Histones are thought to have specific roles in mammalian spermatogenesis, because several subtypes of histones emerge that are post-translationally modified during spermatogenesis. Though regular assembly of nucleosome is guaranteed by histone chaperones, their involvement in spermatogenesis is yet to be characterized. Here we identified a histone chaperone-related factor, which we designated as CCG1-interacting factor A-II (CIA-II), through interaction with bromodomains of TAF\(\text{II}\)\text{250/CCG1}, which is the largest subunit of human transcription initiation factor IID (TFIID). We found that human CIA-II (hCIA-II) localizes in HeLa nuclei and is highly expressed in testis and other proliferating cell-containing tissues. Expression of mouse CIA-II (mCIA-II) does not occur in the germ cell-lacking testes of adult WBB6F1-W\(^{W}\) mutant mice, indicating its expression in testis to be specific to germ cells. Fractionation of testicular germ cells revealed that mCIA-II transcripts accumulate in pachytene spermatocytes but not in spermatids. In addition, the mCIA-II transcripts in testis were present as early as 4 days after birth and decreased at 56 days after birth. These findings indicate that mCIA-II expression in testis is restricted to premeiotic to meiotic stages during spermatogenesis. Also, we found that hCIA-II interacts with histone H3 in vivo and with histones H3/H4 in vitro and that it facilitates supercoiling of circular DNA when it is incubated with core histones and topoisomerase I in vitro. These data suggest that CIA-II is a histone chaperone and is implicated in the regulation of mammalian spermatogenesis.

Eukaryotic genomic DNA is packaged into a nucleoprotein complex of nucleosomes and forms chromatin structure in a nucleus (1). Conversion of nucleosome structure is a critical step in the regulation of DNA-mediated transactions (2–4). In addition to histone-modifying enzymes (i.e. acetyltransferase, deacetylase, and methylase) and chromatin-remodeling factors addition to histone-modifying enzymes, histone chaperone is a type of protein that modulates formation of nucleosomes (5, 6). It binds to core histones and facilitates conversion of the topology of the relaxed form of a circular plasmid DNA into the supercoiled form in the presence of topoisomerase I and core histones (5). This conversion activity is considered to stimulate assembly of histones and DNA and is therefore called nucleosome assembly activity. Such activity was confirmed by micrococcal nuclease digestion assay, where DNA ladders were detected as a gel electrophoresis pattern when histones and DNA were incubated with a histone chaperone (5). This category of proteins with nucleosome assembly activity includes nucleoplasmin (7), nucleosome assembly protein-I (NAP-I)\(^{1}\) (8, 9), template-activating factor-I (TAF-I) (10), suppressor of Ty 6 (11), and CCG1-interacting factor A (CIA) (12).

CIA is the most conserved histone chaperone characterized to date (12). It was identified as human factor that interacts with human cell cycle gene 1 (hCCG1), which is the largest subunit of hTFIID (12, 13). TFIID is the key factor responsible for specific binding with the TATA box, which is the basal sequence observed within the promoter of typical eukaryotic genes transcribed by RNA polymerase II (14). CCG1 associates with TATA box-binding protein (TBP) (15) and inhibits binding of TBP onto TATA box via its N-terminal region (16, 17). To date, CIA is the only histone chaperone known to associate with TFIID, through interaction with the bromodomain-containing domain of CCG1 (12, 13).

The gene encoding CIA counterpart in Saccharomyces cerevisiae was genetically identified as anti-silencing function-1 (ASF1) that causes derepression of genes located at silenced loci when it is exogenously overexpressed (18–20). Yeast Asf1p was shown to have nucleosome assembly activity as well, and its highly conserved domain is essential for histone binding, nucleosome assembly, cell growth, and anti-silencing activities, indicating preservation of the function of CIA/Asf1p throughout evolution (20). Through such activities, CIA is thought to contribute to chromatin DNA-mediated reactions such as gene transcription (13) as well as DNA replication (21) and DNA repair (18).

Involvement of Asf1p in gene transcription is suggested by its suppressor of Ty phenotype-causing activity (13). More importantly, Asf1p physically associates with yeast bromodomain factor 1 (yBd1p) (13). It has been reported that yBd1p acts as the missing bromodomain in yCCG1/TAF\(\text{250}\)/145, which is the counterpart for hCCG1 in S. cerevisiae but lacks C-terminal

\(^{1}\) The abbreviations used are: NAP, nucleosome assembly protein; CCG1, cell cycle gene 1; CIA, CCG1-interacting factor A; TFIID, transcription initiation factor IID; h, human; m, mouse; TBP, TATA box-binding protein; TAF\(\text{II}\)/TAF\(\text{250}\), TBP-associated factor; ASF1, anti-silencing function 1; PBS, phosphate-buffered saline; GST, glutathione S-transferase; X-gal, 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside.

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Histone Chaperone Implicated in Spermatogenesis

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—We isolated the cDNA of CIA-II by using a yeast two-hybrid screen (12, 38, 39), searching for factors that interact with bromodomain (residues 1342–1629) of human CCG1, a subunit of CIA-II. The human peripheral lymphocyte cDNA library fused to the Gal4 activation domain in pACT was co-transformed with the pAS1-CYH2 plasmid (12) in Y190 yeast cells. Yeast transformants (~0.9 × 10^6 cells) were cultured on Trp(-) Leu(-) plates and replicated on Trp(-) Leu(-) His(-) plates containing 25 mg 3-aminotriazole. The resultant colonies were tested for β-galactosidase activity by using X-gal (Wako). This assay for detecting specific interactions was done as previously described (12, 36). By this screening, we isolated two independent clones that have an ~1.6 kb insert DNA that contains CIA-II sequences. We sequenced one of the clones (BRD 101) and found that this insert DNA consists of 1659 nucleotides and contains the full-length sequence of CIA-II. The entire coding sequence of CIA-II cDNA was also identified as anti-silencing function 1B (ASF1B) (40) or hypothetical protein FLJ10604 (GenBankTM accession numbers AF279307 and NM_018154, respectively). For confirmation of protein-protein interaction, we constructed plasmid with “bait” cDNA in “prey” vector (pACTII) and plasmid with “prey” cDNA in “bait” vector (pAS1-CYH2) by excising the inserted cDNA with appropriate restriction enzymes and then ligating it with the other vector in its open reading frame. The co-transformants of pAS1-CYH2 and pACTII derivatives were spotted on filters (PROTRAN BA 85; Schleicher & Schuell) and tested for β-galactosidase activity. We used a cDNA for histone H3.3 (residues 559) labeled with [32P]dCTP. Filters were washed three times at room temperature in a solution of 2× SSC and 0.1% SDS for 10 min and then twice at 1× SSC and 0.1% SDS for 20 min. Autoradiography was performed with intensifying screens at ~80 °C for 6 days. Mouse RNA was prepared by the acid guanidium/phenol/chloroform (AGPC) method (43). Poly(A)+ RNA was isolated by using Oligotex-dT30 (Takara Shuzo) according to the manufacturer’s recommendations. RNA was electrophoresed on a 1.5% agarose-formaldehyde gel and transferred to a nylon filter (Gene Screen plus; PerkinElmer Life Sciences). Filters for the mouse Northern blot analysis (multiple tissue Northern blot; Clontech) were hybridized at 42 °C in a solution containing 50% formamide, 10× Denhardt’s solution, 5× SSPE, 2% SDS, and 100 µg/ml salmon sperm DNA for 16 h with either the [32P]dCTP-labeled human CIA-II XbaI-XhoI fragment (+780 to +1671), human CIA HindIII-XhoI fragment (+534 to +950), or β-actin gene. The filters were washed three times at room temperature in a solution of 2× SSC and 0.1% SDS for 10 min and then twice at 1× SSC and 0.1% SDS for 20 min. Autoradiography was performed with intensifying screens at ~80 °C for 6 days. Mouse RNA was prepared by the acid guanidium/phenol/chloroform (AGPC) method (43). Poly(A)+ RNA was isolated by using Oligotex-dT30 (Takara Shuzo) according to the manufacturer’s recommendations. RNA was electrophoresed on a 1.5% agarose-formaldehyde gel and transferred to a nylon filter (Gene Screen plus; PerkinElmer Life Sciences). Filters for the mouse Northern blot analysis (multiple tissue Northern blot; Clontech) were hybridized at 42 °C in a solution containing 50% formamide, 10× Denhardt’s solution, 5× SSPE, 2% SDS, and 100 µg/ml salmon sperm DNA for 12 h using the mouse CIA-II fusion protein (Clontech) and the EcoRI-XhoI fragment of mouse S-II-T1 gene (41) as controls.

Recombinant Proteins—cDNA encoding full-length of human CIA-II was subcloned into pGEX-5X-2 (Amersham Biosciences) to produce the GST-CIA-II fusion protein. CIA-II was expressed in Escherichia coli BL21 (DE3) and extracted in BA-500 (20× Tris-HCl, pH 7.9, 10% glycerol, 0.1% Nonidet P-40, and 0.35% 2-mercaptoethanol; BA-500 contains 500 µmol NaCl) containing 1 mol methylsulfonyl fluoride and 20 µmol of leupeptin and pepstatin A. The extract was then incubated with glutathione-Sepharose resin (Amersham Biosciences) equilibrated with BA-500, washed with BA-500, and eluted with BA-0 (i.e., no NaCl) containing 20 mol reduced glutathione. The eluted fraction was loaded on a Poros HQ column (PerSeptive Biosystems) equilibrated with BA-0 and eluted with a linear gradient from 0.2 to 1 mol NaCl. GST-CIA-II protein was eluted at about 0.4 mol NaCl. Core histones...
Identification of Histone Chaperone-related Factor CIA-II—

In a previous study, we isolated human cDNA for histone chaperone CIA by a yeast two-hybrid screen using cDNA that encodes the bromodomain-containing fragment of human CCG1 (CCG1-BrD) as bait (12). From this screening, we simultaneously obtained an ~1.6 kbp insert cDNA that encodes the polypeptide consisting of 202 amino acids in the correct coding frame. Because this sequence is similar to that of human CIA, we designated this putative product as CIA-II. As shown in the assay (Fig. 1A), the colony that co-expresses the full-length of CIA-II fused to Gal4 activation domain and CCG1-BrD fused to Gal4 DNA-binding domain exhibits dark blue color in the X-gal assay (38). This assay also showed that the combination of SNF1-SNF4 and CCG1-BrD-CIA was a positive control of interaction and that other combinations yielding white colonies indicated negative interaction between exogenous products in yeast cells. As judged from the colony’s color, interaction between CIA-II and CCG1-BrD was apparently as strong as that between CIA and CCG1-BrD (Fig. 1A). Furthermore, we tested the interaction of CIA-II with other baits, such as TAFI18 (i.e., hTAF18), or with other domains of hCCG1, such as high mobility group (HMG) box-like domain, but none of them detects any positive interaction (data not shown). To further exclude the possibility that these interactions might be false positives because of artificial expression, we reconstructed plasmid with bait cDNA in prey vector (pACTII) and that with prey cDNA in bait vector (pAS1-CYH2). The results (Fig. 1B) showed that the co-transformants of CIA-II fused to Gal4 DNA-binding domain and CCG1-BrD fused to Gal4 activation domain did indeed form a dark blue colony in the X-gal assay, hence suggesting two-hybrid interaction exists between CIA-II and CCG1-BrD in vivo.

Structural Features of CIA-II—Fig. 1C shows the deduced amino acid sequence of human CIA-II aligned with those of human CIA. The amino acid sequence of CIA-II is highly similar to that of human CIA from the BLAST homology search. Sequence comparison between CIA-II and CIA suggests that CIA-II possesses 71% sequence identity with CIA. Based on sequence similarity between CIA-II and CIA, we divided the CIA-II and CIA sequences into three segments with 1–36, 37–153, and 154–202 (or 204 in CIA) (Fig. 1C; see also Fig. 4A). The N-terminal segment (residues 1–36) is well conserved, and its sequences exhibit 67% identity to each other. The central segment (residues 37–153) is highly conserved between CIA and CIA-II, and 91% of their amino acid sequences are identical to each other without any gap. Also, this central segment is evolutionarily conserved from yeast to human, although there are two gap sequences in cia1, which is the Schizosaccharomyces pombe orthologue of CIA (20). On the other hand, the C-terminal segment (residues 154–202 in CIA-II and 154–204 in CIA) is less conserved with 29% identity and has one gap. Interestingly, this C-terminal segment of CIA-II contains 7 acidic residues (14%), whereas human CIA possesses 10 residues (20%). Therefore, it suggests that the C-terminal region rich in anionic residues is specific to protozoan CIA orthologues (i.e. S. cerevisiae Asf1p and S. pombe cia1) (20).

Histone-binding Activity of CIA-II—Because CIA-II is homologous to CIA, a function of CIA-II is thought to be a chaperone for histones. To clarify this, we investigated histone-interactive activity of CIA-II by yeast two-hybrid assay using cDNA for histone H3 that associates with human CIA (12). Plasmid encoding CIA-II fused to the Gal4 DNA-binding domain was co-transformed with plasmid encoding histone H3 fused to the Gal4 activation domain (Fig. 2A). Interaction between CIA-II and histone H3 is positive as indicated by the dark blue colony in the β-galactosidase assay (Fig. 2A). Strength of this interaction between CIA-II and histone H3 is thought to be almost the same as that between CIA and histone H3. Next, to determine whether this interaction of CIA-II is direct, we did an in vitro binding assay of CIA-II against core histones. Recombinant CIA-II fused to GST was expressed in E. coli and

H2A, H2B, H3, and H4 from HeLa cells were purified as a mixture using a hydroxyapatite column (Bio-Rad) (44).

GST Pull-down Assay—The method for the GST pull-down assay was described previously (12). Glutathione-Sepharose resin (Amersham Biosciences) was equilibrated with the binding buffer (pH 7.5, 25 mM HEPES-NaOH, 200 mM KCl, 13 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40, and 0.3% 2-mercaptoethanol), and purified recombinant protein (0.2 µg) was bound to the resin. HeLa core histones (0.6 µg) were then added and incubated at 4 °C for 2 h, with rotation. The resin was washed in the same buffer, and bound proteins were eluted with 15 µl of SDS loading buffer and subjected to SDS-PAGE.

Plasmid Superciling Assay—Nucleosome assembly reactions were performed as follows (11). Covalently closed circular plasmid DNA pBluescript II SK (–) (2.96 kbp) was relaxed by wheat germ topoisomerase I (Promega). Relaxed circular DNA (0.1 µg; with 5 units of topoisomerase I) was combined with purified recombinant GST or GST-CIA protein (0.5 µg) and HeLa core histones (0.3 µg) in the assembly buffer (pH 7.5, 50 mM NaCl, 100 mM NaCl, 2 mM MgCl2, 0.5 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin) containing 2 mM ATP. Reaction mixtures were incubated at 37 °C for 1 h and then incubated for 30 min to stop the reaction after adding an equal volume of the stop A buffer (20 mM EDTA, pH 8.0, 1% SDS, and 200 µg/ml proteinase K). Plasmid was extracted by phenol-chloroform and precipitated with ethanol. Purified plasmid was subjected to 12% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Identification of Histone Chaperone-related Factor CIA-II

The method for the GST pull-down assay

FIG. 1. Identification of histone chaperone CIA-related factor CIA-II. A, identification of CIA-II as an interactor of human CCG1 bromodomains. SNF4, hCIA-II, and hCIA were fused to the Gal4 activation domain, and SNF1 and the bromodomain-containing segment of human CCG1 (CCG1-BrD) were fused to the Gal4 DNA-binding domain. Interactions of these factors were tested by β-galactosidase assay in the yeast two-hybrid system. Combination of SNF1-SNF4 represents a positive control in the assay, which forms a dark blue colony. B, confirmation of two-hybrid interactions. SNF1, hCIA-II, and hCIA were fused to the Gal4 DNA-binding domain, and SNF4 and CCG1-BrD were fused to the Gal4 activation domain. C, amino acid sequences of hCIA-II. The deduced amino acid sequence of human CIA-II (upper) is aligned with that of human CIA (lower). The open reading frame of hCIA-II (GenBank accession number AB104486) encodes a protein of 202 amino acids. Numbers on the right side indicate the positions of amino acid. Conserved amino acids are denoted by dots, and gaps are denoted by dashes.
Histone Chaperone Implicated in Spermatogenesis

Histones were incubated with GST (lane 3), or GST-CIA (lane 4) immobilized with glutathione-Sepharose resin as shown in Fig. 2C. The adsorbed histones were eluted with SDS sample buffer and analyzed by using 15% SDS-PAGE. The Coomassie Brilliant Blue-stained gel (Fig. 2C) showed that GST-CIA-II bound preferentially to core histones H3/H4 (lane 3) but not with histones H2A/H2B, whereas GST did not bind preferentially to core histones (lane 2), suggesting that CIA-II can bind to histones H3/H4 in vitro. Therefore, we conclude that interaction between CIA-II and core histones is direct.

Nucleosome Assembly Activity of CIA-II—Based on its observed histone-interactive activity (Fig. 2, A and C), CIA-II might also exhibit nucleosome assembly activity. Therefore, to determine whether CIA-II has core histone-dependent nucleosome assembly activity, we did a plasmid supercoiling assay in vitro. As shown in Fig. 2D, GST (lane 5), GST-CIA-II (lane 6), or GST-CIA (lane 7) was mixed with relaxed circular plasmid and HeLa core histones and tested for nucleosome assembly activity. In this assay, negative supercoils were introduced into plasmid DNA by the actions of topoisomerase I when it forms nucleosomes. We found that CIA-II stimulates supercoiling of plasmid DNA and that the activity of CIA-II to yield supercoiled DNA is comparable with that of other histone chaperones such as NAP-I (lane 4) or CIA (lane 7). These results indicate that CIA-II exhibits nucleosome assembly activity, and, indeed, belongs to a family of histone chaperones.

Subcellular Localization of CIA-II—Although CIA-II is thus demonstrated to be another member of histone chaperone, its cellular role is yet to be characterized. To discriminate whether CIA-II is involved in the assembly of newly synthesized histones in the cytosols or regulates nucleosome DNA-mediated events in the nucleus, we further investigated subcellular localization of CIA-II. Gal4-fused CIA-II was transfected to HeLa cells, and its subcellular localization was visualized using X-gal (Fig. 2E). Gal4 protein was diffused in both nucleus and cytosols in cells that were transfected with vector plasmid, whereas Gal4-TBP was localized in nuclei. Gal4-CIA-II was found to specifically localize in the nucleus of HeLa cells, although slight diffusion of Gal4-CIA-II into cytosol was detected in some cells (Fig. 2E). Furthermore, Gal4-CIA-II was localized in the nucleus. These results suggest that the involvement of histone chaperone CIA-II in the assembly of nucleosome in nuclei is similar to that of CIA and that it is unlikely that CIA-II contributes to structural maintenance of newly synthesized histones in cytosols.

Tissue Distribution of CIA and CIA-II—Why does the human genome contain two genes encoding closely related nuclear histone chaperones CIA and CIA-II? One possibility is that these histone chaperones are preferentially utilized in different cells or different developmental stages in multicellular organisms. To test this hypothesis, we examined the distribution of CIA-II and CIA in 16 different human tissues by Northern blot analysis. We detected two different subtypes of hCIA transcripts distributed almost ubiquitously in the tissues analyzed (Fig. 3, middle panel). On the other hand, hCIA-II transcripts were, to our surprise, detected only in restricted tissues such as testis, thymus, small intestine, and colon (upper panel). The amount of loaded poly(A)+ RNA was confirmed by Northern blot analysis using β-actin as a control probe (lower panel).

Restricted Expression of mCIA-II in Testicular Germ Cells—Expression of hCIA-II was the highest in testis (Fig. 3, lane 4). Testis consists of a variety of germ cells and somatic cells, such as Sertoli cells, Leytig cells, and macrophages. To determine whether expression of CIA-II in testis is restricted to germ or somatic cells, we first screened cDNA encoding CIA-II or CIA from mouse poly(A)+ RNA by RT-PCR. As schematically...
shown in Fig. 4A, DNA primers were designed to hybridize the gene segment that corresponds to the highly conserved region of both CIA-II and CIA (see “Experimental Procedures”). We obtained two kinds of mouse cDNA; one sequence was similar to that of hCIA-II and the other to that of hCIA. Next, we purified poly(A)+ RNA from the testes of WBB6F1-W/W mutant mice and WBB6F1+/+ wild-type mice and compared the amount of mCIA-II transcripts by Northern blotting using isolated mCIA-II cDNA as a probe. The testes of adult WBB6F1-W/W mice contain somatic cells but lack testicular germ cells because of a mutation in the e-kit gene (45). As shown in Fig. 4B, the mCIA-II transcripts were not detected in the testes of adult WBB6F1-W/W mutant mice (upper panel, lane 2), whereas abundant mCIA-II transcripts were detected in those of wild-type mice (lane 1). This RNA-blotted filter was also hybridized with cDNA encoding transcription elongation factor S-II-T1 as the positive control probe (middle panel), because S-II-T1 is specifically expressed in germ cells in testis (41, 46), and is lost in the testis of WBB6F1-W/W mutant mice (41). In addition, hybridization was done using β-actin gene to demonstrate that loaded amounts of RNA from WBB6F1-W/W mutant and wild-type mice were almost equivalent (lower panel). Taken together, these results indicate that expression of mCIA-II in testis is specific to testicular germ cells.

Expression of mCIA-II in Meiotic Germ Cells—Male germ cells differentiate dramatically through both mitosis and meiosis (47). Therefore, to determine in which kinds of germ cells CIA-II is expressed, we analyzed the accumulation of CIA-II transcripts in different types of differentiated germ cells by using centrifugal elutriation of mouse testicular cells. This size-fractionation can yield different fractions such as enriched in pachytene spermatocytes, round spermatids, elongated spermatids, and residual bodies (41). Fig. 4C indicates components of each fraction and of the whole testis for comparison. Using these cell fractions, we examined the expression level of mCIA-II in differentiated spermatogenic cells by Northern blot analysis (Fig. 4D, upper panel). In this study, we found that CIA-II transcripts were specifically accumulated in the fraction of pachytene spermatocytes (lane 1) and in the whole testis (lane 5) but not in the fraction enriched in round spermatids, elongated spermatids, or residual bodies (lanes 2–4, respectively). In this fractionation experiment, the amount of β-actin transcripts was kept at the same level in each fraction (lower panel), and S-II-T1 was detected in every fraction (middle panel). This method of cell fractionation could not exclude contamination of small amounts (no more than 5%) of somatic Sertoli cells and spermatids (42). However, because mCIA-II expression in spermatids fractions (lanes 2 and 3) was less than the detection limits, expression of mCIA-II transcripts in the fraction of pachytene spermatocytes is attributable to pachytene spermatocytes themselves. Thus, we conclude that mCIA-II is expressed in pachytene spermatocytes but is not expressed in spermatids.

Existence of mCIA-II in Premeiotic Germ Cells—Premeiotic histones are involved in the early stage of spermatogenesis, and their function in spermatogenesis has been studied by using CIA (46). CIA-II expression is restricted to meiotic germ cells, and its expression is different from that of CIA (46). In this study, we investigated the expression of mCIA-II in premeiotic germ cells. As an example, we used CIA-II as a probe (Fig. 4A, upper panel). This hybridization was done using four different stages of male germ cells. Twenty micrograms of total RNA prepared from fractions rich in pachytene spermatocytes (a), round spermatids (b), elongated spermatids (c), and residual bodies (d) were compared with that of whole testis (e). D, accumulation of mCIA-II transcripts at different developmental stages of testicular germ cells. Northern blot analysis was done using four different stages of male germ cells. Twenty micrograms of total RNA prepared from fractions rich in pachytene spermatocytes (a), round spermatids (b), elongated spermatids (c), and residual bodies (d) were compared with that of whole testis (e).
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In this study, we identified testicular germ cell-type histone chaperone CIA-II. The sequence and functional studies showed that CIA-II is highly homologous to previously characterized histone chaperone CIA (12, 13, 20). Through molecular cloning and functional characterization of CIA-II as a histone chaperone, this study revealed the following five aspects of CIA-related factors: 1) There are multiple members of CCG1-interactive histone chaperones in mammals; 2) the primary structures of CIA and CIA-II could be divided into three segments, respectively, at amino acid positions 36/37 and 153/154; 3) the central segment (residues 37–153) of CIA/CIA-II is evolutionarily most highly conserved among known histone chaperones; 4) the C-terminal segment is less conserved between CIA-II and CIA, and it is evolutionarily distinct; and 5) both CIA-II and CIA are histone H3/H4-interactive nuclear factors that exhibit nucleosome assembly activity. In addition to these structural and functional aspects, we identified that CIA-II is unique in its temporal/spatial regulation of expression, whereas CIA is ubiquitously found in adult tissues. According to our expression analysis, we found that CIA-II expression in testis is restricted to premeiotic and meiotic germ cells. However, expression of CIA-II is not limited to testis only but is also detected in proliferating cell-containing tissues such as thymus, small intestine, and colon among human tissues. Furthermore, expression of CIA-II in these tissues is several times greater than that of CIA (Fig. 3). Therefore, CIA-II might be transcribed within proliferating somatic cells in these tissues as well as in premeiotic germ cells. This raises the possibility that CIA-II facilitates nucleosome assembly reaction in the course of DNA replication during mitotic cell division of both somatic and germinal cells. To date, tissue distribution or temporal regulation of histone chaperones is poorly understood. In this study, we conclude that CIA-II mRNA is expressed in spermatogonia and spermatocytes among male germ cells. Before this study, tissue distribution of family members of NAP-I was only characterized among mammalian histone chaperones. Nucleosome assembly protein-1-like 2 (Nap1l2) that controls neurulation is specifically expressed in neurons and binds to condensing chromatin during S phase (49). Another member of the NAP-I family, MB20, which is specifically expressed in the brain, is distributed in both the cytoplasm and the nucleus and binds to histones (50). Testis-specific protein Y-encoded (TSPY), which is also structurally related to nucleosome assembly protein NAP-I, is expressed in spermatogonia and in early testicular carcinoma (51). However, it still remained unclear whether these NAP-I-related factors possess nucleosome assembly activities. Thus, our study is the first to indicate identification of temporally and spatially regulated nuclear histone chaperone.

**DISCUSSION**


**Fig. 5. Premeiotic onset of mCIA-II during testicular germ cell maturation.** A, mCIA-II expression during testis maturation. Total RNA was harvested from mouse testes. The age is listed below the autoradiograms as indicator of the stage of sexual development. Ages of mice used for analysis are 4, 7, 10, 14, 17, 21, 24, 28, 35, and 56 days after birth. Each lane contains 2 μg of poly(A)+ RNA. mCIA-II (upper), testis-specific mS-II-T1 (middle), or human β-actin cDNA (lower) were used as probes. Onset of spermatogonia, pachytene spermatocytes, and spermatids in the testes of wild-type mice is indicated by arrows at the top. B, schematic representation of expression profiles of CIA-II mRNA during spermatogenesis. The cell type in which CIA-II is assumed to exist is shown by a parenthesis on the bottom. A linear arrow denotes direction of differentiation. A curved arrow indicates that spermatogonia can proliferate.

Spermatogonium is difficult to fractionate by centrifugal elution; therefore, we could not determine whether CIA-II is expressed in such an early spermatogenic cell. To check whether CIA-II is expressed in spermatogonia, we evaluated the expression profiles of mCIA-II mRNA in the testes of newborn mice because emergence of testicular germ cells is distinctly regulated after birth. For example, spermatogonia, pachytene spermatocytes and round spermatids are known to emerge at 4, 10, and 17 days after birth, respectively (47, 48). We thus tested Northern blot analysis using poly(A)+ RNA prepared from testes of 4-day-old to 56-day-old mice (Fig. 5A). During this observation period, β-actin transcripts were constantly detected, and loaded poly(A)+ RNA was almost equivalent in each lane (Fig. 5A, lower panel). Emergence of S-II-T1 mRNA (Fig. 5A, middle panel) at around 10–14 days after birth was used as the control of onset of pachytene spermatocytes (41). The Northern blot data using mCIA-II cDNA as a probe shows that mCIA-II transcripts were already visible in the testes of 4-day-old mouse (Fig. 5A, upper panel). Considering that mCIA-II is transcribed in a germ cell-specific manner in mouse testis (Fig. 4B), transcription of mCIA-II occurs in spermatogonium, which is the unique germ cell found in a testis of this age. The testis of 7-day-old mouse contains early spermatocytes such as leptotene spermatocytes and zygote spermatocytes in addition to premeiotic spermatogonia. Because expression level of mCIA-II in the 7-day-old testis is apparently greater than that in 4 days after birth, transcription of mCIA-II seems to be continued in these spermatocytes. In addition to leptotene and zygote spermatocytes, pachytene spermatocytes are the major germ cells found in the testes of 14- to 35-day-old mice. The mRNA level of mCIA-II is relatively high and constant in those periods, consistent with our observation that mCIA-II mRNA is detected in fractionated pachytene spermatocytes (Fig. 4D). The mCIA-II transcription in the testes of 56-day-old adult mice decreased significantly as compared with those of younger mice (Fig. 5A). In adult (56 days old) testis, spermatids were most abundant (~70%) among male germ cells. Therefore, the mCIA-II gene seems to be scarcely transcribed within spermatids. Also, it is consistent with the observation that mCIA-II transcript is not detected in the cell fractions in spermatids (Fig. 4D). Based on these expression analyses, we thus postulate that expression of mCIA-II transcripts in testis occurs in premeiotic to meiotic germ cells as schematically shown in Fig. 5B.
Interestingly, CIA-II as well as CIA is phosphorylated by tyrosine phosphorylation, acetylation of itself, and protein modification (e.g., trimethylation of lysine). Domain analysis of CIA-II responsible for binding with cell cycle-regulated histones, assembly of a testis-specific nucleosome that will utilize testis-specific histone chaperone CIA-II, presumably in collaboration with CIA-I, will provide insight into the regulation of differentiation, cell death, or maturation of sperm.

Activity of histone chaperone CIA-II might be regulated by protein modification (e.g., phosphorylation, acetylation) of itself. Interestingly, CIA-II as well as CIA is phosphorylated by tyrosine kinases in both in vitro and in vivo. The protein level of CIA-II in HeLa cells seems to be virtually constant throughout the cell cycle (40). Phosphorylation of CIA-II is not detected during the cell cycle, whereas a change in CIA phosphorylation is detected during S phase (40). However, considering that the expression of CIA-II is regulated in both a temporal- and spatial-specific manner, CIA-II might be phosphorylated in testicular germ cells and proliferating somatic cells. The activity of CIA-II might also be controlled by protein modification of its interactive proteins such as core histones H3/H4 (37).

CIA-II might also be controlled by protein modification of its activity by creating CIA-II-manipulated animals will provide insight into the regulation of differentiation, cell death, or maturation of sperm.

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