Different Roles of N-terminal and C-terminal Halves of HIRA in Transcription Regulation of Cell Cycle-related Genes That Contribute to Control of Vertebrate Cell Growth*

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We reported previously that chicken HIRA, a homolog of Saccharomyces cerevisiae transcriptional co-repressors Hir1p and Hir2p, possesses seven WD dipeptide motifs and an LXXLL motif in its N-terminal and C-terminal halves, respectively, required for transcription regulations. Here, by using the gene targeting technique, we generated the homozygous HIRA-deficient DT40 mutant ΔHIRA. The HIRA deficiency caused slightly delayed cell growth and affected the opposite transcriptions of cell cycle-related genes, i.e. repressions for P18, CDC25b, and BCL-2, activations for P19 and cyclin A, and histones H2A, H2B, H3, and H4. These altered expressions were completely revived by the artificial stable expression of hemagglutinin-tagged HIRA in ΔHIRA. The ability to rescue the delayed growth rate was preferentially aided by the N-terminal half instead of the C-terminal half. We cloned the chicken P18 genomic DNA, and we established that its promoter was located surrounding the sequence GCGGGCGC at positions −1157 to −1150. Chromatin immunoprecipitation assay revealed that the N-terminal half interacted directly or indirectly with the putative promoter region of the p18 gene, resulting in up-regulation of the gene. These results indicated that the N-terminal half of HIRA should contribute positively to the growth rate via up-regulation of a set of cell cycle-related genes, whereas the C-terminal half down-regulated another set of them without exhibiting any effect on the cell growth.

Two transcriptional co-repressors, Hir1p and Hir2p, that appear to act on the chromatin structure in Saccharomyces cerevisiae repress the transcription of one copy each of histone H2A and H2B genes located at the HTA1–HTB1 locus throughout the cell cycle and transiently recruit a SWI/SNF chromatin remodeling complex, thereby leading to transcription activation at this locus at the G1/S transition (1–4). A deficiency of the hir genes or cac1, -2, and -3 genes encoding S. cerevisiae homologs of p150, p60, and p48 subunits of CAF-1 (chromatin assembly factor-1) (5–11) results in a synergistic defect in silencing at both telomerases and silent mating type loci (3, 12). For such silencing, CAF-1 mutants devoid of PCNA2-binding ability require Hir/Asf-1 (anti-silencing function-1) proteins (13). Moreover, Hir and CAF-1 proteins have been reported to be involved in building functional kinetochores in S. cerevisiae (14). The Schizosaccharomyces pombe Hir-like protein Hip1 is required for the periodic expression of histone genes and contributes to the function of complex centromeres (15).

The mammalian HIRA family (HIR is an acronym for histone regulator) has been named for its homology to Hir1p and Hir2p and was cloned from the DiGeorge syndrome (DGS) critical region (16–19). This DGS candidate gene is required for cardiac outflow tract septation (19) and is expressed in embryonic structures affected in human CATCH22 patients (20). Targeted mutagenesis of the HIRA gene has been reported to result in gastrulation defects and patterning abnormalities of mesodermal derivatives prior to early embryonic lethality (21). HIRA directly interacts with the transcription factor Pax3 haplo-insufficiency that results in the mouse splotch and human Waardenburg syndrome (WSI and WSII) phenotypes (22). These results, together with others, suggested that altered stoichiometry of HIRA-containing complexes should be important for the development of structures affected in WS and DGS.

The relationship in yeast indicated that HIRA and CAF-1 could play overlapping roles in chromatin dynamics and was consistent with other findings that mammalian HIRA exhibited binding ability as to histones (23) and ASF-1 (24), and ASF-1 then interacted directly with CAF-1p60 (25, 26). The HIRA protein family with seven WD repeat domains was predicted to form a β-propeller structure, which may be involved in protein-protein interactions, in its N-terminal half most similar to Hir1p and CAF-1p60 (2, 19, 21). The C-terminal half, similar to Hir2p, is responsible for its binding ability as to homeodomains, HIRA-interacting protein 3 (HIRIP3), and core histones (22, 23). Hence, as with CAF-1 and ASF-1, HIRA is known to act as a histone deposition factor (chaperone).

In recent years, the participatory roles of the mammalian HIRA family in chromatin assembly, cell cycle progression, and transcription regulation of histone genes have gradually been elucidated. The ectopic expression of HIRA, a novel cyclin-cdk2 substrate, in cells blocks S phase progression (27, 28). The ectopic expression of human HIRA represses transcription of histones, and in turn this triggers a concerted block of DNA synthesis. Coupling of DNA synthesis and histone synthesis in S phase is independent of cyclin/cdk2 activity and presumably

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*The abbreviations used are: PCNA, proliferating cell nuclear antigen; DGS, DiGeorge syndrome; Chip, chromatin immunoprecipitation; GAPDH, glyceraldehydephosphate dehydrogenase; Luc, luciferase; neo, neomycin; RT, reverse transcription; WS, Waardenburg syndrome; HA, hemagglutinin; WT, wild type; AMBSF, 4-(2-amino-ethyl)-benzolsulfonylfluoridochloride.
contributes to the prompt and orderly assembly of newly replicated DNA into chromatin (29). The Xenopus HIRA facilitates chromatin assembly independent of DNA synthesis in vitro, indicating that HIRA functions as a histone chaperone for DNA replication-uncoupled nucleosome assembly (30). This notion is further supported by a finding that CAF-1 and HIRA are contained in the histone H3.1 (replication-coupled) and H3.3 (replication-uncoupled) complexes, respectively (31). On the other hand, we cloned cDNA encoding chicken HIRA, and we demonstrated that WD dipeptide motifs in the N-terminal half of HIRA and an LXXLL motif in the C-terminal half are necessary for its in vitro and in vivo interactions with CAF-1p48 and histone deacetylase-2 (HDAC-2), respectively. Furthermore, our results revealed that the WD dipeptide motifs and the LXXLL motif are individually necessary for global transcription regulation in vivo, indicating that the two halves should be differentially involved in numerous DNA-utilizing processes, probably through distinct participations in transcription regulations (32, 33).

In this study we generated the homozygous HIRA-deficient DT40 mutant ΔHIRA. The HIRA deficiency caused not only the delayed cell growth but also the opposing influences on transcriptions of cell cycle-related genes, i.e. repression for P18, CDC25B, and BCL-2 and activation for P19, cyclin A, and core histones. These altered transcription patterns and delayed growth rates were completely revived by the expression of HA-tagged HIRA protein in ΔHIRA. Most interestingly, the compensatory ability for delayed growth rate was preferentially based on the N-terminal half of HIRA but not on the C-terminal half. The putative promoter of the chicken P18 gene was located surrounding the sequence GCGGGCGC at positions −1157 to −1150. The N-terminal half of HIRA then interacted with this putative promoter region and thereby up-regulated the P18 gene. Based on these results, we propose possible involvements of the N-terminal and C-terminal halves of HIRA in both cell growth and transcription regulations of cell cycle-related genes.

**EXPERIMENTAL PROCEDURES**

**Cloning and Mapping of Chicken Genomic HIRA and P18 Genes—** By using chicken HIRA cDNA (33) and 700-bp P18 cDNA fragments amplified by reverse transcription (RT)-PCR as probes, genomic DNA clones specific for HIRA and P18 were isolated on the screening of a DT40 genomic DNA library in a library as described (34, 35). The organizations of the genomic HIRA and P18 genes obtained were determined by the PCR and partial sequencing protocol (Amersham Biosciences).

**Cell Cultures—**DT40 cells and all subclones were cultured in Dulbecco’s modified medium as described (34, 35). At the indicated times, cells were counted to determine the growth rate.

**Gene Constructs, Transfection, Mutant Isolation, and Southern Blotting—** We constructed two targeting vectors pB(II)HIRA.neo and pΔHIRA/hisD for the disruption of the HIRA gene as follows. First, we cloned the 4.6-kb NotI/EcoRI fragment containing exons 1 and 2 from the HIRA genomic clone into pBluescript II (Stratagene) to yield pB(II)HIRA-4.6kb. The 7-kb region of the genomic HIRA gene containing exons 7–12 was amplified by PCR, using a sense primer from exon 7 (5’-GAAAACTTGCACTTGTGAAAGGAGGATCAT-3’), an antisense primer from exon 12 (5’-CTCTACTGCCATGGTGAGTTCTCGTCTGCAGCAG-3’), and the genomic clone as a template. After blunt-ending with a blunt-ending kit (Takara), this fragment was cloned into the HincII-digested pBluescript II to yield pB(II)HIRA-7kb. From the pB(II)HIRA-7kb plasmid, we excised the 2.3-kb EcoRV fragment of the genomic HIRA gene containing exon 10, and we then inserted it into EcoRV-digested pB(II)HIRA-4.6kb to yield pB(II)HIRA-6.9kb plasmid.

Finally, we inserted a blunt-ended cassette carrying the neo or hisD gene driven by the chicken β-actin promoter (35) into the EcoRI/T4 polymerase site of pB(II)HIRA-6.9kb plasmid to yield pΔHIRA.neo or pΔHIRA/hisD.

**Transfection was carried out essentially as described (36, 37).** To obtain ΔHIRA cells, transfectants with the pΔHIRA.neo construct were first selected in medium containing 2 mg of neomycin (neo; G-418) per ml. We transfected the pΔHIRA/hisD construct into clones, in which one of the two HIRA alleles had already been disrupted, and we selected stable transfectants in medium containing 0.8 mg of histidinol and 2 mg of neo per ml, respectively.

Genomic DNA from DT40 and drug-resistant clones was digested with EcoRI, separated in a 0.8% agarose gel, transferred to a Hybond N membrane, and then hybridized with 32P-labeled probe HIRA-5’, HIRA-3’, neo, or hisD as described (34, 35, 38).

**Semi-quantitative RT-PCR—** Total RNA was isolated from exponentially growing DT40 and ΔHIRA cells (~1 × 107) with or without appropriate HIRA derivatives, using TRIzol Reagent kit (Invitrogen). Reverse transcription was performed with a first strand cDNA synthesis kit (ReverTra Ace-α; Toyobo) at 42 °C for 20 min, followed by heating at 99 °C for 5 min. PCRs were carried out at 96 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s for the indicated cycles for appropriate genes, using appropriate sense primers and antisense primers. Chicken GAPDH gene was used as the internal control. The nucleotide sequences of all amplified RT-PCR products were confirmed as described (39).

**Expression of Exogenous HA-tagged HIRA in HIRA-deficient Clones—** ΔHIRA cells (cl2-14) were stably transfected with a linearized pβ-HA-HIRA, its derivates, or vector alone pβ-HA(+/−), which are expressed under the control of the β-actin promoter as described (35), and then stable transfectants were selected in medium containing 0.4 mg/ml puromycin (Sigma). Of the clones obtained, some clones (designated by appropriate names for HA-HIRA and its derivates) that overexpressed the HA-tagged HIRA and its derivates in the absence of puromycin were further selected. The other vectors, such as a linearized pTet-HA-HIRA, its derivates, and pTet-TA-bleo, which are expressed under the control of the tetracycline (tet) operator and cytomegalovirus minimal promoter (35, 40), were stably co-transfected into ΔHIRA cells (cl2-14), and then the transfected clones were selected in medium containing 0.3 mg/ml Zeocin (Stratagene). Of the clones obtained, some clones (designated by appropriate names for HA-HIRA and its derivates) that overexpressed the HA-tagged HIRA and its derivates in the absence or presence of tet were further selected.

The expression of recombinant HIRA and its derivates in ΔHIRA cells was verified by Western blotting using anti-HA antisera as a primary antibody. The expression was confirmed by RT-PCR using primer pairs HIRA-5’ (obtained using a sense primer 5’-GAAAAGCCCATATTTTCGATGGACATT-3’ from exon 2 and an antisense primer 5’-CTAAAGTTGTTCCATCGAGAACGACTC-3’ from exon 4), using a Superscript One-step RT-PCR kit (Takara) under the following conditions: 96 °C for 1 min, followed by the treatment of 96 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s for the indicated cycles.

**Luciferase Activity Assay—** To identify the putative promoter region(s) of the P18 gene, we first amplified the 2.6-kb upstream SmaI/Ncol fragment by PCR using the P18 genomic clone as a template, and we then cloned the amplified fragment into the Xhol/Ncol sites of the luciferase (Luc) reporter vector pGL3-Basic (Promega) to yield a parental pGL3-Basic p18 (WT) plasmid. By using this parental plasmid, we constructed its derivative plasmids, carrying appropriate 5’-truncated fragments, i.e. pGL3-Basic p18 (Apal/Ncol), pGL3-Basic p18 (HindIII/Ncol), pGL3-Basic p18 (Bsu36I/Ncol), pGL3-Basic p18 (PstI/Ncol), pGL3-Basic p18 (SalI/HindIII), and pGL3-Basic p18 (ΔHindIII/
Bsu36I) lacking internal HindIII/Bsu36I fragment, by combined digestions with appropriate restriction enzymes. From pGL3-Basic p18 (WT) as a wild type plasmid, we also constructed three point mutation plasmids, pGL3-Basic p18-PM1-(GT-CA), pGL3-Basic p18-PM2-(GC-AT), and pGL3-Basic p18-PM3-(GT-CA, GC-AT) by PCR with a QuickChange mutagenesis kit (Stratagene). All resultant constructs were verified by sequencing to ensure their junctions and correct incorporations of point mutations.

HeLa cells (60–70% confluence of 24-well culture plates) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine and then were transfected with 2 μg of pGL3-reporter constructs or pGL3-control vector and 20 ng of the pRL-CMV-Luc plasmid (Promega) as an internal control, using the FuGENE 6 transfection reagent (Roche Applied Science). To study the effects of HIRA and its derivates on the P18 promoter activity, HeLa cells were co-transfected with 1 μg of pGL3-reporter constructs carrying the 5′-flanking region of the P18 gene and its derivates, 1 μg of pβ-HA-HIRA and/or its derivates, and 20 ng of the pRL-CMV-Luc plasmid. Cells were harvested 36 h after transfection, and then Luc activity was assayed as recommended by the supplier. Each transfection was performed in duplicate and repeated at least three times. The relative Luc activity was normalized by dividing the measurement for the firefly Luc activity with that for the Renilla Luc activity.

Chromatin Immunoprecipitation (Chip) Assay—Chip assay was performed as described (41). DT40 and ΔHIRA cells containing HA-tagged HIRA or its derivates were grown until 10^8 in two 15-cm dishes and then cross-linked by addition of formaldehyde (to a final concentration of 1%) at room temperature for 10 min. After quenching of the reaction by addition of Gly to 0.125 M at room temperature for 5 min, cells were washed with 0.1% phosphate-buffered saline containing 0.1% AMBSF (Merck) and 0.5% protease inhibitors mixture (Sigma), collected by centrifugation at 2,000 rpm for 5 min, resuspended in 0.4 ml of sonication buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.2% SDS, 5 mM EDTA, 1% Triton X-100, 0.1% AMBSF (Merck), and 0.5% protease inhibitors mixture (Sigma)), and sonicated using Bioruptor (Cosmo Bio), a device for cell disruption, on cold water (6–8 °C) so that the average length of chromatin DNA fragments was 250–1000 bp. After centrifugation at 15,000 rpm for 15 min at 4 °C, the supernatant containing the DNA-protein complexes was collected, and then 350 μl of buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM
EDTA, 1% Triton X-100, 0.1% AMBSF (Merck) and 0.5% protease inhibitors mixture (Sigma) was added, and then aliquots (40 µl) of the resultant solution were used as input. Immunoprecipitations were carried out, using 50 µl of a 50% slurry of anti-HA-conjugated agarose beads (Santa Cruz Biotechnology) and/or anti-FLAG-conjugated agarose beads (Sigma) as a negative control for 2 h at 4°C, and finally the immunoprecipitate was pelleted at 12,000 rpm for 20 s. The immunoprecipitated material was washed 11 times with a washing buffer and eluted with an elution buffer (0.1% NaHCO₃ and 1% SDS). The cross-link was reversed by addition of NaCl (to a final concentration of 200 mM), incubation at 65°C for 4–6 h, and treatment with 2 µl of 20 mg/ml proteinase K (Takara) at 55°C for 1 h. The resultant samples were then extracted with phenol/chloroform followed by ethanol precipitation. Pellets were resuspended in 50 µl of TE buffer containing 100 µg/ml RNase and assayed by semi-quantitative PCR with Ex TaqHS DNA polymerase (Takara) using the following primers: p18UR1, 5’-TGGTAGCAGGGTGTTGGT-3’ and 5’-CATATTAAGACCTTGTGGCG-3’; p18UR2, 5’-GATAAGCCGCGACCGTGAC-3’ and 5’-AGCGGAGCCGAGAATAACTT-3’. RT-PCR was performed with an input of 50 ng of total RNA. The PCR conditions were 1 min at 94°C followed by 25 cycles of 15 s at 94°C, 30 s at 60°C and 1 min at 72°C. Each experiment was performed at least three times.

RESULTS AND DISCUSSION

Generation of Homozygous HIRA-deficient DT40 Mutants—To clarify the molecular mechanism underlying the participation of HIRA on the cell growth and transcription regulations in vivo, we generated the homozygous HIRA-deficient DT40 mutant. By using the chicken HIRA cDNA (33) as a probe, we first screened a DT40 genomic library to isolate 11 different genomic DNAs specific for the chicken HIRA. Detailed analyses, including the PCR method followed by sequencing, revealed that the partial genomic DNA of about 20 kb in length completely hybridized to the 9-kb EcoRI fragment, in addition to the endogenous 7-kb EcoRI fragment, originating from the upstream of exon 1, hybridized to the 12.5-kb EcoRI fragment (Fig. 1, A–C). After integration of this targeting vector into the remaining HIRA allele, in two of the stable transfectants selected with neo, probe HIRA-5’, originating from the upstream of exon 1, hybridized to the 12.5-kb EcoRI fragment, in addition to the endogenous 7-kb EcoRI fragment (Fig. 1, A–d and B). This result was confirmed by the finding that probe neo only hybridized to the 12.5-kb EcoRI fragment. One of the neo-resistant clones (+/–, cl2) was chosen for second round of transfection with the pΔHIRA/neo construct (Fig. 1A–a). After integration of this targeting vector into the HIRA locus, in two of the stable transfectants selected with neo, probe HIRA-5’, originating from the upstream of exon 1, hybridized to the 12.5-kb EcoRI fragment, in addition to the endogenous 7-kb EcoRI fragment (Fig. 1, A–d and B). This result was confirmed by the finding that probe neo only hybridized to the 12.5-kb EcoRI fragment. One of the neo-resistant clones (+/–, cl2) was chosen for second round of transfection with the pΔHIRA/neo construct (Fig. 1A–a).

Effect of the HIRA Deficiency on Expressions of Core Histone and Cell Cycle-related and Apoptosis-related Genes—Because HIRA has been reported to repress transcriptions of core histone genes (29), we examined whether or not the HIRA deficiency affects the expressions of these

FIGURE 2. Influence of HIRA deficiency on gene expressions of core histones and cell cycle-related and apoptosis-related factors. Total RNAs were extracted from DT40 (+/+), ΔHIRA (cl2-14; –/–), ΔHIRA carrying pβ-HA-2 (cl2-14-pβ-HA-2; –/–), and ΔHIRA carrying pβ-HA-HIRAF (cl2-14-pβ-HA-HIRAF; –/–), and the mRNA levels were then determined by RT-PCR or semi-quantitative RT-PCR using the appropriate primers. A, RT-PCR for core histones H2A, H2B, H3, and H4 and GAPDH. The 28 S and 18 S rRNA bands stained by ethidium bromide show almost equal loading of total RNAs used for RT-PCR. B, RT-PCR for cell cycle-related and apoptosis-related factors such as cyclins B2, B3, C, D1, D2, D3, E, and G1; c-Jun, HST2, E2F-1, E2F-4, PCNA, P130, P107, RB, bcl-xl, cdc2 and 20, and cdk2, 5–7, and ASP19, and P27, and GAPDH. C, semi-quantitative RT-PCR for cell cycle-related and apoptosis-related factors such as P18, BCL-2, CDC25B, cyclin A, and P19, together with GAPDH.
elevated expressions of all core histone genes were mostly suppressed in the presence of full-length HA-tagged HIRA expressed stably in ΔHIRA cells (cl2-14-pβ-HAHIRAF-6), although the mutant cells transfected with vector pβ-HA-2 as a control showed no influence on all these genes (cl2-14-pβ-HA-2), indicating that HIRA surely exhibits the ability to repress the transcriptions of core histone genes.

To determine whether or not the HIRA deficiency influences the transcriptions of cell cycle-related and apoptosis-related genes, we car-
ried out RT-PCR on total mRNA prepared from DT40 and ΔHIRA cell lines, using appropriate primers (39). No changes were detected in the steady-state mRNA levels of the various genes examined, i.e. cyclin B2, cyclin B3, cyclin C, cyclin D1, cyclin D2, cyclin D3, cyclin E, cyclin G1, c-JUN, HST2, E2F-1, E2F-4, PCNA, P130, P107, Rb, BCL-XL, CDC2, CDC20, CDK-2, CDK-5, CDK-6, CDK-7, CDK ASP19, and P27, as well as GAPDH as a control (Fig. 2B). On the other hand, because the deficiency of HIRA influenced oppositely on the transcriptions of remaining cell cycle-related genes, we carried out semi-quantitative RT-PCR on these genes (Fig. 2C). In ΔHIRA cells (cl2-14 and cl2-14-pβ-HA-2), the mRNA levels of P18, CDC25B, and BCL-2 remarkably decreased (to ~20, ~30, and less than 10%), but those of cyclin A and P19 obviously increased (to ~200 and more than 300%). All these altered expressions of genes were obviously complemented in the presence of full-length HA-tagged HIRA expressed stably in ΔHIRA cells (cl2-14-pβ-HA-HIRAF-6), indicating that HIRA was responsible for up- or down-regulating these cell cycle-related and apoptosis-related genes.

N-terminal or C-terminal Half of HIRA Up-regulates or Down-regulates the Genes of P18, CDC25B, and BCL-2—Our previous results showed that the N-terminal and C-terminal halves of HIRA not only interact differentially with CAF-1p48 and HDAC-1/2 in vivo and in vitro but also control individually the global transcription in vivo (32, 33). To clarify how these two halves are involved in the altered expressions of cell cycle-related and apoptosis-related genes, we constructed three different types of ΔHIRA cell lines, expressing stably HA-tagged full-length HIRA (cl2-14-ptet-HAHIRAF-3), N-terminal half (amino acids 1–443) (cl2-14-ptet-HAHIRAN-1), and C-terminal half (amino acids 444–...
The N-terminus and C-terminus of HIRA affect transcription regulations of differentially expressed cell cycle-genes. Two former constructs, but not the latter one, in the absence of tet, were almost similar to that of endogenous HIRA in DT40 cells and disappeared completely in the presence of the drug, like in a control null mutant (c12-14) (Fig. 3a). These findings were confirmed by Western blotting using anti-HA antibody (Fig. 3A). The steady-state mRNA levels of P18, BCL-2, and CDC25B in ΔHIRA cells carrying ptet-HAHIRAF-3 were virtually the same as DT40 cells, but the addition of tet led to a decrease in the tune of levels as seen in a control null mutant (Fig. 3b, 1st, 2nd, 7th to 10th, 15th, and 16th lanes). On the other hand, the steady-state mRNA levels of cyclin A and P19, as well as those of histones H2A, H2B, H3, and H4, without tet in ΔHIRA cells carrying ptet-HAHIRAF-3 remained unchanged and similar to the levels of those in DT40 cells but conversely increased, in the presence of the drug, to the levels in the control null mutant (Fig. 3, B-c and B-d, 1st, 2nd, 7th to 10th, 15th, and 16th lanes). These findings roughly agreed with those in Fig. 2 and proved that HIRA preferentially up-regulates the expressions of P18, BCL-2, and CDC25B genes but down-regulates those of cyclin A, P19, and histone H2A, H2B, H3, and H4 genes.

Next, we examined the influence of the N-terminal and C-terminal halves on transcription regulations of differentially expressed cell cycle-related and apoptosis-related genes. The steady-state mRNA levels of P18, BCL-2, and CDC25B in ΔHIRA cells carrying ptet-HAHIRAN-1 were maintained but decreased to the levels of the control null mutant when treated with the drug (Fig. 3B, b, 1st to 4th and 9th to 12th lanes). Similarly, the transcript levels of cyclin A and P19, as well as those of histones H2A, H2B, H3, and H4, in ΔHIRA cells transfected with ptet-HAHIRAN-1, remained the same as found in the control null mutant and did not change even in the presence of the drug (Fig. 3, B-c and B-d, 1st to 4th and 9th to 12th lanes). On the other hand, when compared with DT40 cells, ΔHIRA cells with ptet-HAHIRAC-13 showed diminished mRNA levels of P18, BCL-2, and CDC25B and maintained almost similar levels to those in the control null mutant, without any change even in the presence of the drug (Fig. 3B-b, 1st, 2nd, 5th, 6th, 9th, 10th, 13th, and 14th lanes). Although ΔHIRA cells carrying ptet-HAHIRAC-13 exhibited no alterations in expressions of cyclin A and P19, like those of histones H2A, H2B, H3, and H4, in the absence of tet, the tet treatment increased their expression at the levels of the control null mutant (Fig. 3, B-c and B-d, 1st, 2nd, 5th, 6th, 9th, 10th, 13th, and 14th lanes). Therefore, the N-terminal and C-terminal halves deliver individually different influences on transcription regulations, i.e., the former up-regulates the expressions of P18, BCL-2, and CDC25B genes, and the latter down-regulates those of cyclin A, P19, and histones H2A, H2B, H3, and H4 genes.
The N-terminal Half of HIRA Is Involved in Control of the Growth Rate—The growth rate of ΔHIRA cells (cl2-14) decreased slightly, the doubling times being about 15 h (Fig. 3C–b) compared with that of the GCN5-deficient mutant (17 h) (39). To confirm this result and, if so, to assess which half of the N and C terminus of HIRA participates in controlling the growth rate, we transfected pΔHIRA(F), pΔHIRA(N), and pΔHIRA(C), as well as vector pΔHA-2 (lane 4), into ΔHIRA cells (cl2-14) to yield three different ΔHIRA cell lines for stable expressions of full-length and N-terminal and C-terminal halves of HIRA, respectively. RT-PCR using HIRA-5′ primers and/or Western blotting using anti-HA antibody showed that three resultant cell lines, cl2-14-pΔβ-HAHIRAF, cl2-14-pΔβ-HAHIRAN, and cl2-14-pΔβ-HAHIRAC, respectively, stably expressed the full-length N-terminal half and C-terminal half of HIRA (Fig. 3C–a), and these results agreed nearly with those in Fig. 3, A and B-a.

As shown in Fig. 3C–b, the stable expression of HA-tagged full-length HIRA in ΔHIRA cells could recover the delayed growth rate to the same level as that of DT40 cells and thus compensated for the loss of the endogenous HIRA function, but the clone transfected with the vector cl2-14-pΔβ-HA-2 (−/−) could not. The stable expression of the HA-tagged N-terminal half of HIRA, but not the C-terminal half, in ΔHIRA cells rescued the delayed growth rate similar to that of both wild type DT40 and ΔHIRA cells with the stably expressed HA-tagged full-length HIRA. These results revealed not only that HIRA is certainly involved in control of the growth rate but also that its participation in growth kinetics is preferentially attributable to its N-terminal half rather than its C-terminal half.
evident in HIRA-deficient cells. This can be explained as DT40 cells contain BCL-XL, another apoptosis-related gene, that participates mainly in programming the apoptosis pathway, and its transcription is then preferentially controlled by GCN5 (39).

Identification of a Putative Upstream Promoter Region of the P18 Gene—It was most likely that the participation of HIRA (specially its N-terminal half) in controlling the growth rate should be the outcome from its role in transcription regulations of the cell cycle-related and apoptosis-related genes, such as P18, BCL-2, and CDC25B genes. As a first step to answer to this question, hereafter we focused on a representative P18 gene, because of both its noticeable influence by the HIRA deficiency and the advantage over other altered genes to be cloned, despite an inhibitor of cdks. We isolated the chicken P18 genomic DNA from a DT40 genomic library, using the 700-bp P18 cDNA fragment amplified by RT-PCR as in Fig. 2c as a probe. Detailed analyses, including the PCR method followed by sequencing, revealed that the P18 genomic DNA comprises two exons and is about 4.7-kb in length, as schematically depicted in Fig. 4A. To determine putative promoter region(s) of the P18 gene, a series of 5’-, internal, and 3’-truncated fragments of its 2.6-kb upstream Smal/NcoI fragment as a parental one was constructed and ligated upstream of the Luc gene in the pGL3-Basic vector. The effects of these on the P18 promoter activity were studied as an ability to activate or suppress the expression of the reporter gene in HeLa cells (Fig. 4B). Because the vector lacks promoter and enhancer sequences, the Luc activity was possible only when the DNA sequence examined possesses a promoter.

The Luc activity of the parental pGL3-Basic p18 (WT), carrying the 2.6-kb Smal/NcoI fragment, was comparable (~53%) with that of the pGL3-Basic vector driven by the SV40 promoter (Fig. 4B, lanes 1 and 2), indicating that the 2.6-kb upstream fragment of the P18 gene contained a potential promoter element(s). The Luc activities (~63 and ~60%) of two mutants pGL3-Basic p18 (ApaI/NcoI) and pGL3-Basic p18 (HindIII/NcoI), lacking fragments from positions −2557 to −1241, were virtually similar to that of the parental one (Fig. 4B, lanes 3 and 4). As expected, the mutant pGL3-Basic p18 (Smal/HindIII), bearing the fragment from positions −2557 to −1241, exhibited no Luc activity (Fig. 4B, lane 7), indicating that the region between positions −2557 and −1241 contained no promoter sequences. On the other hand, the mutant pGL3-Basic p18 (Bsu36I/NcoI), carrying the fragment from positions −970 to −1, as well as another mutant pGL3-Basic p18 (PstI/NcoI), carrying the fragment from positions −284 to −1, showed no activity (Fig. 4B, lanes 5 and 6), indicating that the putative promoter sequence of the P18 gene should be located within the region between positions −1240 and −971. This result was confirmed by the finding that the mutant pGL3-Basic p18 (ΔHindIII/Bsu36I), carrying two fragments from positions −2557 to −1241 and positions −970 to −1 but lacking the fragment from positions −1240 to −971, lost the Luc activity completely (Fig. 4B, lane 8). As shown in Fig. 4C, two sequences, GGTTGG and GCCGGCCGC, were located at positions −1203 to −1198 and −1157 to −1150 in this putative promoter region.

Promoter Element of the P18 Gene and Transcription Activation Ability of N-terminal Half of HIRA—Not only to identify the minimal promoter element of the P18 gene but also to determine whether or not the N-terminal half of HIRA possesses transcription activation ability for this element, we generated three mutant plasmids pGL3-Basic p18-PM1-(GT-CA), pGL3-Basic p18-PM2-(GC-AT), and pGL3-Basic p18-PM3-(GT-CA, GC-AT), respectively, possessing putative mutant promoters PM1, PM2, and PM3, with point mutations at appropriate positions depicted in Fig. 5A. Relative promoter activities of these three PM1, PM2, and PM3 promoters in cells expressing only endogenous HIRA were ~45, ~58, and ~79%, respectively, that of wild type promoter (Fig. 5B).

We then studied the effects of the full-length N-terminal half (amino acids 1–443) and C-terminal half (amino acids 444–1019) of HIRA on the activity of the wild type and putative mutant promoters. As shown in Fig. 5C, for the wild type promoter the full length activated the promoter activity to ~400% that of the control (Fig. 5C, lanes 1 and 4). The N-terminal half increased the promoter activity to ~400% (Fig. 5C, lane 2), which was comparable with that of the full-length, although the C-terminal half exhibited no effect (lane 3). The effect of the N-terminal half on the activity of the PM1 promoter, carrying mutations within the

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**FIGURE 7.** A model for different participations of N-terminal and C-terminal halves of HIRA in control of vertebrate cell growth.
sequence GGTTGG, was comparable with that of the full-length promoter (Fig. 5D, lanes 2 and 4), and then the enhancement was almost similar to that for the wild type promoter, whereas the C-terminal half exhibited no effect (lane 3). On the other hand, for the PM2 promoter, carrying mutations within the sequence GCGGCGCCG, neither the N-terminal half, the C-terminal half, nor full length of HIRA exhibited any effects on the promoter activity (Fig. 5E, lanes 2–4). As expected, no effects of the N-terminal half, the C-terminal half, and full length were also observed for the activity of the PM3 promoter, carrying mutations within both the sequences GGTTGG and GCGGCGCCG (Fig. 5F, lanes 2–4). These results indicate not only that the promoter element of the P18 gene should be located surrounding the sequence GCGGCGCCG at positions 1157 to 1150, but also that the N-terminal half, like the full length, instead of C-terminal half, possessed its ability to activate the P18 gene promoter, when the nucleotide sequence is wild type form but not mutant one, whereas the C-terminal half exhibits no influence at all. However, these results did not reflect each promoter activity in cells expressing only endogenous HIRA. A difference in the affinities for endogenous HIRA and exogenous HIRA to these elements cannot be ruled out.

The N-terminal Half of HIRA Interacts with Putative Promoter Region of the P18 Gene—Next, we examined the interaction of HIRA (and its N-terminal and C-terminal halves) with the putative promoter region of the P18 gene. We chose four upstream regions, UR1, UR2 that mostly overlapped the putative promoter, UR3, and UR4, which were located at positions −429 to −379, −1269 to −1113, −1563 to −1390, and −2126 to −1961, respectively, because of their advantages over other regions to be amplified by PCR (Fig. 6B). We then constructed three different types of ∆HIRA cell lines, cl2-14-pβ-HA-HIRA(full), cl2-14-pβ-HA-HIRA-(1–443), and cl2-14-pβ-HA-HIRA-(444–1019), respectively, which expressed stably HA-tagged full-length, N-terminal half, and C-terminal half of HIRA under the control of the β-actin promoter.

To determine whether or not HIRA and its derivatives interact with these upstream regions of the P18 gene, the Chip assay was performed on these three ∆HIRA cell lines with the control cl2-14-pβ-HA-2(–) clone. In three cases of the chromatin complex after sonication, the complex with anti-HA beads, the eluate from the complex with anti-HA beads, but not in the case of complex with the anti-HA beads after elution, Western blotting using anti-HA antibody showed that N-terminal half, the C-terminal half, and the full length were well expressed in the corresponding clones (Fig. 6A, columns 1–2). The Chip assay performed on cl2-14-pβ-HA-HIRA(full), using anti-HA antibody and semi-quantitative PCR, showed positive bands for the upstream regions UR2 and UR3 that was close to UR2 but not for UR1 and UR4 (Fig. 6C). Similarly, the Chip assay carried out on cl2-14-pβ-HA-HIRA-(1–443) generated positive bands for upstream regions UR2 and UR3 but not for UR1 and UR4, although the assay performed on cl2-14-pβ-HA-HIRA-(444–1019), like on control clone cl2-14-pβ-HA-2(–), showed no bands for all upstream regions UR1, UR2, UR3, and UR4. The control Chip assay, using anti-FLAG antibody instead of anti-HA antibody, also showed no bands for all four upstream regions. These results suggest not only that the full-length of HIRA interacts directly or indirectly with the upstream region UR2 at positions −1269 to −1113, which mostly overlapped the putative promoter region at positions −1240 to −971, but also that this interaction is preferentially attributable to the N-terminal half. The reason for the positive signal for the upstream region UR3 might be that UR3 could also have cross-linked with HIRA or the N-terminal half (probably the presumable complex containing it) and then was easily amplified by PCR, because it is closely located to UR2, and the average length of the chromatin DNA complex was 250–1000 bp. Specifically, the participation of the N-terminal half of HIRA (and also the full-length) in the up-regulation of the P18 gene is based on its binding ability to the putative promoter region at positions −1240 to −971, probably to the promoter element at positions −1157 to −1150.

In summary, based on these results and others reported previously (31, 32), we propose a model for the possible role of HIRA linked to a fine control of the growth of vertebrate cells (Fig. 7). HIRA is doubly and oppositely involved in the transcription regulations of cell cycle-related and apoptosis-related genes, as well as core histone genes, and these different participations are then divided into two ways because of the N-terminal and C-terminal halves, respectively. The N-terminal half binds to the putative promoter region (probably the minimal promoter element) of the P18 gene, as well as promoters of CDC25B and BCL-2 genes, directly or indirectly through formation of complex(es) with or without CAF-1p48 and/or other proteins, resulting in up-regulations of these cell cycle-related and apoptosis-related genes. On the other hand, the C-terminal half possibly binds to the putative promoter regions of the P19 and cyclin A genes, like those of histones H2A, H2B, H3, and H4 genes, directly or indirectly through formation of complex(es) with or without HDAC-2 (and/or HDAC-1) and/or other proteins, resulting in down-regulations of genes encoding these cell cycle-related factors and core histones. Thus, HIRA contributes to a minute control of the growth (and also cell cycle) of vertebrate cells, preferentially through transcription regulations of a set of the cell cycle-related genes, with the fine-tuning of distinct participations of its N-terminal and C-terminal halves. On the other hand, it is unlikely that the BCL-2 gene, activated by HIRA, acts as a prime candidate for switching apoptotic DT40 cell death. Rather, the BCL-XL gene of DT40 cells, the transcription of which is preferentially controlled by GCN5, is likely to be a predominant factor linked to an apoptosis process. The influence on the down-regulation of all core histone genes by HIRA remains to be resolved. To clarify exactly how the HIRA participates in the fine control of both the cell cycle progression and the growth of vertebrate cells, we are now cloning the genomic CDC25B DNA and studying the interaction of the N-terminal half of HIRA with its promoter region, because it predominantly facilitates the cell cycle progression through dephosphorylation of cdks.

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Different Roles of N-terminal and C-terminal Halves of HIRA in Transcription Regulation of Cell Cycle-related Genes That Contribute to Control of Vertebrate Cell Growth

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