The NOT4 protein is a component of the CCR4-NOT complex, a global regulator of RNA polymerase II transcription. Human NOT4 (hNOT4) contains a RING finger motif of the C4C4 type. We expressed and purified the N-terminal region of hNOT4 (residues 1–78) encompassing the RING finger motif and determined the solution structure by heteronuclear NMR. NMR experiments using a 113Cd-substituted hNOT4 RING finger showed that two metal ions are bound through cysteine residues in a cross-brace manner. The three-dimensional structure of the hNOT4 RING finger was refined with root mean square deviation values of 0.58 ± 0.13 Å for the backbone atoms and 1.08 ± 0.12 Å for heavy atoms. The hNOT4 RING finger consists of an α-helix and three long loops that are stabilized by zinc coordination. The overall folding of the hNOT4 RING finger is similar to that of the C3HC4 RING fingers. The relative orientation of the two zinc-chelating loops and the α-helix is well conserved. However, for the other regions, the secondary structural elements are distinct.

The CCR4-NOT complex was first detected in Saccharomyces cerevisiae as a global transcription regulator, affecting transcription of multiple functionally unrelated genes positively as well as negatively (1). The complex consists of CCR4 (carbon catabolite repressor 4), CAF1 (CCR4-associated factor 1, also known as POP2), the five NOT proteins (NOT1–5), and several unidentified proteins (1). The yeast NOT genes have been identified in a screen for elevated HIS3 expression (2–4). The HIS3 gene contains two core promoters, TC, a TATA-less element, and TR, a canonical TATA sequence (5, 6). Mutations in NOT genes selectively elevate transcription from TC (2–4). Besides repressing genes involved in histidine biosynthesis (HIS3 and HIS4), NOT proteins also affect transcription of genes involved in pheromone response (STE4), nuclear fusion (BIR1), and RNA polymerase II transcription (TBP) (2, 3). The CCR4 gene product regulates expression of ADH2 and other genes involved in nonfermentative growth, cell wall integrity, and ion sensitivity (7–9). CCR4 exists in a complex with other proteins (10), and two-hybrid screening with CCR4 identified CAF1 (11, 12) and DBF2 (a cell cycle-regulated kinase) (9, 13) as binding partners. Recently, it was found that CCR4 and CAF1 reside with the NOT proteins in a 1.2-MDa complex (1). Besides physical interactions between CCR4, CAF1, and NOT proteins, there is also a functional association. Mutations in the NOT, CCR4, and CAF1 genes lead to similar, but not identical, phenotypes (1, 14). Interestingly, mutations in NOT1, NOT3, NOT5, and CAF1 genes suppressed a mutation in SRB4, which is an essential component of the RNA polymerase II holoenzyme and required for the expression of most protein-coding genes. This suggests that the yeast CCR4-NOT complex has a very general role in RNA polymerase II transcription (15).

Recently, the human counterpart of the yeast CCR4-NOT complex has been identified (16). cDNAs for four subunits, hNOT2, hNOT3, hNOT4, and human CALIF (CAF1-like factor), were isolated and characterized. Like yeast NOT4, hNOT4 interacts with yeast NOT1 and an N-terminally truncated hNOT1 protein, and hNOT4 complements a not4-null mutation in yeast (16). Human NOT4 contains two protein motifs in its N-terminal region, a RING finger and an RNA recognition motif (16). The N-terminal part of the protein is evolutionarily conserved, in contrast to the C-terminal part (16). The RNA recognition motif has been implicated in binding of single-stranded nucleic acids (reviewed in Ref. 17). The RING finger is found in a large number of proteins in animals, plants, and viruses involved in distinct cellular functions (reviewed in Refs. 18 and 19). RING fingers are thought to mediate protein-protein interactions, and RING finger proteins are often found in large multiprotein complexes (reviewed in Refs. 18–20).

Recently, an increasing amount of data showed that several RING finger-containing proteins function as E3 ubiquitin ligases, which target proteins for degradation (reviewed in Ref. 21). Examples include the proto-oncogene product c-Cbl (22), which ubiquitinates receptor protein-tyrosine kinases and the SCF complex, containing the RING finger Rbx1 protein, which targets several proteins, including G1 cyclins for degradation (23, 24). Possibly, NOT4 also functions as an E3 ligase.
The RING finger motif can be defined by the consensus sequence Cys-X2-Cys-X9–30-Cys-X1–3-His-X2–3 (Cys/His)-X2–4-Cys-X2–4-Cys, in which X can be any amino acid. It binds two zinc atoms using its cysteine and histidine residues (reviewed in Refs. 18 and 19). By primary sequence comparison, RING finger variants have been identified in which the zinc-coordinating ligands have been replaced with other residues (25, 26). The structure of three C4HC4 RING fingers has been solved to date (reviewed in Ref. 27). The solution structures of the immediate-early EHV-1 protein from equine herpesvirus (IEEHHV) and the acute promyelocytic leukemia protooncogene product (PML) have been solved by NMR methods (28, 29), and the crystal structures of the immunoglobulin gene recombination enzyme RAG1 (30) and the c-Bl RING finger bound to ubiquitin-conjugating enzyme UbcH7 (31) have been solved by x-ray diffraction. The RING finger structures of RAG1 and IEEHHV are remarkably similar, but differ considerably from the PML RING finger structure. Despite this, all C4HC4 structures possess some common features, the most obvious being the coordination of the two zinc atoms in a cross-brace configuration. In this system, Cys4, Cys2, Cys3, and Cys6 coordinate the first zinc atom, and Cys3, His1, Cys7, and Cys8 share the second zinc atom. The inter-zinc distance in all three structures is 14 Å.

The consensus sequence for the RING finger of NOT4 orthologs is Cys-X2-Cys-X1–3-Cys-X2–4-Cys-X2–4-Cys (Cys-X1–16-Cys-X2–4-Cys). It constitutes a novel RING finger variant of a C4C4 type in which His3 is replaced with cysteine. Also, the spacing between the fourth and fifth metal-coordinating residues is different. To investigate whether this motif in NOT4 can adopt a RING finger conformation, we determined its structure by NMR methods. We found that the overall fold of the NOT4 RING finger resembles that of the C4HC4 RING fingers. However, important differences especially in the secondary structure elements are notable.

MATERIALS AND METHODS

Plasmids—pET15b-hNOT4-N78 encodes the first 78 amino acids of hNOT4. This N-terminally truncated, C-terminally His-tagged protein was overexpressed in E. coli DH5α, with an N-terminal 23-residue His6 tag. His6-NOT4-N78 was expressed and purified to homogeneity by inclusion body solubilization and refolding, followed by Ni2+-affinity chromatography (QIAGEN Inc.) equilibrated in lysis buffer containing 300 mM KCl. After harvesting, cells were washed with phosphate-buffered saline and resuspended in lysis buffer (100 mM Tris-HCl (pH 7.9), 20 mM EDTA, 100 mM KCl, 1 mM imidazole, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, 0.2 mM sodium bisulfite, 1 µM aprotinin, 1 µM/ml leupeptin, and 1 µM/ml pepstatin). Lysate was mixed with 1 µM isopropyl-β-D-thiogalactopyranoside and 10 mM ZnCl2 for 1 h at 37 °C. After harvesting, cells were washed with phosphate-buffered saline and suspended in lysis buffer (100 mM Tris-HCl (pH 7.9), 20% sucrose, 1 mM EDTA, 100 mM ZnCl2, 1 mM imidazole, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, 0.2 mM sodium bisulfite, 1 µM aprotinin, 1 µM/ml leupeptin, and 1 µM/ml pepstatin). Lysome was added to a final concentration of 200 µM/ml, followed by one freeze-thaw cycle to lyse the cells. After addition of KCl to 300 mM, the lysate was centrifuged for 1 h at 250,000 × g. The cleared lysate was applied to a Ni2+–nitrilotriacetic acid-agarose column (QIA-GEN Inc.) equilibrated in buffer A containing 1 and 16 mM imidazole, and protein was eluted in buffer A using a linear gradient from 16 to 400 mM imidazole. Peak fractions were pooled and dialyzed against buffer B (20 mM potassium phosphate (pH 7.0), 10% glycerol, 50 mM KCl, 20 mM ZnCl2, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM β-mercaptoethanol, 0.2 mM sodium bisulfite, 1 µM aprotinin, 1 µM/ml leupeptin, and 1 µM/ml pepstatin) containing 1 and 16 mM imidazole. The protein was eluted in buffer C using a linear gradient from 100 to 500 mM KCl, and was purified to homogeneity as judged by Coomassie Blue staining of protein gels. hNOT4-N78 was concentrated using a Centricon spin dialysis tube (10-kDa cutoff, Amicon, Inc.). The protein was analyzed using the program REGINE (39). The Ramachandran plot of Fig. 4 was produced using the program MOLMOL (45).

RESULTS AND DISCUSSION

Expression and Assignments—The first 78 residues of hNOT4, which contain the C4C4 RING finger, were fused to an N-terminal His4 tag. This protein was overexpressed and 15N- and 13C-isotopically labeled in bacteria and purified to a final concentration of 150 µM in 10% [13C]glycerol and 15N-[13C]glycerol. The purified protein was dialyzed against buffer A containing 50 mM KCl, 10% glycerol, 50 mM KCl, 20 mM ZnCl2, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.2 mM sodium bisulfite, 1 µM aprotinin, and 1 µM/ml leupeptin containing 100 mM KCl (buffer Bap) and applied to a MonoQ HR 10/10 column (Amersham Pharma BioTech) equilibrated in buffer Bap. After washing with buffer Bap, the column was developed using a linear gradient from 100 to 500 mM KCl. hNOT4-N78 eluted from the column at ~170 mM KCl and was purified to homogeneity as judged by Coomassie Blue staining of protein gels. hNOT4-N78 was concentrated using a Centricon spin dialysis tube (10-kDa cutoff, Amicon, Inc.). The protein was analyzed using the program REGINE (39). The Ramachandran plot of Fig. 4 was produced using the program MOLMOL (45).
belonging to the algorithm (46).

asterisks. Sequence alignment was performed using the ClustalW algorithm (46). yNOT4, yeast NOT4.

homogeneity as described under “Materials and Methods.” The sequence of the first 78 residues of hNOT4 is shown in Fig. 1 and is compared with the corresponding region in yeast NOT4 and with the C3HC4 RING fingers of IEEHV and RAG1.

Sequence-specific assignments were obtained by the analysis of three-dimensional CBCA(CO)NH and three-dimensional HNCA(CO)HA spectra and by the application of a three-dimensional CDCA(NCO)CAHA spectrum (33) for 11 proline residues. The assignments for 10 residues of the 23-residue His6-tagged region were also obtained. H-1^13N CORRELATIONS for the remaining residues in the His6-tagged region were not observed, and the resonances for those residues were therefore left unassigned. Stereospecific assignments for methyl protons in the prochiral center of Val122, Leu167, Leu171, and Leu182 were obtained. In addition to this, 14 out of 64 β-methylene protons were also obtained.

Metal-binding Sites—To determine the number of metal ions present in the hNOT4 RING finger and the coordination system, we performed 113Cd-1H HSQC experiments using hNOT4-N78 in which the zinc ions were replaced with cadmium ions. 113Cd-substituted hNOT4-N78 was obtained by adding 113Cd-EDTA to a final concentration of 4 mM to zine-containing hNOT4-N78, and the exchange of zinc with 113Cd was monitored using 1H-1^15N HSQC spectra.

Since most of the resonances were shifted by the substitution, the assignments of 1H and 1^15N resonances of 113Cd-hNOT4-N78 had to be confirmed by three-dimensional NOESY-(1H,1^15N)-HSQC and three-dimensional TOCSY-(1H,1^15N)-HSQC spectra. Fig. 2A shows the chemical shift differences between 113Cd-hNOT4-N78 and Zn-hNOT4-N78. Cys17, Cys31, and Cys56 displayed the largest differences in both the 1H and 1^15N chemical shifts, which is correlated with hydrogen bonding from amide protons to the sulfur atoms of the zinc cluster (see below). Residues other than cysteines displayed only a slight change in chemical shifts, showing that exchange took place without breaking the integrity of the whole structure. Fig. 2B shows the two-dimensional 113Cd-1H HSQC spectrum of 113Cd-hNOT4-N78 displaying the correlation between two cadmium resonances and the 1H chemical shifts belonging to the β protons of the coordinating cysteine residues. The observed chemical shift values of cadmium resonances, 687.5 and 714.4 ppm, are both typical for cadmium(II) coordinated by four sulfur atoms. These results show that the NOT4 RING finger contains two zinc ions, which are ligated in a cross-brace manner, similar to the canonical RING fingers.

Structure of hNOT4-N78—The three-dimensional structure was determined for the zinc-containing form of hNOT4-N78. In total, 397 distance restraints (20 intraresidue, 171 sequential, 74 medium-range, and 132 long-range) and 32 angle constraints (19 φ and 13 χ) obtained from various two- and three-dimensional spectra were used for the structure calculations. Four hydrogen bond restraints, which were identified in a long-range HNCO spectrum, were also included. Finally, 200 structures were calculated, and 30 structures with a low energy were selected. Fig. 3A shows the superposition of the backbone atoms of these 30 calculated structures, and a summary of structural statistics is given in Table I and Fig. 3 (B–D). The region between residues 12 and 61 is well structured, whereas the N- and C-terminal parts are disordered due to lack of NOEs. Root mean square deviation values in the structured region versus the mean coordinates are 0.58 ± 0.13 Å for the backbone atoms and 1.08 ± 0.12 Å for all heavy atoms.

The structure of hNOT4-N78 consists of three long loops, L1 (residues 12–22), L2 (residues 27–38), and L3 (residues 49–61), and an α-helix (residues 39–48) between the second and third loops. Of the 8 cysteine residues that are involved in zinc coordination, the first (Cys14, Cys17), second (Cys31, Cys56), and forth (Cys53, Cys56) pairs are located in L1, L2, and L3, respectively. The remaining 2 cysteines (Cys41, Cys43) are located at the end of L2 and in the α-helix. Region 23–26 is recognized as a helical turn in L5 out of 30 calculated structures using secondary structure analysis in PROCHECK-NMR. All proline residues were found to have a trans-configuration on the basis of the observation of NOEs between the αC–αC and δH protons. The three loops are stabilized by the coordination with the zinc ions and by hydrophobic interactions. Leu167 in L1 and Pro45 in L3 form a hydrophobic area with Ile37 in L2 and Ile45 belonging to the α-helix. The conformations of both L1 and L3 are re-
Markably similar, Val12–Pro20 can be superimposed on Gly51–Tyr60 with a root mean square deviation of 0.18 Å versus the mean coordinates. Consistent with this, hydrogen bonds were also identified for CO(Val12)–NH(Leu21) in L1 and CO(Gly51)–NH(Tyr60) in L3 on the basis of hydrogen bond J-couplings (36) in a long-range HNCO spectrum.

The Ramachandran plot for residues 12–61 of the 30 calculated structures is shown in Fig. 4A. In addition to Gly34 and Gly51, Met18 in L1 and Arg57 in L3 also have positive φ angles in all 30 structures, although their ψ angles were not refined well. These positive φ angles of Met18 and Arg57 were confirmed by observation of cross-correlated relaxation of HN–N and HN–Ha dipolar interactions of the multiple lines (47). For this, we measured the intensity ratio of the 15N-coupled amide proton resonances in 15N-labeled hNOT4-N78. Fig. 4B shows the NH multiplets of these residues together with those of Glu49 and Leu52, which have negative φ angles. For Met18 and Arg57, the peak heights of the inner two multiplet components were lower than those of the outer two multiplet components were lower than those of the outer two multiplet components.

The distance between the two zinc atoms is 14.9 ± 0.3 Å, which is slightly longer than the well conserved value of 14 Å as found in the C3HC4 RING finger structures of PML (29), IEEHV (28), RAG1 (30), and Cbl (31). This could be due to the fact that there is a 4-residue spacing between Cys33 and Cys38 in hNOT4-N78, whereas only 2 residues separate the same zinc-ligating residues in the other three C3HC4 RING fingers.

Several NH protons surrounding the metal atoms display large upfield shifts in the amide proton resonance positions upon exchanging zinc with cadmium, as shown in Fig. 2A. These protons were found within a short distance of the sulfur atoms of the cysteines involved in metal ligation in 30 structures. For example, the distances between NH and the sulfur atoms are 2.90 ± 0.02 Å for NH(Cys17) and Cys14, 2.45 ± 0.04 Å for NH(Cys17)–S(Cys31), 2.55 ± 0.08 Å for NH(Cys17)–S(Cys31), and 2.54 ± 0.31 Å for NH(Cys17)–S(Cys31). The chemical shift of the amide proton is sensitive to hydrogen bond length (37), and substitution of zinc in all 30 structures, although their ψ angles were not refined well. These positive φ angles of Met18 and Arg57 were confirmed by observation of cross-correlated relaxation of HN–N and HN–Ha dipolar interactions of the multiple lines (47). For this, we measured the intensity ratio of the 15N-coupled amide proton resonances in 15N-labeled hNOT4-N78. Fig. 4B shows the NH multiplets of these residues together with those of Glu49 and Leu52, which have negative φ angles. For Met18 and Arg57, the peak heights of the inner two multiplet components were lower than those of the outer two multiplet components.
with cadmium causes a small change in hydrogen bond length due to the increase in the metal–sulfur bond length (48, 49). It is likely that these observed large chemical shift changes upon exchanging zinc with cadmium reflect the presence of hydrogen bonds from NH protons to sulfur atoms. These hydrogen bonds could contribute to stabilize the coordination of the zinc ion.

Comparison with C\(_{3}H_{4}\) RING Finger Structures—Fig. 5 shows a schematic drawing of the RING finger structures of hNOT4-N78, IEEHV, and RAG1. The α-helix in hNOT4-N78 is well conserved in IEEHV and RAG1. However, the β-sheet that exists in both IEEHV and RAG1 is not present in hNOT4-N78. The region corresponding to the third strand of the β-sheet in IEEHV, which is absent in RAG1, is unstructured in hNOT4-N78. The region corresponding to the first and second β-strands adopts a loop conformation in hNOT4-N78. Although these regions have a different conformation in hNOT4-N78 and the other two RING fingers, they are located in a similar position and have a similar orientation.

The root mean square deviation values of C-α atoms between the mean coordinates of the C\(_{3}H_{4}\) RING finger and C\(_{3}H_{4}\) RING finger structures are 1.7 Å for IEEHV (45 C-α atoms; Protein Data Bank code 1CHC), 1.6 Å for RAG1 (45 C-α atoms; Protein Data Bank code 1RMD), and 1.8 Å for c-Ring (45 C-α atoms; Protein Data Bank code 1FBV). Despite the differences in secondary structural elements, the overall structure of hNOT4-N78 is quite similar to the structures of IEEHV, RAG1, and c-Ring.

In the hydrophobic region of the C\(_{3}H_{4}\) RING finger, 2 central residues are well conserved (Phe\(^{28}\) and Ile\(^{33}\) in IEEHV, Phe\(^{309}\) and Ile\(^{314}\) in RAG1, and Met\(^{309}\) and Leu\(^{405}\) in c-Cbl). In hNOT4-N78, the backbone atoms of Ile\(^{37}\) and Trp\(^{42}\) are located in a similar position in space. The orientation of the side chains, however, is slightly different. The side chain of Ile\(^{37}\) is still positioned in the core, similar to the conserved Phe\(^{28}\) of IEEHV and Phe\(^{109}\) of RAG1; but the side chain of Trp\(^{42}\) is pointing away from Ile\(^{37}\), although they have limited contact. Instead, the side chain of Ile\(^{45}\) is pointing toward the side chain of Ile\(^{37}\) and participating in the hydrophobic core, taking over the role of Trp\(^{42}\). Consistent with this, Ile\(^{45}\) is invariant in all known NOT4 orthologs to date (Fig. 1 and data not shown).

Recently, the crystal structure of the c-Cbl RING finger bound to the ubiquitin-conjugating enzyme UbcH7 has been reported (31). The UbcH7-binding site on the c-Cbl RING finger is provided by the shallow groove formed by the α-helix and two zinc-chelating loops. It is interesting to note that this region is well conserved with the hNOT4 C\(_{3}H_{4}\) RING finger in structural but not chemical terms. Also, hNOT4 has a shallow groove that is formed by Leu\(^{16}\) in L1; Pro\(^{34}\) in L3; and Arg\(^{44}\), Ile\(^{45}\), and Glu\(^{49}\) in the α-helix. Although L1 and L3 come close to each other, there is a groove between the two loops and the α-helix. It is important to note that although the structure is conserved, the side chains are not. Ile\(^{38}\), Ser\(^{307}\), Trp\(^{408}\), and Ser\(^{411}\) which form the binding site for UbcH7 in the c-Cbl RING, are replaced by Leu\(^{16}\) Arg\(^{44}\) Ile\(^{45}\), and Asp\(^{48}\), respectively, in hNOT4.

Implication for Function—So far, the exact role of the hNOT4 RING finger domain in the CCR4-NOT complex is unclear. Yeast complementation analysis showed that unlike full-length hNOT4, a hNOT4 protein lacking the RING finger motif does not complement its yeast counterpart. Surprisingly, the RING finger domain of hNOT4 is not required for interaction with hNOT1, as this is mediated by the nonconserved C-terminal part of hNOT4 (data not shown). Analogous to the RING finger of c-RING (22, 31), the hNOT4 RING finger may serve in a role as a E3 ligase in (poly)ubiquitination of proteins. In accordance with this proposal, we have identified in yeast two-hybrid screens components of the ubiquitin pathway as NOT4 RING interaction partners. The structure of the hNOT4 RING finger displays the features that were observed in the Cbl-UbcH7 interaction. The described structure of the hNOT4 C\(_{3}H_{4}\) RING finger allows the rational design of phenotypic mutations that affect these interactions and subsequent testing of the effects on the in vivo function of NOT4. This should provide a better understanding of transcription regulation by the CCR4-NOT complex.

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The Structure of the C₄C₄RING Finger of Human NOT4 Reveals Features Distinct from Those of C₃HC₄ RING Fingers
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