amino acids at its N-terminal and C-terminal ends. In addition, within the considerably constant background in the corresponding regions of the three chicken enzymes, chHDAC-3 is noticeably different from the other two, especially in the N-terminal region of approximately 140 amino acids and the C-terminal region of approximately 100 amino acids.

As described previously (26), we have isolated two different genomic DNAs specific for chHDAC-1 and -2. Further detailed analysis involving the PCR method followed by sequencing revealed not only that another genomic DNA obtained was specific for chHDAC-3 but also that it comprised 14 exons and was 15 kb in length (see the middle panel of Fig. 3A). Moreover, sequence analysis revealed that the 5’-upstream region of the gene was rich in GC, and typical TATA boxes were not observed in the region (data not shown).

**HDAC Activity in the chHDAC-3 Immunoprecipitate and Subcellular Localization of the Enzyme**—Western blotting using anti-chHDAC-3 antisera against the recombinant chHDAc-3 C-terminal peptide (of amino acids 363–428)–GST fusion protein, which was absorbed with C-terminal regions of chHDAC-1 and -2, revealed a protein of 45 kDa (p45) in DT40 cells (Fig. 2A). The radioimmunoprecipitation experiment, after labeling with [35S]Met and [35S]Cys, also revealed the p45 protein in the immunoprecipitate with anti-chHDAC-3 antisera (Fig. 2B). The block + (Fig. 2B) experiment established that the 45-kDa protein did not appear when anti-chHDAC-3 antisera was preincubated with the chHDAC-3 C-terminal peptide–GST fusion protein. Moreover, no other polypeptides co-precipitated specifically with anti-chHDAC-3 antisera were present in the chHDAC-3 immunoprecipitate.

Next, we examined whether or not the HDAC activity was present in the chHDAC-3 immunoprecipitate prepared from DT40 cell extracts. As shown in Fig. 2C, as expected, this immunocomplex exhibited significant HDAC activity compared with the precipitate with the control preimmune antisera. To confirm the HDAC activity in the immunoprecipitate, we examined the influence of TSA. Treatment with TSA reduced the enzyme activity to a background level, indicating that the activity is really due to chHDAC-3.

In the immunofluorescence microscopy involving anti-chHDAC-3 antisera we examined the subcellular localization of chHDAC-3 in DT40 cells. As shown in upper panels 1 and 2 of Fig. 2D, chHDAC-3 was localized in both nuclei and the cytoplasm, although both chHDAC-1 and -2 were preferentially localized in nuclei (26). To confirm this result, we constructed a chimeric plasmid expressing the C-terminal region of chHDAC-3 fused to green fluorescent protein and transiently overexpressed the green fluorescent protein–chHDAC-3 fusion protein in the DT40 cell line. Inspection of the resultant cells by fluorescence microscopy revealed that chHDAC-3 was certainly localized in nuclei and the cytoplasm (data not shown).

**Generation of the Conditional chHDAC-3-deficient Mutant**—To generate homozygous chHDAC-3-deficient mutants, we first introduced the pΔchHD3-3/neo construct into DT40 cells (Fig. 3, A and D). As expected, after integration of this target vector into the chHDAC-3 locus in two of the stable transfectants selected with G-418 (+/-; cl 17 and cl 18), probe A, originating from the middle of intron 2, newly hybridized to the 5.0-kb BamHI fragment in addition to the endogenous 12.5-kb BamHI fragment. To obtain a homozygous chHDAC-3-deficient mutant, we transfected two of the G-418-resistant clones with the pΔchHD3-3/hisD construct. Genomic DNAs were isolated from the G-418 and histidinol-resistant transfectants 10-fold more than those examined on other genomic loci (26–29, 33)
and were analyzed by Southern blotting. However, for all clones examined, the expected 15.0-kb BamHI fragment was not observed, and the 12.5-kb BamHI fragment did not disappear (data not shown). All these unsuccessful experiments strongly suggested that the disruption of both alleles of chHDAC-3 appeared to be lethal for DT40 cells.

Next, one of the heterozygous chHDAC-3-deficient mutants (1/2\(D\)chHDAC-3; cl 18) was transfected with the tet-responsive chHDAC-3 expression vector, and then 1/2\(D\)chHDAC-3/FHDAC3 subclones carrying the construct bearing the FLAG sequence integrated randomly on the chromosomes were obtained in the presence of phleomycin (Fig. 3, B and C). As shown in Fig. 3C, in four of the clones obtained (cl 18-tet1 to 4), the FLAG-tagged chHDAC-3 chimeric protein, FHDAC3, was detected with anti-FLAG antiserum but had disappeared at 48 h after the addition of tet, indicating that the amounts of the chimeric protein in the DT40 subclones could be controlled.

Finally, we transfected the pDchHD-3/bsd construct into one of 1/2\(D\)chHDAC-3/FHDAC3 (cl 18-tet2) (Fig. 3, A and B). Genomic DNAs were isolated from G-418 and histidinol-resistant transfectants and then analyzed by Southern blotting (Fig. 3D). As expected, after integration of this targeting vector into the remaining endogenous chHDAC-3 locus in one (with three more clones) of the stable transfectants selected (1/2\(D\)chHDAC-3/FHDAC3; cl 18-tet2), probe A newly hybridized to the 15.0-kb BamHI fragment in addition to the 5.0-kb BamHI fragment, and then the endogenous 12.5-kb BamHI fragment disappeared. On the other hand, in one of the stable transfectants (1/2\(D\)chHDAC-3/FHDAC3; cl 18-tet2-1), the 15.0-kb BamHI fragment was not detected, and the 12.5-kb BamHI fragment did not disappear, indicating that the residual endogenous chHDAC-3 allele was not disrupted in this clone, as in most of the remaining clones. As expected, probe B, derived from neo, hybridized to the 9.0-kb BamHI fragment in the two clones, but probe C, derived from hisD, hybridized to the 15.0-kb BamHI fragment in the cl 18-tet2 clone but not in the cl 18-tet2-1 clone. Thus, the cl 18-tet2-1 clone (with three more clones) was really the conditional homozygous chHDAC-3/FHDAC3 mutant. Taken together, these results indicated not only that chHDAC-3 is essential for the viability of DT40 cells, but also that FHDAC3 expressed from the transgene is capable of providing the chHDAC-3 function(s) required for the viability.

**Inhibition of Expression of FHDAC3 Alters the Growth and Cell-cycle Distribution of the Conditional chHDAC-3-deficient Mutant**—We studied the possible influence of the addition of tet on the growth of chHDAC-3-deficient mutants. The dou-
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Expression, immunoprecipitation, deacetylation activity, and subcellular localization of chHDAC-3. A, Western blotting. Total cellular proteins were prepared from DT40 cells. Aliquots were subjected to 7% SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane, and then the proteins were detected with anti-chHDAC-3 antiserum and horseradish peroxidase-conjugated goat anti-rabbit IgG. The preimmune serum was used as a control. The band corresponding to chHDAC-3 is indicated by p45. B, radioimmunoprecipitation. The immunoprecipitation of chHDAC-3 from [35S]Met- and [35S]Cys-labeled DT40 cells was performed with anti-chHDAC-3 antiserum, and then the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Block + corresponds to the addition of the cognate immunogen for the first antibody. The polypeptide corresponding to chHDAC-3, the molecular weight of which is 45 kDa, is indicated by p45. C, histone deacetylase activity. A cell extract was prepared from DT40 cells followed by immunoprecipitation using anti-chHDAC-3 antiserum. An in vitro HDAC assay was performed for the resultant immunoprecipitate. Preimmune serum was used as a control. TSA + indicates that the immunoprecipitated proteins were pretreated with 100 nM TSA before being assayed for HDAC activity. The amount of released [3H]acetic acid is correlated with the immunoprecipitated HDAC activity. Results shown represent average values for three independent experiments.

In vivo HDAC activity in tet+ and tet− cells. For this purpose, we constructed vectors expressing C-terminal- and N-terminal-truncated mutants of HA-tagged chHDAC-3 under the control of the β-actin promoter together with ones expressing HA-tagged chHDAC-1, 2, and 3. All the constructs contained a cassette of the puromycin-resistant gene driven by the SV40 promoter in their 3’-downstream regions for selection with the drug. Although Western blotting involving anti-HA antiserum revealed that all the HA-tagged chHDAC-3-related proteins (including the missense mutants mentioned later) were almost equally transiently expressed in ΔchHDAC-3/ΔFHDAC3, it was difficult to measure quantitatively their deacetylation activity, because their amounts were very low (data not shown). Therefore in COS7 cells we first confirmed the transient expression of all these proteins at almost equal levels (Fig. 5C), and then measured their deacetylation activity toward acetylated core histones (Fig. 5B). Three
FIG. 3. Generation of the homozygous ΔchHDAC-3/FHDAC3 mutant. A, genomic organization and schematic diagram of the homologous recombination, resulting in disruption of the first and second alleles of chHDAC-3. a, the targeting vector, the pΔchHD-3/neo, or pΔchHD-3/hisD.
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N-terminal-truncated mutants, pβHAHDAC3-(24–428), pβHAHDAC3-(58–428), and pβHAHDAC3-(78–428), exhibited almost the same deacetylation activity (70–80%) as that of the parental pβHAHDAC3 construct. However, the remaining N-terminal-truncated mutant, pβHAHDAC3-(188–428), lacked the activity completely, indicating that the active domain for the deacetylation was contained in the region comprising amino acids 78–187 of chHDAC-3. These findings agreed with the finding that the active domain for the deacetylation of acetylated core histones is highly conserved in the internal regions surrounding amino acids 100–200 of other HDAC family members (45). On the other hand, one C-terminal-truncated mutant, pβHAHDAC3-(1–418), exhibited approximately 70% that of the parental pβHAHDAC3 construct. The activity of another C-terminal mutant, pβHAHDAC3-(1–388), was lower than that of the parental construct, the decrease being approximately 80%. The remaining C-terminal mutant, pβHAHDAC3-(1–322), exhibited no activity. These findings indicated that the C-terminal region of amino acids 323–428, especially amino acids 323–388, is also involved in the deacetylation activity of chHDAC-3.

Next, we transfected ΔchHDAC-3/FHDAC3 with these truncated constructs to determine whether or not they can complement the tet-induced lethality by means of a colony formation experiment (Fig. 5A). Results obtained in the presence of puromycin and in the absence of tet revealed not only the transfection with an almost equal frequency (71–143% of the control pβHASVpuro construct), regardless of kinds of the HA-tagged chHDAC-3-related genes, but also no dominant-negative effects of the mutant proteins produced from them. And then the complementation ability of these chHDAC-3 mutant proteins was measured in the presence of both tet and puromycin. The N-terminal-truncated mutant, pβHAHDAC3-(24–428), exhibited no complementation ability, indicating that the region comprising amino acids 1–23 of chHDAC-3 was necessary for cell viability. As easily expected, three other N-terminal mutants, pβHAHDAC3-(58–428), pβHAHDAC3-(78–428), and pβHAHDAC3-(188–428), exhibited no complementation ability. On the other hand, one C-terminal-truncated mutant, pβHAHDAC3-(1–418), exhibited almost similar complementation ability (~67%) toward the parental pβHAHDAC3 construct. However, two other C-terminal-truncated mutants, pβHAHDAC3-(1–388) and pβHAHDAC3-(1–322), exhibited no complementation ability. These findings suggested that the region comprising amino acids 389–418 of chHDAC-3 was also necessary for the viability of DT40 cells. Furthermore, either of two control constructs, pβHAHADC1 and pβHAHADC2, exhibited no complementation ability, indicating that both chHDAC-1 and -2 have no ability to compensate for this specific chHDAC-3 function.

A possible tyrosine phosphorylation signal for YSrp (45) was present at positions 404–407 in the essential C-terminal region of chHDAC-3 but absent in the corresponding regions of both chHDAC-1 and -2. To determine whether or not the specific chHDAC-3 function accompanied the tyrosine phosphorylation of this particular site, we constructed a vector expressing the single missense mutant protein of HA-tagged chHDAC-3, pβHAHDAC3-(Y404F), and then assayed its complementation ability (Fig. 5). The Y404F mutant protein transiently expressed exhibited similar deacetylation activity to that of the parental HA-tagged chHDAC-3 (Fig. 5B). This Y404F mutant protein of chHDAC-3 exhibited almost similar complementation ability (~70%) to the parental protein (Fig. 5A), indicating that the tyrosine phosphorylation of this possible signal, if it occurred, did not accompany the specific chHDAC-3 function.

Deacetylation Activity and Nuclear Export Signal of chHDAC-3 Required for Cell Viability—The active domain for the deacetylation of acetylated core histones is highly conserved surrounding positions 100 to 200 in chHDAC-3, as in other HDAC family members; also, two His residues at positions 135 and 193, important for the deacetylation activity, are also conserved. To determine whether or not the deacetylation activity of chHDAC-3 is necessary for the cell viability, we constructed vectors expressing two single missense mutant proteins of HA-tagged chHDAC-3, pβHAHDAC3-(H135A) and pβHAHDAC3-(H193F), identical to the conserved deacetylation active domain, transfecting ΔchHDAC-3/FHDAC3 with these two constructs, and then assayed their complementation ability (Fig. 5). The deacetylation activity of the two mutant proteins transiently expressed was severely reduced or had disappeared (Fig. 5B). The two single missense mutant proteins exhibited no complementation ability (Fig. 5A), indicating that the chHDAC-3 function specific for the cell viability should accompany the histone deacetylation activity.

On the other hand, a typical nuclear export signal (NES) of LALTVHVLHYGL (46, 47) was present at positions 29–41 just behind the essential N-terminal region of chHDAC-3, but the NES sequence was somewhat different in the corresponding regions of chHDAC-1 and -2, which were preferentially localized in the cytoplasm (26). Four Leu residues exist at positions 29, 31, 35, and 38 in chHDAC-3 and are thought to be important for the NES function. In the cases of both chHDAC-1 and -2, the two former Leu residues are simultaneously substituted by Ile and Met, respectively (see Fig. 1). We examined whether or not this typical NES was involved in the specific chHDAC-3 function for cell viability. For this purpose, we constructed a vector expressing the double missense mutant protein of HA-tagged chHDAC-3, pβHAHDAC3-(L291I/L31M), and then assayed its complementation ability (Fig. 5). The histone deacetylation activity of this double missense mutant protein transiently expressed was unchanged (Fig. 5B). This double missense mutation caused a prominent decrease in the complementation ability (~5% of the parental pβHAHDAC3 construct) (Fig. 5A), suggesting the necessity of the proper NES sequence of chHDAC-3 for the full cell viability.

DISCUSSION

A novel chicken HDAC, chHDAC-3, like most HDAC family members including chHDAC-1 and -2, possesses a highly conserved active domain for the deacetylation of core histones in its internal region (Fig. 1) (45) and really participates in the
Fig. 4. Influences of inhibition of the expression of FHDAC3 on the growth and cell cycle of chHDAC-3-deficient mutants. A, growth curves of chHDAC-3-deficient DT40 subclones. Three chHDAC-3-deficient DT40 subclones were cultured at $1 \times 10^4$ cells/ml in the absence or presence of tet. Dotted lines represent the growth from days 3 to 4. At day 3, the cells were transferred to fresh media at $1 \times 10^4$ cells/ml and continuously grown. The cell numbers determined at the indicated times are plotted on a log scale. The values are the averages for three independent experiments. The symbols for the cell lines are shown in the inset. B, the effect of the addition of tet on the expression of FHDAC3 in ΔchHDAC-3/FHDAC3. FHDAC3 in ΔchHDAC-3/FHDAC3 (cl 18-tet2−5) was analyzed by Western blotting involving anti-chHDAC-3 antiserum.
deacetylation of acetylated core histones (Fig. 2C). On the other
hand, there are various differences in characteristics between
chHDAC-3 and both chHDAC-1 and -2, as follows. First, chHDAC-3 lacks two regions corresponding to the N-terminal seg-
mament of 6–7 amino acids and the C-terminal segment of ap-
proximately 50–60 amino acids of chHDAC-1 and -2,
respectively. Second, within the corresponding regions of these
three chHDACs, chHDAC-3 is considerably distinct from the
other two in both the approximately 140 N-terminal amino acid
sequence and the approximately 100 C-terminal amino acid
sequence. Third, a possible phosphorylation signal of YSRP
(45) is present at positions 404–407 in the C-terminal region of
chHDAC-3 but not in the corresponding regions of chHDAC-1 and -2. Fourth, a typical NES of LALTHSLVLHYGL (46, 47) is
present at positions 29–41 in the N-terminal region of chHDAC-
3, but the NES sequence is somewhat distinct in the
corresponding regions of chHDAC-1 and -2, although a nuclear
localization signal (48) that should be present in these three
chHDACs has not yet been identified. As a result, there is a
difference in their subcellular localization, i.e. the former
should shuttle between nuclei and the cytoplasm (Fig. 2D), but
the latter are preferentially localized in nuclei (26). Fifth, no
other polypeptides co-precipitating specifically with anti-chH-
DAC-3 antiserum were present in the immunocomplex (Fig.
2B), although several polypeptides, probably including chicken
homologs of mSin3A, Mad, SMRT, or N-coR, were co-precipi-
tated with anti-chHDAC-1 or 2 antiserum (26).

All our attempts involving the conventional gene targeting
technique to generate the homozygous DT40 mutant devoid of
two chHDAC-3 alleles were unsuccessful, indicating that chH-
DAC-3 should be essential as to the viability of DT40 cells,
whereas chHDAC-1 and -2 are nonessential (26). To assess the
role of chHDAC-3 in vivo, we generated a tet-responsive, con-
tditional, homozygous chHDAC-3-deficient mutant, D
chHDAC-3/FHDAC3, wherein the level of the product of the chHDAC-3
transgene, the FLAG-tagged chHDAC-3 protein (FHDAC3),
could be easily controlled (Figs. 3C and 4B). Upon inhibition of
the expression of the chHDAC-3 transgene by the addition of
tet, FHDAC3 disappeared in less than 24 h, but ΔchHDAC-3/
FHDAC3 grew normally by day 2 (Fig. 4, A and B). Thereafter,

as in Fig. 2A, at the indicated times after the addition of tet. Endogenous chHDAC-3 in DT40 cells was also analyzed. C, the effect of the addition of tet on the cell-cycle distribution of ΔchHDAC-3/FHDAC3. At the indicated times after the addition of tet, cellular DNA contents were determined
by flow cytometry after a pulse BrdUrd-labeling. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated tyramide-anti-BrdUrd (γ
axis, log scale) to detect BrdUrd incorporation and with propidium iodide (PI) to detect total DNA (α axis, linear scale). A typical experiment is
shown. The R1 box represents G1 cells, the R2 box represents S cells with a high uptake of BrdUrd, the R3 box represents G2/M cells, the R4 box
represents S cells without an uptake of BrdUrd, and the R5 box represents apoptotic cells. The numbers given on the boxes indicate the average
percentages of gated events for three independent experiments.
Histone Deacetylase-3 Deletion Is Lethal

However, the growth of the mutant was delayed. The number of mutant cells in the S or G2/M phase of the cell cycle decreased, cells containing less than a diploid amount of DNA appeared by 72 h, and then the number of dying cells increased (Fig. 4C).

To clarify whether or not the inhibition of the expression of chHDAC-3 (FHDAC3) caused alterations in the level of general histone deacetylation, we first determined the amounts of acetylated core histones within bulk chromosomal histone preparations derived from DT40 cells in the absence of tet and ΔchHDAC-3/FHDAC3 after treatment with the drug. Inspection of the chromosomal histone preparations on an acid-urea gel followed by Western blotting involving antibodies against particular Lys residues Lys-8 and Lys-12 of histone H4 (49) revealed insignificant changes in the deacetylation levels of chromosomal core histones (data not shown). These results suggested not only that the chHDAC-3 mutation did not cause alterations in the global deacetylation of chromosomal core histones but also that the mutations should only affect the deacetylation of core histones involved in particular, narrow regions of chromatin if chHDAC-3 targets chromosomal core histones. Therefore, to clarify this possibility, we next compared total cellular proteins in the presence or absence of tet. The electrophoretic patterns on two-dimensional PAGE of proteins obtained in the presence of the drug were virtually the same as those in the control case, indicating that the chHDAC-3 mutation resulted in insignificant changes in the amounts of most major cellular proteins (data not shown). Moreover, the chHDAC-3 depletion caused no alterations in the expression of the cell cycle-related genes examined, i.e., the bcl-2, bcl-X, c-Rel, NFκB, p50, c-fos, and E2F genes and the genes encoding cyclins A1, A2, B2, B3, C1, D1, D2, and E (data not shown). The chHDAC-3 mutation is, therefore, likely to influence other types of cell functions or the expression of other cell growth-related genes, i.e., ones encoding the machineries for chromatin (nucleosome) assembly, DNA replication, transcription, and/or translation. However, the expression of genes examined encoding chHDAC-1, chHDAC-2, chCAF-1p48, and H1 and core histones was unchanged (data not shown). An initial step for resolving such a most fundamental question was to clarify how only chHDAC-3 was involved in the control of cell proliferation, even though the chHDAC family contains at least two more different members. This particular participation of chHDAC-3 should be based on the difference(s) in the above-mentioned characteristics of chHDAC-3 and both chHDAC-1 and -2.

The complementation experiment, involving truncated chHDAC-3 mutants, revealed that both the 1–23 N-terminal amino acid sequence and the 389–417 C-terminal amino acid sequence were essential for the viability of DT40 cells (Fig. 5A). Nine of the essential N-terminal 22 amino acids (except the putative initiation Met) were different from the corresponding ones of chHDAC-1 and -2 (see Fig. 1). The sequence of GSEE in the essential C-terminal region of chHDAC-3 comprising 29 amino acids was deleted in the corresponding regions of chH- DAC-1 and -2, and 26 of those of chHDAC-3, including the above-mentioned four amino acids, were different from the corresponding ones of the two other chHDACs. Because the number of acidic amino acids (Asp and Glu) and that of basic amino acids (Lys, Arg, and His) in the essential C-terminal sequence were 11 and 3, respectively, this particular region of chHDAC-3 should be strongly acidic. Then, to determine whether or not the tyrosine phosphorylation of the possible phosphorylation site at positions 404–407 in the essential C-terminal region of chHDAC-3, but not in the corresponding regions of chHDAC-1 and -2, was necessary for the cell viability, we performed a complementation experiment involving the single missense mutation (Y404F) (Fig. 5A). Contrary to our expectation, the chHDAC-3 function specific for the cell viability did not accompany the tyrosine phosphorylation of this particular site. However, it is likely that the essential C-terminal region of chHDAC-3 participates in the cell viability through the deacetylation activity, which depends on the C-terminal region of amino acids 323–428, whereas the participation of the essential N-terminal region of amino acids 1–23 does not accompany the deacetylation activity (see Fig. 5, A and B).

Moreover, to determine whether or not the deacetylation activity of chHDAC-3 really accompanied the cell viability, we performed a complementation experiment involving two single missense mutations (H135A) and (H193F) (Fig. 5, A and B). As expected, substitution of the essential His residues at positions 135 and 193, respectively, to Ala and Tyr resulted in a decrease or loss of the deacetylation activity. In parallel, both the single missense mutations caused a lack of the complementation ability. These results revealed that the deacetylation activity of chHDAC-3, based on the conserved active domain, was certainly necessary for its specific function in the cell viability.

We next examined whether or not the typical NES at positions 29–41 just behind the essential N-terminal region of chHDAC-3 was necessary for the cell viability. For this purpose, we performed a complementation experiment involving a double missense mutation (L29I,L31M) (Fig. 5, A and B). Interestingly, the double substitution of the conserved Leu residues at positions 29 and 31 to Ile and Met, respectively, which are identical to the amino acids at the corresponding positions of both chHDAC-1 and -2, resulted in an extensive loss of the complementation ability but not the deacetylation activity. On the other hand, an immunofluorescence involving anti-chHDAC-3 antiserum established that chHDAC-3 was localized in both nuclei and the cytoplasm (Fig. 2D), although chHDAC-1 and -2 are preferentially localized in nuclei (26). In addition, overexpressed FHDAC3 was localized in nuclei, as in the cytoplasm, but exhibited no influence on the cell growth (Figs. 2D and 4A), indicating that the reduced complementation ability of the double missense mutation (L29I,L31M) should be due to decreased nuclear export rather than increased accumulation in nuclei. Taken together, these results suggested not only that the proper NES sequence should be required for the nuclear export of chHDAC-3 and should be a significant requirement for its specific function but also that the Leu residues at definite positions could not be completely compensated for by even the amino acids located at the corresponding positions of other HDAC family members.

Based on the results obtained for chHDAC-3 together with those obtained for chHDAC-1 and -2 (26), we propose a molecular basis for the differences in the functional specificities of these multiple chHDACs in vivo. The highly conserved internal domains of the chHDAC family members should participate in their general roles; for instance, all of them should universally catalyze the removal of acetyl group(s) of the Lys residue(s) of core histones and interact with both the small subunit of the chicken chromatin assembly factor-1 (chCAF-1p48) (50) and the retinoblastoma protein (22, 23). Conversely, the variable N-terminal and C-terminal domains of the enzymes should be involved in their individual, particular roles. If the three chHDAC members separately catalyze the removal of acetyl group(s) of the different acetylated Lys residue(s) of core histones, although a final conclusion is not possible at present, the specificity should be mostly determined based on these two variable domains. For instance, of the two chHDACs, chHDAC-2 controls the amount of the IgM H-chain at the steps of both transcription of its gene and alternative processing of its
pre-mRNA, although chHDAC-1 merely affects gene expression in DT40 cells (26). The difference in the participation of these two enzymes should be mainly due to the differences in both the 50–60 C-terminal amino acid sequence and the formation of complexes with certain transcription factors. On the other hand, the smallest member, chHDAC-3, is essentially involved in the cell viability. This particular participation is probably mainly due to the N-terminal and C-terminal regions, which are considerably distinct in amino acid sequences from those of two other members, chHDAC-1 and -2. As an example, the ability of only chHDAC-3, i.e. not chHDAC-1 and -2, to export from nuclei to the cytoplasm should be due to the proper NES sequence in the variable N-terminal region. As a possible result, chHDAC-3, as a scavenger in the cytoplasm but not in nuclei, even if it acts differently in the two subcellular fractions, probably deacetylates acetyl groups from the acetylated Lys residue(s) of core histones before they assemble to form the nucleosome. However, the disruption of yeast Rpd3, which is homologous to mammalian HDAC-3 as well as other types of yeast HDACs, resulted in no alterations in the cell viability. It was reported only recently that a strong hypomorphic mutation of yeast HDACs, resulted in no alterations in the cell viability. It should be clarified by further studies.

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