Novel RING Finger Proteins, Air1p and Air2p, Interact with Hmt1p and Inhibit the Arginine Methylation of Npl3p*

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Heterogeneous nuclear ribonucleoproteins (hnRNPs) are involved in the mRNA processing and export and are post-translationally modified by methylation at arginine residues in their arginine-glycine-rich (RGG) domains. We screened the factors that can interact with the RGG domain of Npl3p only in the presence of Hmt1p with the two-hybrid system in Saccharomyces cerevisiae. An isolated clone, YIL079, encodes a novel RING finger protein that was not directly bound to Npl3p but associated with the N terminus of Hmt1p. Thus, we designated the gene product Air1p (arginine methyltransferase-interacting RING finger protein). Air1p inhibited the Hmt1p-mediated methylation of Npl3p in vitro. Overexpression of Air1p repressed the Hmt1p-dependent growth of cells. Since homology searches indicate that the YDL175 gene product has significant identity (45%) with Air1p, we designated the gene AIR2. Air2p also has a RING finger domain and was bound to Hmt1p. Although single disruption of either gene gave no effect on the cell growth, cells lacking Air1p and Air2p grew at an extremely slow rate with accumulated poly(A)* RNA in the nucleus. Thus, Air1p and Air2p may affect mRNA transport by regulating the arginine methylation state of heterogeneous nuclear ribonucleoproteins.

Various types of protein modifications, such as phosphorylation, acetylation, glycosylation, and myristoylation, are now recognized in a number of eukaryotic proteins, and their functional significance has been exhibited. A potential role of protein methylation as a post-translational modification in signal transduction, however, has not been explored with the same breadth and intensity that protein phosphorylation has enjoyed in recent years. The methyl group, donated by SAM, is transferred to carboxyl groups or to the side chain nitrogens of the amino acid lysine, arginine, or histidine. Evidence for the post-translational methylation of arginine residues in proteins was first provided by the presence of radioactive species chromatographing at positions near that of arginine in acid hydrolysates of isolated calf thymus nuclei incubated with [methyl-14C]SAM (2). The products of the methylation reaction were determined to be N^G-monomethylarginine and N^G,N^G-dimethylarginine (3). Type I protein arginine methyltransferases methylate arginine residues to form asymmetric N^G,N^ε-dimethylarginine residues, and type II enzymes catalyze the formation of symmetric N^G,N^G-dimethylarginine residues. Genes encoding type I methyltransferases have been identified in yeast (4, 5), rats (6), and humans (7), although type II protein arginine methyltransferases have not been cloned. In addition to these classical members, a coactivator-associated arginine methyltransferase 1 (CARM1) was recently cloned as a binding protein to p160 coactivators that include SRC1, GRIP/TIF2, and pCIP/RAC3/ACTR/AIB1/TRAM1 by using the yeast two-hybrid system to screen a mouse embryo cDNA library (8).

Although histones and myelin basic proteins were initially used as substrates to determine the arginine methyltransferase activity, hnRNPs are now known to be more efficient for substrates (9). hnRNPs are abundant RNA-binding proteins, and more than 20 different hnRNPs, designated A–U, have been identified (10, 11). hnRNPs are associated with nascent polymerase II transcripts and involved in many steps of mRNA processing and nuclear transport (10–12). In Saccharomyces cerevisiae, temperature-sensitive mutants of one such protein, Npl3p, display defects in export of mRNA from the nucleus (13–15). Npl3p is a yeast homologue of the prototypical hnRN, A1, which has two RNA recognition motifs of 90 residues each and an arginine-glycine-rich C-terminal domain termed the “RGG box” (10). In addition to the ability of RNA recognition motifs, the RGG box domain (or RGG domain) has been shown to mediate RNA-protein interactions (16, 17), and arginine methylation typically occurs within the regions with the RGG domain in hnRN A1 and Npl3p (6, 9, 18). Several other potential substrates for the enzyme including fibrillarin and nucleolin have a similar RGG methylation consensus: [G/F/G]-GRGG(G/F) (19).

Although the significance of arginine methylation in the RGG domain has not been revealed fully, Shen et al. (20) recently reported that Npl3p cannot exit the nucleus efficiently in cells lacking a major arginine methyltransferase, Hmt1p/ Rmt1p, and overexpression of Hmt1p enhances Npl3p export. The export of another RNA-binding protein known to shuttle from the nucleus to the cytoplasm, Hrp1p, is also impaired when HMT1 is missing (20, 21). Thus, the efficient export of at least two shuttling hnRNPs implicated in RNA processing and export requires arginine methylation in the RGG domain.

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‡ The abbreviations used are: SAM, S-adenosyl-L-methionine; hnRNP, heterogeneous nuclear ribonucleoprotein; GST, glutathione S-transferase; FMSF, phenylmethylsulfonyl fluoride; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; GFP, green fluorescent protein; PCR, polymerase chain reaction; kb, kilobase(s).
Inhibition of Arginine Methylation of Npl3p by Air1p

**TABLE I**

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| L40    | MATa his3-Δ200 trp1-901 leu2-3,112 ade2 lys (lexAop)4-HIS3 URA3 (lexAop)8-ileZ | Ref. 25 |
| ETM43  | 5-fluoro-orotic acid (5-FOX) resistance of L40 | This study |
| ETM63  | ETM43 hmt1-1:CgURA3 | This study |
| W303   | MATa/MATa ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-1,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100 | Ref. 24 |
| W303-1A| MATa ade2-1 his3-11,15 leu2-3,112 trp1-1/can1-100 | Ref. 24 |
| W303-1B| MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1-1 can1-100 | Ref. 24 |
| ETM52  | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1-1 can1-100 air1/CgURA3 | This study |
| ETM56  | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1-1 can1-100 air2/CgTRP1 | This study |
| ETM159 | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1-1 can1-100 air2/CgURA3 | This study |
| PSY1023| MATa npf13-17 ura3-52 leu-3 his3 lys1-1 ade2-1 ade8 can1-100 | Ref. 20 |

**TABLE II**

| Plasmid | Features | Source or reference |
|---------|----------|---------------------|
| pBTM116 | 2 µm TRP1 | Ref. 25 |
| pBTM16a | 2 µm TRP1 | Y. Mukai |
| pBTM116NPL3 | pBTM116 + NPL3 (residues 281–414) | This study |
| pACTII | 2 µm LEU2 | Y. Mukai |
| pACTIIAIR2 | pACTII + AIR2 | This study |
| pYE52 | 2 µm URA3 | This study |
| pYES2AIR1 | pYES2 + AIR1 | This study |
| pNB1 | 2 µm HIS3 | This study |
| pNB1HA-HMT1 | pNB1 + HA tag + HMT1 | This study |

These findings gave us the idea that unidentified protein(s) may interact with the RGG domain in a methylation-dependent manner as a regulator of the hnRNP shuttling. In support of this possibility, Michael et al. (22) reported that a part of C-terminal RGG domain is sufficient for its nuclear export when hnRNP A1 shuttles from the nucleus to the cytoplasm. In these experiments, the C-terminal RGG domain is sufficient for its nuclear export as a regulator of the hnRNP shuttling. In support of this possibility, Michael et al. (22) reported that a part of C-terminal RGG domain is sufficient for its nuclear export when hnRNP A1 shuttles from the nucleus to the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**Microorganisms and Plasmids**—The genotypes and sources of *S. cerevisiae* strains used in this study are listed in Table I. Yeast strains were grown in standard YPD (1% Bacto-yeast extract, 2% Bactopeptone, 2% glucose, and 0.04% adenine sulfate) or synthetic complete media lacking the appropriate amino acids (23). Sporulation medium was purchased from Bio 101.

The plasmids used for yeast in this study are listed in Table II. A PCR product of 0.4 kb encoding amino acid residues 281–414 of Npl3p was inserted into the BamHI–NotI gap of pBTM116 (25), named pBTM116NPL3S. A 1.0-kb PCR-amplified fragment containing the Air2p-coding region was inserted into the NotI–XhoI gap of pACTII (26), designated pACTIIAIR2. A PCR-amplified 1.1-kb fragment containing the Air1p-coding region was inserted into the BamHI–XhoI gap of pYES2 (Invitrogen), designated pYES2AIR1. Three genes described above were PCR-amplified from the genome of *S. cerevisiae* strain S288C. pCgURA3 and pCgTRP1, plasmids for gene disruption in yeast, above were PCR-amplified from the genome of *S. cerevisiae* strain S288C. pCgURA3 and pCgTRP1, plasmids for gene disruption in yeast, were kindly provided by Arisawa (27).

**Expression of Proteins in Bacteria**—*E. coli* DH5α carrying the pGEX fusion constructs was grown in Luria-Bertani medium at 37 °C and induced with 0.5 mM IPTG for 5–6 h at 30 °C. Bacteria were harvested by centrifugation and lysed in resuspension buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM PMSF, and 1% Triton X-100). The cells were ruptured by sonication on ice, using an ultrasonic disruptor (UP-201; Tomy). Sonication (five pulses of 1 min each) was carried out at 50% duty cycle. After centrifugation at 6,000 × g for 30 min at 4 °C, the supernatant was mixed with glutathione beads at 4 °C overnight. The protein-bound beads were washed repeatedly with the same resuspension buffer. GST fusion proteins were then eluted a few times with 30 mM reduced glutathione in the resuspension buffer and dialyzed overnight against dialysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol) at 4 °C. Proteins were analyzed by SDS-PAGE, and appropriate fractions were stored at −80 °C.

**GST Pull-down Experiments**—For in vitro protein–protein interaction assay, 3 µg of GST or GST fusion proteins were incubated for 1.5 h at 4 °C with 10 µl of glutathione-Sepharose beads in the binding buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, and 1% Triton X-100). After washing with the binding buffer once, the beads were incubated for 6 h at 4 °C with 0.5 µg of TG-tagged proteins in the binding buffer. The beads were then washed five times with the binding buffer, and retained proteins were eluted with 30 µl reduced glutathione in the binding buffer.

**Protein Analysis**—The aliquots of GST pull-down experiments were boiled in loading buffer and electrophoresed on sodium SDS-polyacrylamide gel. Separated proteins on SDS-PAGE were transferred to nitrocellulose membranes, which were then blocked in 5% dry milk in phosphate-buffered saline and probed with anti-GST rabbit polyclonal (Z-5; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-T7 antibody (Novagen). After washing in phosphate-buffered saline five times, the membranes were probed with an alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse antibody (Zymed Laboratories Inc.), respectively. After washing with Tris-buffered saline with 0.1% Tween 20 times, proteins were visualized.
For the staining of HA-tagged proteins in yeast extracts, anti-HA polyclonal antibody (Medical and Biological Laboratories) was used.

**Yeast Protein Extraction**—Yeast proteins were extracted as described (30). Each yeast strain was grown at 30 °C in galactose-containing synthetic medium. Cells were harvested to the optical density of 1.0 at 600 nm in the medium (1 ml), resuspended in 100 μl of sample buffer (60 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.0025% bromphenol blue), and heated immediately at 95 °C for 5 min. The extracted proteins were obtained in the supernatants following the centrifugation of the extracts, and the aliquots were analyzed.

**Gene Disruptions**—Disruption of the HMT1 gene was performed as described (31). Briefly, two oligonucleotides were synthesized: olig146 and olig147 (see below). Oligonucleotide olig147 contains 50 nucleotides of the HMT1 5′-untranslated sequence followed by 20 nucleotides of the sequence from the 3′-end of CgURA3 (27). Oligonucleotide olig146 contains 50 nucleotides of the translated sequences of the HMT1 followed by 21 nucleotides from the 5′-end of CgURA3. The PCR products were amplified with pCgURA3 as a template and olig146 and olig147 as primers and transformed in ETM43. We selected Ura+ transformants, and the structure of the disrupted genes was verified by PCR.

To construct a null mutant of AIR1 or AIR2, we used two pairs of oligonucleotides, olig454-455 (see below) or olig451-452 (see below), as primers and pCgURA3 or pCgTRP1 as templates, respectively. Diploid strain W303 was transformed with the PCR products. Posttransformation and tetrad analysis yielded a haploid strain containing air1::CgURA3 or air2::CgTRP1, designated ETM52 or ETM56, respectively. To create the parental strain of double disruption mutant air1::CgURA3 and air2::CgTRP1, ETM52 was mated with ETM56. The diploids were selected, tetrad s were dissected, and Trp+/Ura+ spores were selected, as ETM159.

The primers used for gene disruption were olig146 (5′-CACCATAATTTCTTTCTTTTTACTTATGTAATGACGCTCTTACGCTATGACAGTATTACG-3′), olig147 (5′-ATGATGGAGGACCTTGCGGAGAAAGATGCGCCCTTACAATGATCGCTACAGGTTTTAAA-3′), olig451 (5′-TTTTACCACTAAATGATCGCTATGACCGGCCTTTTTCTCTCTCAGCAGGGTAAAAT-3′), olig452 (5′-ACCGTGAACCGACGCTACGTGTATTTGATGTTCGAAATG-3′), olig453 (5′-AACGGAACCTATGGGCTCCTTTCTGTTGTTGTTGTACCTGTTACCTCCAGCTCAGCGTTGGTAAAAT-3′), and olig455 (5′-AAAGGGTGGTAAAAACACTCTTCTTTTTATAGGAGCAGCTGTATTATAACAGCAGGATGTTACG-3′).

**Fluorescent in Situ Hybridization**—Each strain was grown to midlog phase in YPD at 30 °C. The cells were then subjected to in situ hybridization as described (32) with some modification. Fixation of the cells was done with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 6.0) for 1 h at room temperature. After washing three times in 0.1 M sodium phosphate buffer, 105 cells were treated with 2 μl of β-mercaptoethanol and 20 μl of 2 mg/ml E. coli RNase 100T (Seikagaku Corp.) in 200 μl of sorbitol buffer (1.2 M sorbitol, 0.1 M sodium phosphate buffer) for 20–30 min at 37 °C. Resultant spheroplasts were washed three times in ice-cold sorbitol buffer and were put into the wells of Teflon-faced multiwell slides (Nalge Nunc International) coated with poly-l-lysine (M, 150,000–300,000; Sigma). After 30 min, nonadhered cells were removed by aspiration, and the slides were plunged into 70, 90, and 100% ethanol for 5 min successively. The slides were then dried completely to avoid condensation and incubated in 4× prehybridization buffer containing 4× SSC, 5× Denhardt’s solution, and 1 mg/ml tRNA at room temperature for 30 min. Hybridization was denatured (4) with some modification. 1× phosphate-buffered saline) and were examined under BX-50 (Olympus). The slides were mounted with a mounting medium (90% glycerol, 10% polyvinylalcohol, and 0.0025% bromphenol blue), and heated immediately at 95 °C for 5 min. The extracted proteins were obtained in the supernatants following the centrifugation of the extracts, and the aliquots were analyzed.

**In Vitro Methylation Assay—**In vitro methylation assay was done as described (4) with some modification. 1 μg of GST-Npl3p was incubated at 30 °C for 45 min in a 20-μl reaction containing 50 mM Na-MOPS (pH 7.2), 300 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10% glycerol, 0.5 μl of GST-Histone 1D, 0.4 μM methyl-[3H]SAM (specific activity of 1.3 Ci/mmol, NEN Life Science Products), and the indicated quantities of T7-Air1p or BSA (as a control). The reactions were terminated by adding SDS sample buffer and boiling for 5 min. 50% of each reaction solution was then loaded onto 15% SDS-polyacrylamide gel and subjected to electrophoresis. Gels were stained with Coomassie Blue R-250 to visualize protein bands and then fluorographed. The results represent 2-day exposures at −80 °C.

**RESULTS**

**Isolation of Npl3p-interacting Proteins by Two-hybrid Screening**—We screened ~7 × 106 transformants of yeast cDNA library using a bait of RGG domain of Npl3p (Fig. 1A) as described under “Experimental Procedures.” The results yielded nine different genes summarized in Table III. One clone is, as expected, Hmt1p/Rmt1p, and seven clones encode RNA-interacting proteins. Moreover, five of seven have the regions with RGG repeats, the RGG domain. High frequencies of the RGG proteins can be explained by the interaction between the RGG domains, since Cartegni et al. (44) reported that hnRNP A1 proteins interact with each other with the RGG domain. The other clone is the YIL079 gene, which encodes an unknown open reading frame. According to the data of the Yeast Proteome Database, the open reading frame is 1080 nucleotides in length, encoding a basic (predicted pI = 9.2) protein of 360 amino acid residues with a predicted molecular mass of 41,647 kDa. Analysis of the predicted amino acid sequence of the gene product indicates that it contains the KRING finger domain near the N terminus (45).

Next, we examined the interaction of the listed proteins with Npl3p in an hmt1 disruptant as a host for two-hybrid system to test their Hmt1p-dependent interaction. The growth of the hmt1 disruptant was similar to or even faster than that of the wild-type strain on a synthetic complete plate lacking histidine, when eight of the nine clones were introduced as preys (Fig. 1B and data not shown), indicating their Hmt1p-independent interaction with Npl3p. In contrast, the hmt1-disrupted strain introducing NPL3 (nuclear protein localization 2) and YIL079 could not survive in the medium lacking histidine, suggesting that the YIL079 gene product can interact with Npl3p only in the presence of Hmt1p (Fig. 1B).

**YIL079 Gene Product Interacts with Hmt1p but Not with Npl3p in Vitro**—The observation shown in Fig. 1B that the interaction of Npl3p with the YIL079 gene product, Yil079p, depends on the presence of Hmt1p implies two possibilities: (i) Npl3p interacts with Yil079p mediated by Hmt1p, or (ii) the methylated state of Npl3p mediated by Hmt1p is required for Npl3p in vitro.

*Inhibition of Arginine Methylation of Npl3p by Air1p*

**Fig. 1.** A, schematic representation of full-length Npl3p (top) or the C terminus of Npl3p (bottom), the latter of which is used as a bait in the two-hybrid screening. B, dot-spot analysis for comparison of interaction in wild-type host or hmt1-deprived host between Npl3p and the clones segregated in a two-hybrid assay. On each plate, the upper two spots represent interactions in the hmt1-deprived host. Precultures were diluted in growth medium, and equivalent amounts of cells (diluted in 10−3 steps) were spotted onto synthetic complete medium plates lacking tryptophan and leucine or lacking tryptophan, leucine, and histidine. Plates were incubated for 3 days at 30 °C. In the clones, only YIL079 and SUB2 (a control for comparison with the case of YIL079) were shown.
Inhibition of Arginine Methylation of Npl3p by Air1p

**TABLE III**

**Summary of Two-hybrid data**

As a bait, pBTM116NPL3S was prepared, in which the C terminus of Npl3p was fused in frame to LexA in the vector pBTM116 (25), and we screened the yeast cDNA library with the vector in a yeast two-hybrid assay. Asterisks indicate genes encoding protein with RGG repeats. aa, amino acids; RRM, RNA recognition motif.

| Gene      | Number of aa | Regions with prey | Functions                                                                 | References |
|-----------|--------------|-------------------|--------------------------------------------------------------------------|------------|
| HMT1      | 348          | 13–348            | Protein arginine methyltransferase                                        | 4, 5       |
| YIL079 (AIR1) | 360        | 8–360             | Unknown open reading frame with RING-type zinc finger domain              | 33         |
| NAB2      | 524          | 8–524             | Nuclear poly(A)-binding protein, required for proper polyadenylation of pre-mRNA and for mRNA export | 34, 35     |
| GBP2      | 427          | 1–143             | G strand binding protein with 2 RRM                                       | 36, 37     |
| GAR1      | 204          | Total             | Protein involved in RNA processing and also associated with small nuclear RNAs | 38         |
| DBP2      | 545          | Total             | ATP-dependent RNA helicase of DEAD box family                             | 39         |
| NSR1      | 413          | 18–413            | Nucleolar protein involved in processing 20 to 15 S rRNA with 2 RRM       | 36, 40     |
| SUB2      | 445          | Total             | Protein with similarity to nuclear RNA helicases ( DEAD/DEAH family); possible involvement with pre-mRNA splicing | 41, 42     |
| RML2      | 393          | 34–393            | Mitochondrial ribosomal protein                                          | 43         |

**Fig. 2. Air1p binds to Hmt1p in vitro.** A, 0.5 μg of T7-tagged proteins were suspended in binding buffer and incubated with glutathione-Sepharose beads bound to 3 μg of GST or GST fusion proteins. The beads were washed, and T7-tagged proteins were analyzed by 15% SDS-PAGE and visualized. B, schematic representation of wild-type (WT) and deletion mutants of Hmt1p used in C. WT Hmt1p is the full-length 348-amino acid protein (4, 5). Shaded boxes (I, post I, II, and III) indicate conserved regions within most of the SAM-dependent methyltransferases (46). The numbers in parentheses indicate amino acid present in the deletion mutants. C, differential in vitro binding of Air1p to wild-type and deletion mutants of Hmt1p. The indicated GST fusion proteins (shown by the arrowheads) were immobilized on glutathione-Sepharose resin and analyzed for T7-Air1p binding as shown in A. The proteins were detected by Western blot analysis.

T7-tagged Yil079p, however, did not bind to GST-Npl3p (Fig. 2A, lane 4). Instead, this protein bound to GST-Hmt1p, but not to GST alone (Fig. 2A, lanes 2 and 5). Although we tried to detect the possibility of methylation-dependent interaction of Npl3p and Yil079p, we could not find any direct interaction between them even after methylation (data not shown). Thus, we designated the YIL079 gene product Air1p (arginine methyltransferase-interacting RING finger protein).

To identify the portion of Hmt1p required for binding to Air1p, we prepared deletion mutants of Hmt1p fused to GST (Fig. 2B). Hmt1p is one of the SAM-dependent methyltransferases, and motifs I, post-I, II, and III are conserved among most of them (46). The mutant constructs were analyzed in vitro in a GST pull-down assay as mentioned under “Experimental Procedures” (Fig. 2C). The lower panel of Fig. 2C shows GST fusion proteins stained with anti-GST rabbit serum, and the upper panel shows the precipitated T7-Air1p recognized with anti-T7 antibody. T7-Air1p was allowed to bind to wild type and Δ1 (residues 1–207), but not to Δ2 (residues 56–348) and Δ3 (residues 56–207), indicating that the N terminus of Hmt1p is essential for the binding to Air1p. Air1p inhibits methylation of Npl3p mediated by Hmt1p—To examine the effect of Air1p on the methyltransferase activity of Hmt1p, GST-Hmt1p and GST-Npl3p were incubated with [methyl-3H]SAM with the indicated amount of recombinant T7-Air1p or BSA, and the status of arginine methylation of GST-Npl3p was examined. The methylation of Npl3p was inhibited by T7-Air1p in a dosage-dependent manner, whereas BSA did not affect the methylation of Npl3p (Fig. 3A).

Next, we examined whether Air1p would inhibit the function of Hmt1p even in vivo. The growth of an npl3 mutant strain, npl3–17, was arrested at the sensitive temperature (34.5 °C) (Fig. 3B). As Shen et al. (20) have reported, the growth defect of the npl3–17 strain was rescued by overexpression of Hmt1p, suggesting that hypermethylation by Hmt1p complements the function of mutated Npl3p. However, when Air1p was overexpressed with Hmt1p in the npl3–17 strain, the growth rescue by Hmt1p disappeared (Fig. 3B). Although we examined the effect of Air1p overexpression on the expression level of Hmt1p in an npl3–17 strain, the expression of Hmt1p was not affected by Air1p (Fig. 3C). These indicated that Air1p could antagonize the methylation of Npl3p by Hmt1p both in vitro and in vivo.

YDL175 Gene Product Is a Homologue of Air1p in S. cerevisiae—Homology searches using BLAST 2.0 indicate that YDL175 gene product in S. cerevisiae has significant identity (45%) with Air1p (Fig. 4A). The gene product also has the RING finger domain, and a cysteine-rich region at the N terminus side including the RING finger domain is highly homologous to amino acid residues 32–217 of Air1p (68%). There were no other clearly homologous proteins in any species in our homology search. In two-hybrid system, cells that expressed Npl3p as a bait and the YDL175 gene product as a prey could grow up in the medium lacking histidine only in the presence of Hmt1p (Fig. 4, B and C), indicating that the interaction of the gene product with Npl3p requires Hmt1p. Indeed, the gene product also bound to Hmt1p in vitro (data not shown). So we designated it as Air2p.

**Air1p and Air2p Have Functional Redundancy on Cell Growth**—To clarify the physiological role of Air1p and Air2p, we examined the growth of air1, air2, and air1 air2 disruptant strains. The air1:CgURA3 and air2:CgTRP1 constructs were targeted to the AIR1 and AIR2 chromosomal loci in the diploid W303 by homologous recombination, respectively, and correct replacements were verified by PCR (data not shown). After sporulation and tetrad dissection, the transformed diploid yielded four viable spores with normal growth from each tetrad, with two being prototrophic and two auxotrophic for uracil and tryptophan, respectively. Single disruption of either AIR1 or AIR2 had no effect on cell growth (Fig. 5). To test whether Air1p and Air2p act redundantly, we obtained the air1 air2 double disruptant. The diploid was constructed by air1 and air2 single disruptants, and tetrad analysis was conducted. Deletion of both AIR1 and AIR2 caused a strong growth defect.
of cells at 30 °C (Fig. 5).

To find the reason for the growth defect of the double disruptants, we analyzed the subcellular localization of poly(A)1 RNA in the wild-type or mutant strains by in situ hybridization using Cy3-labeled oligo(dT)50. However, while each single disruptant showed the similar cytosolic poly(A)1 RNA localization (Fig. 6, H and J) as that of wild type (Fig. 6B), clear nuclear accumulation of mRNA was observed in the air1 air2 double disruptants (Fig. 6E).

**DISCUSSION**

Since the post-translational methylation of protein arginine residues was revealed (2), their activity has been researched (46). Type I protein arginine N-methyltransferases methylate protein arginine residues to form NG,NG-dimethylarginine residues (asymmetric). Hmt1p/Rmt1p was isolated from *S. cerevisiae* as a protein arginine methyltransferase that catalyzes both the NG-monomeric and NG,NG-asymmetric dimethylation of arginine residues (4, 5). Hmt1p may be the one and only type I protein arginine N-methyltransferase present in the yeast, because the mutation of the gene of *S. cerevisiae* eliminates many NG,NG-dimethylarginine residues (5). In contrast to the yeast, at least two active type I arginine N-methyltransferases, PRMT1 and PRMT3, have been identified in mammals (6, 7, 47). One of the fundamental observations reported in recent years, as mentioned in the Introduction, is the possibility of facilitating mRNA export from the nucleus into the cytoplasm. In the process of the revelation of mRNA export, we cloned *HMT1* and *AIR1* using Npl3p as a bait in the two-hybrid screening. An in vitro binding assay showed that Air1p interacts with the RGG domain of Npl3p bridged by Hmt1p. McBride et al. (48) reported that the N-terminal E18V substitution of Hmt1p affected methylation of Npl3p but not of other substrates and suggested that Glu18 is involved in substrate binding or positioning. In support of this finding, the N-terminal deletion mutant of Hmt1p used in this study, D2 (residues
56–348), could not methylate Npl3p in vitro (data not shown).

The inhibitory effect of Air1p on the enzyme activity of Hmt1p was detected by in vitro methylation assay of Npl3p. The methylation of Npl3p is blocked by Air1p in a dose-dependent manner, and overexpression of Air1p inhibited the Hmt1p-dependent growth in vivo. In this case, it is possible that Air1p suppresses the expression of Hmt1p followed by the elimination of the rescue by Hmt1p in an npl3–17 strain. However, since overproduction of Air1p did not affect the amount of exogenous Hmt1p introduced by a high copy plasmid, the suppression of Hmt1p-dependent rescue by overproduction of Air1p is not due to the reduced expression of Hmt1p. Hmt1p has been shown to facilitate the export of Npl3p in nucleocytoplasmic transport (20). Our observation that growth and mRNA transport were affected in cells lacking AIR1 and AIR2 suggests to us that the regulated methylation of Npl3p may be crucial for mRNA transport. When we checked the localization of the GFP-Npl3p in wild type and air1 air2 double disruptant, GFP-Npl3p was localized in the nucleus in both strains, and any difference of the expression pattern was not observed (data not shown). The regulatory mechanism of Air1p and Air2p on mRNA transport should be further elucidated.

It should be noted that Liu and Dreyfuss (9) reported that arginine methylation was not observed in yeast extracts when recombinant hnRNP A1 was used as a substrate, the contrary to the extensive in vivo methylation of hnRNPs in HeLa cells. The inconsistent observations could be explained by the presence of endogenous inhibitory factors like Air1p and Air2p in yeast. An unidentified signal transduction system could be involved in the regulation of protein methylation mediated by Hmt1p as suggested in mammalian cells (1). PRMT1 has been reported to interact with interferon receptor and to affect the proliferation induced by interferon (49). PRMT1 also interacts with TIS21 and BTG1, which are immediate early genes and have an antiproliferative effect, and the methylation activity was modulated by the interaction (6).

Air1p and Air2p have sequence similarity, especially in their N-terminal RING finger domain. The RING finger domain consisting of the amino acid sequence Cys-X_{2}–Cys-X_{9–30}–Cys–X_{1–2}–His–X_{2}–Cys–X_{2}–Cys–X_{4–48}–Cys–X_{2}–Cys is a cysteine-rich zinc-binding domain ubiquitous in species ranging from plants to mammals and has been found in several proteins, which have important physiological roles in various biological phenomena (44). The RING finger domain is now believed to be a...
motif for protein-protein interaction. We could not detect any homologous proteins in data bases of other species, but some proteins have conserved cysteine and histidine over the RING finger domain. Identification of the mammalian homologues of Air1p or Air2p may pave the way to understand the signal-mediated regulation of methyltransferases in mammals.

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