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Mutation of the Yeast ε-COP Gene *ANU2* Causes Abnormal Nuclear Morphology and Defects in Intracellular Vesicular Transport

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ABSTRACT. **Previously we reported an original method of visualizing the shape of yeast nuclei by the expression of green fluorescent protein (GFP)-tagged *Xenopus* nucleoplasmin in *Saccharomyces cerevisiae*. To identify components that determine nuclear structure, we searched for mutants exhibiting abnormal nuclear morphology from a collection of temperature-sensitive yeast strains expressing GFP-tagged nucleoplasmin. Four *anu* mutant strains (*anu1-1, 2-1, 3-1* and *4-1*; ANU=abnormal nuclear morphology) that exhibited strikingly different nuclear morphologies at the restrictive temperature as compared to the wild-type were isolated. The nuclei of these mutants were irregularly shaped and often consisted of multiple lobes. *ANU1, 3* and *4* were found to encode known factors Sec24p, Sec13p and Sec18p, respectively, all of which are involved in the formation or fusion of intracellular membrane vesicles of protein transport between the endoplasmic reticulum (ER) and the Golgi apparatus. On the other hand, *ANU2* was not well characterized. Disruption of *ANU2* (Δ*anu2*) was not lethal but conferred temperature-sensitivity for growth. Electron microscopic analysis of *anu2-1* cells revealed not only the abnormal nuclear morphology but also excessive accumulation of ER membranes. In addition, both *anu2-1* and Δ*anu2* cells were defective in protein transport between the ER and the Golgi, suggesting that Anu2p has an important role in vesicular transport in the early secretory pathway. Here we show that *ANU2* encodes a 34 kDa polypeptide, which shares a 20% sequence identity with the mammalian ε-COP. Our results suggest that Anu2p is the yeast homologue of mammalian ε-COP and the abrupt accumulation of the ER membrane caused by a blockage of the early protein transport pathway leads to alteration of nuclear morphology of the budding yeast cells.**

Key words: coatomer/COPI/vesicle transport/nucleus/GFP

In eukaryotic cells, the transport of materials between membrane-bound organelles occurs through the formation and fusion of small transport vesicles. One of the best understood examples of intracellular transport is the flow of materials between the endoplasmic reticulum (ER) and the Golgi apparatus in the yeast *Saccharomyces cerevisiae*. This transport is mediated by two pathways, the ER-to-Golgi anterograde pathway and the Golgi-to-ER retrograde pathway. In the anterograde pathway, it is generally accepted that most, if not all, vesicle formation is promoted by a set of proteins which forms a coat around vesicles budding from the ER, collectively called COPII proteins (4, 23). Initially identified in yeast cells, the COPII proteins are composed of the small GTPase Sar1p and two complexes, the Sec13p-Sec31p complex and the Sec23p-Sec24p complex (3, 19, 29, 33, 42, 43). In addition, two other ER proteins, Sec12p (guanine-nucleotide-exchange factor for Sar1p) and Sec16p are known to be involved in the formation of COPII-

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Abbreviations: ER, endoplasmic reticulum; NSF, *N*-ethylmaleimide sensitive factor; GFP, green fluorescent protein; NPC, nuclear pore complex; ORF, open reading frame; GST, glutathione S-transferase; EMS, ethylmethane sulfonate; CPY, carboxypeptidase Y.

coated vesicles (2, 10, 21, 32). Fusion of ER-derived vesicles to the Golgi consists of multiple steps. One of these steps involves Sec18p (yeast *N*-ethylmaleimide sensitive factor (NSF)), a factor which is also involved in vesicle fusion events in other organelles, and which acts to prime the fusion machinery (14, 16, 21, 51). Another factor, Uso1p, acts in tethering the vesicles to the Golgi (5, 31).

On the other hand, formation of transport vesicles in the Golgi-to-ER retrograde pathway has been proposed to be promoted by a separate set of proteins, collectively called COPI/coatomer (25, 43). COPI/coatomer, which forms coat around vesicles budding from the Golgi, was originally identified as a major component of Golgi-derived vesicles in mammalian cells (28). COPI/coatomer is composed of seven subunits; α- (160 kDa), β- (110 kDa), β'- (102 kDa), γ- (98 kDa), δ- (61 kDa), ε- (35 kDa) and ζ-COP (20 kDa) (52). The structure of coatomer is evolutionally conserved between yeast and mammals. Coatomer purified from yeast closely resembles its mammalian counterpart in its subunit composition, which consists of peptides with molecular mass of 150, 110, 105, 73, 35 and 25 kDa (20). Moreover, the yeast homologues of all except ε-COP have been identified, viz. Ret1p/Cop1p, Sec26p, Sec27p, Sec21p, Ret2p and Ret3p/Yzc1p, which correspond to α-, β-, β'-, γ-, δ- and ζ-COP, respectively (7, 8, 13, 20, 25, 53). Yeast cells with mutations in various coatomer subunits are defective in their ability to retrieve certain classes of proteins from the Golgi to the ER (1, 20, 25, 26, 53).

In one of our previous studies, a fusion protein composed of green fluorescent protein (GFP) and *Xenopus* nucleoplasmin was expressed in yeast cells in order to visualize the yeast nuclei (27). Nucleoplasmin is a nucleoprotein carrying a bipartite nuclear localization signal (40). This method is more suitable to observe nuclear shape than nuclei staining by DNA fluorochromes such as 4′,6′-diamidino-2-phenylindole because the latter results in high background staining of mitochondrial DNA and requires cell permeabilization which often distorts the shape of the nuclei. Using this method, we previously reported that a mutant of the *NSP1* gene (34), which encodes a subunit of the nuclear pore complex (NPC), exhibited an abnormal nuclear morphology (27). Based on this observation, the present study examines a collection of temperature-sensitive mutants expressing GFP-tagged nucleoplasmin to screen for abnormal nuclear morphology, with the aim to identify factors required for construction of nuclei, particularly components of the NPC, nuclear membranes or nuclear matrix. Screening a collection of 700 temperature-sensitive yeast strains yielded 4 mutants that developed abnormal nuclear morphology after incubation at the restrictive temperature, 37°C. We designated them as *anu* (abnormal nuclear morphology) mutants. Unexpectedly, all *ANU* genes were found to encode factors related to the formation or fusion of membrane vesicles involved in transport between the ER and the Golgi; *ANU1*, *ANU3* and *ANU4* are identical with *SEC24*, *SEC13* and *SEC18*, respectively. We also describe the characterization of *ANU2*, and propose that Anu2p is the yeast homologue of mammalian ε-COP.

Materials and Methods

Plasmids

The *Bam*HI and *Sma*I sites of pUC119 were converted to *Not*I and *Sal*I sites respectively by insertion of appropriate oligonucleotide linkers. A 4.9-kb long *Xba*I-*Hin*dIII yeast genomic fragment containing *LYS2* was subcloned into the resultant plasmid to generate a yeast integrative vector pKN1. The *Hin*dIII site of pKN1 was converted to *Cla*I by insertion of an oligonucleotide linker to generate another yeast integrative vector pKN1-2. Plasmid pKR1 was used for expression of a fusion protein consisting of a mutant of *Aequorea victoria* GFP, with an amino acid substitution of $Ser^{65} \rightarrow Thr$, and *Xenopus* nucleoplasmin under the *ADH1* promoter in yeast cells. $Ser^{65} \rightarrow Thr$ GFP is one of the most widely utilized GFP variants due to its stronger fluorescence and faster maturation rate relative to the wild-type (18). pKR1 was constructed identically to pAGN1, except that the GFP gene in pKR1 was derived from pQB1 (24, 27). A 3.0-kb long *Xho*I-*Not*I fragment containing the *ADH1* promoter and the GFP-nucleoplasmin fusion gene was excised from pKR1 and subcloned into pKN1 to generate pAGN5. A YCp50-based (*CEN* vector; *URA3*) yeast genomic library (41) was used for screening of genes which complement the temperature-sensitive growth defects of *anu* mutants. One of the plasmids obtained in this screen contained *ANU2* and was named pKR4. A 2.0-kb long yeast genomic fragment containing the *ANU2* (YIL076w) open reading frame (ORF), plus 556 bp long 5′- and a 329 bp long 3′-flanking sequences, was amplified from pKR4 by PCR, creating *Cla*I and *Bam*HI sites at the 5′ and the 3′ ends, respectively. This PCR product was subsequently subcloned into the corresponding sites of pRS316 (*CEN* vector; *URA3*; 45) to obtain pYIL076w. To obtain pRS306-*ANU2*, the 2.0-kb long DNA fragment described above was subcloned into pRS306 (a yeast integrative vector; *URA3*; 45). 5′- and 3′-DNA fragments flanking the *ANU2* ORF were each amplified from pKR4 by PCR, creating appropriate restriction sites at each end (refer to sequences below). These DNA fragments were subsequently subcloned into pRS305 (a yeast integrative vector; *LEU2*; 45) to yield plasmid p*ANU2*-DIS, harboring a sequence as follows: **CTCGAG**TCCAACTGAT — (the 840 bp long 3′-flanking sequence of the *ANU2* ORF in the reverse direction) — TAGCGTAACA**CTGCAG**TTTG ACTTTC — (the 1,115 bp long 5′-flanking sequence of the *ANU2* ORF in the forward direction) — GTATAGGTGC**G**

AGCTC [The cloning sites of pRS305 (*Xho*I, *Pst*I and *Sac*I) are in bold.]. A 0.5-kb long DNA fragment containing the *GAL1* promoter sequence was amplified from pYES2 (Invitrogen, Carlsbad, CA, USA) by PCR, creating appropriate restriction sites at its ends; and the PCR product was subsequently subcloned into pKN1-2 to obtain p_{Gal1p}-N, a yeast inducible expression vector, harboring the sequence **GTCGAC** CTGCAGATCCACTAGTACGGATT — AAAAAACTAT aagcttGGTCGA**GCGGCCGC** [The cloning sites of pKN1-2 (*Sal*I and *Not*I) are in bold, and the *GAL1* promoter sequence is underlined. A unique *Hin*dIII site is in small letters.]. DNA fragments encoding amino acids 1-359 (the entire *ANU2* ORF) and 64-359 of the *ANU2* ORF, respectively, were amplified from pKR4 by PCR, creating *Hin*dIII and *Not*I sites at the 5′ and 3′ ends. Those DNA fragments were each subcloned into p_{GAL1p}-N to obtain p_{GAL1p}-Long and p_{GAL1p}-Short. For bacterial expression of *Schistosoma japonicum* glutathione S-transferase (GST)-tagged Anu2p, a 1.0-kb long DNA fragment encoding amino acids 35-359 of the *ANU2* ORF was amplified from pKR4 by PCR, and the PCR product was subsequently subcloned into pGEX-5X-2 (Amersham Pharmacia Biotech Buckinghamshire, UK) to generate pGST-ANU2p. Plasmid pFIW was used for expression of a fusion between invertase and the C-terminal 111 amino acid residues of Wbp1p under the *ADH1* promoter in yeast cells (53). Plasmid pFIS is identical to pFIW, except that pFIS encodes a fusion of invertase-Wbp1p in which the C-terminal double lysine motif (-K-K-T-N-COOH) was converted to -S-S-T-N-COOH. Both pFIW and pFIS, generous gifts from K. Mihara of Kyushu University, are yeast episomal plasmids containing the *URA3* selectable marker.

Yeast strains and culture methods

YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (Difco Laboratories, Detroit, MI, USA); YPD and YPG media were supplemented with 2% glucose and 2% galactose, respectively. Minimal media (SD) containing 2% glucose and 0.67% Bacto-yeast nitrogen base w/o amino acids (Difco Laboratories) were supplemented with appropriate amino acids. Solid media were supplemented with 2% Bacto-agar (Difco Laboratories). Standard genetic manipulations were performed as described in Kaiser *et al.* 1994.

All yeast strains used in this study were derived from FY strains (generous gifts from F. Winston, Harvard Medical School, Boston, MA, USA); FY8 (*MATα ura3-52 lys2Δ202*), FY23 (*MATa ura3-52 trp1Δ63 leu2Δ1*), FY24 (*MATα ura3-52 trp1Δ63 leu2Δ1*) and FY78 (*MATa his3Δ200*). TKO1 (*MATa ura3-52 lys2Δ202::*pAGN5*::LYS2*) and TKO2 (*MATα his3Δ200 lys2Δ202::*pAGN5*::LYS2*) were used as wild-type strains expressing GFP-nucleoplasmin. To construct TKO1 and TKO2, pAGN5 was linearized within the *LYS2* sequence by digestion with *Bgl*II and introduced into meiotic segregants of a diploid strain (FY8 × FY78). Diploid strain DFY24 (*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 trp1Δ63/TRP HIS/his3Δ200 lys2Δ202/LYS*) was constructed by crossing various FY strains.

For isolation of temperature-sensitive mutant strains, TKO1 was mutagenized with ethylmethane sulfonate (EMS), and standard replica techniques were performed. Seven hundred temperature-sensitive strains were collected, and the mutant phenotype was observed according to the following procedure: each strain was precultured to an OD_{600} of approximately 0.5 at 23°C (permissive temperature) in YPD, shifted to 37°C (restrictive temperature) for 2 h, and observed under fluorescence microscope. Four *anu* mutant strains were obtained, and they were crossed two or three times against TKO2. The resultant strains (TKC3: *anu1-1*, TKE77: *anu2-1*, TKD76: *anu3-1*, TKA33: *anu4-1*) were of the same genotype as TKO1 except for the *anu* mutations.

Integration of the *URA3* marker into the genome adjacent to *ANU2* was performed by transformation of FY24 with pRS306-*ANU2* which had been linearized within the *ANU2* sequence by *Hin*dIII. The resultant strain was named YKIM1 (*MATα ura3-52 trp1Δ63 leu2Δ1 ANU2::*pRS306*::ANU2*). For gene disruption of *ANU2*, p*ANU2*-DIS was linearized by *Pst*I and introduced into DFY24. Tetrad dissection of the resultant transformant yielded YNAR1 (*MATa ura3-52 leu2Δ1 lys2Δ202 anu2::LEU2*). Plasmid p_{GAL1p}-Short and p_{GAL1p}-Long were linearized within the *LYS2* sequence by *Bgl*II and introduced into YNAR1 to generate Y_{GAL1p}-Short (*MATa ura3-52 leu2Δ1 lys2Δ202::*p_{GAL1p}-Short*::LYS2 anu2::LEU2*) and Y_{GAL1p}-long (*MATa ura3-52 leu2Δ1 lys2Δ202::*p_{GAL1p}-Long*::LYS2 anu2::LEU2*), respectively.

Fluorescence and electron microscopy

Microscopic observation of GFP fluorescence was performed using Axiophot (Carl Zeiss, Jena, Germany). Preparation of thin section of yeast cells by the freeze-substituted fixation method was carried out as described. (48), except that Reichert KF80 was used to freeze cells and that thin sections were viewed on a JEOL100CX electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Production of anti-Anu2p antisera

GST-tagged Anu2p was expressed from pGST-Anu2p in *E. coli* XL1-blue and purified by chromatography on immobilized glutathione (Bulk GST Purification Module; Amersham Pharmacia Biotech). Immunization of rabbits and preparation of sera were performed as described in Harlow and Lane (17), using GST-tagged Anu2p.

Detection of Anu2p by Immunoblotting

Yeast cells were cultured to an OD_{600} of 0.5 at 23°C in 10 ml of YPD or YPG media. Cells were then harvested, re-suspended in 10 ml of lysis solution (1% NaOH and 1.2% β-mercaptoethanol) and incubated on ice for 10 min. To precipitate total protein, 650 μl of 60% trichloroacetic acid was

added to the lysates. These precipitates were subjected to Western blot analysis as described in Harlow and Lane (17), using anti-Anu2p antisera (1:100 dilution) and the ECL Western blotting detection kit (Amersham Pharmacia Biotech).

Carboxypeptidase Y (CPY) and invertase-Wbp1p transport assays

To test for intracellular transport of endogenous CPY, pulse and chase experiments were carried out as described (53) using anti-CPY antibody (a generous gift from Dr. A. Nakano, RIKEN, Japan). To assay for Golgi-to-ER retrograde vesicular transport, yeast cells were transformed with plasmid pFIW or pFIS, and each transformant was subjected to pulse and chase experiments as described in (11), using anti-invertase antibody (a generous gift from Dr. K. Mihara, Kyushu University, Japan).

Results

Isolation of anu mutants

A yeast haploid strain (TKO1) expressing GFP-tagged nucleoplasmin under the control of the constitutive *ADH1* promoter was subjected to EMS-mutagenization, and a collection of 700 mutants that exhibit conditional growth phenotype, i.e., no or retarded growth at the restrictive temperature of 37°C was generated. These temperature-sensitive strains were analyzed for abnormal nuclear morphology after 2 h of incubation at 37°C, by observation of GFP-tagged nucleoplasmin fluorescence in the nuclei. Four candidates were obtained, and complementation analysis showed that each mutant was independent and belonged to different complementation groups. Hence, they were designated *anu1-1*, *anu2-1*, *anu3-1* and *anu4-1*. Light microscopic images of wild-type and *anu* mutant cells are shown in Figure 1. In contrast to the wild-type cells, *anu* cells exhibited irregularly shaped nuclei which often consisted of multiple lobes when cultured at the restrictive temperature. However, the cell morphology of *anu* mutants was normal, except that *anu3-1* cells were slightly smaller in size. We believe that the abnormality in nuclear morphology is not due to defects in nuclear division in mitosis, as the cell cycle was not arrested at any specific stage when these cells were cultured at the restrictive temperature (data not shown).

Cloning of ANU genes

All *ANU* genes were cloned by screening a *CEN*-based genomic library for genes that complemented the temperature-sensitive growth of *anu* mutants. All plasmids complementing the *anu3-1* mutant were found to contain the *SEC13* gene. Further subcloning and genetic

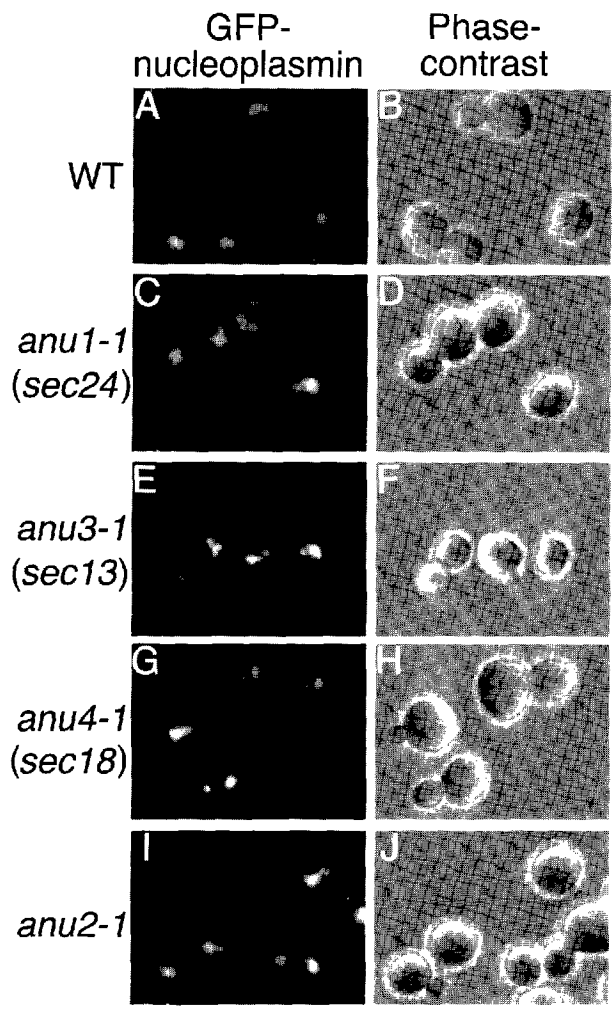

Fig. 1. Mutants exhibiting abnormal nuclear morphology. Wild-type (WT; TKO1; A and B), *anu1-1* (TKC3; C and D), *anu3-1* (TKD76; E and F), *anu4-1* (TKA33; G and H) and *anu2-1* (TKE77; I and J) cells were grown to OD_{600} of approximately 0.5 at 23°C in YPD and shifted to 37°C for 2 h. Green fluorescent signals from cells excited with blue light are shown in panel A, C, E, G and I (Carl Zeiss filter set 09 was used). Panels B, D, F, H and J are phase-contrast images of the same fields of cells as in panels A, C, E, G and I, respectively.

analysis indicated that *ANU3* was identical with *SEC13*. By the same means *ANU4* was found to be identical with *SEC18*. Identification of *ANU1* as *SEC24* will be described elsewhere (H. Higashio, Y. Kimata and K. Kohno, manuscript in preparation.)

We obtained one plasmid, pKR4, that complemented the *anu2-1* mutant. Further DNA restriction and sequencing revealed a single 2.0-kb ORF that complemented *anu2-1* (Fig. 2A). This ORF corresponds to the sequence designated as YIL076w in the Saccharomyces Genome Database (SGD, Stanford Genomic Resources, Stanford University, Stanford, CA, USA).

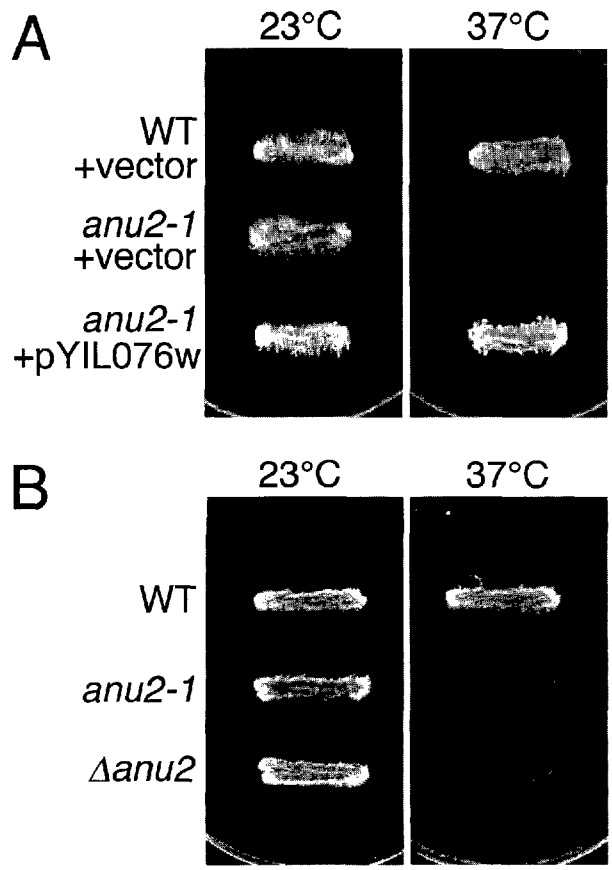

Fig. 2. Temperature-sensitivity of *anu2* mutants and complementation with the *ANU2* gene. (A) Wild type (WT; TKO1) and *anu2-1* (TKE77) cells containing a vector pRS316 or pYIL076w (a plasmid containing YIL076w) were replica-plated onto SD plates, and these plates were incubated at the indicated temperatures for 2 days and subsequently photographed. (B) Wild-type (TKO1), *anu2-1* (TKE77) and Δ*anu2* (YNAR1) cells were replica-plated onto YPD plates, and these plates were incubated at the indicated temperatures for 2 days and subsequently photographed.

YIL076w had not been well characterized, and therefore we examined the functional properties of this gene.

To confirm that YIL076w is *ANU2*, and not a suppressor of the *anu2-1* mutation, integrative genetic mapping was done. The *URA3* gene was integrated adjacent to the YIL076w locus in a wild-type strain for *ANU2* via homologous recombination as described in the Materials and Methods. This strain (YKIM1) was then mated with an *anu2-1* strain, and sporulation and tetrad dissection was performed (60 tetrads). Tetrads were analyzed and the meiotic progeny exhibited a 2:2 segregation pattern of thermosensitive: nonthermosensitive and Ura^-:Ura^+ indicating that ANU2 is identical or closely linked to YIL076W.

Disruption of the ANU2 gene

One copy of the *ANU2* gene was disrupted in a diploid strain, DFY24, by replacing the entire ORF region with the *LEU2* gene. The resultant strain was sporulated, and tetrads were dissected. Most of the tetrads (90%) yielded four viable spores that displayed 2 Leu^+ and 2 Leu^- segregation, indicating that *ANU2* gene is not essential. A haploid strain (YNAR1) carrying the *ANU2* disruption (Δ*anu2*) was obtained and used for characterization below. As shown in Figure 2B, the Δ*anu2* cells did not grow at 37°C, reminiscent of *anu2-1* cells. Like *anu2-1* cells, Δ*anu2* cells exhibited abnormal nuclear morphology when cultured at 37°C, as revealed by the fluorescence of GFP-tagged nucleoplasmin (data not shown). This observation suggested that *anu2-1* mutation resulted in loss of function.

Electron microscopic analysis of anu2-1 cells

To demonstrate the detailed subcellular organization of *anu2-1* cells, we analyzed ultrathin sections of the cells obtained by the freeze-substituted fixation method. This method minimizes artifactual modifications that can occur during fixation by conventional methods. When wild-type cells were incubated at 23°C or 37°C, nuclei appeared round and the ER membrane formed cisterna that protruded from the nuclear outer membrane (Fig. 3A) or single layer localized at the inner surface of the plasma membrane. On the other hand, as indicated in Figure 3B, *anu2-1* cells incubated at the restrictive temperature of 37°C for 2 h accumulated membranes, which appeared to be extension of the ER because of their continuity with the outer nuclear membrane. Furthermore, as shown in Figure 3D, a large space between the inner and outer nuclear membranes was created, probably owing to separation of outer and inner nuclear membranes. A 4 h incubation at 37°C led to even more obvious aberrant nuclear morphology. The cells shown in Figures 3C, E and F seem to contain two nuclei surrounded by one outer nuclear membrane (or it may be just a section of one bent nucleus). This phenotype may reflect the fragmented nuclei observed by fluorescent microscopy of GFP-tagged nucleoplasmin in *anu2-1* cells (Fig. 1). Accumulation of the ER membrane is often seen in mutants of genes involved in vesicle transport between the ER and the Golgi, suggesting the involvement of *ANU2* in the vesicle transport, similar to other *ANU* genes.

Anterograde protein transport in anu mutant cells

As described above, *ANU1, 3* and *4* encode important factors in the secretory pathway. One possible explanation of the accumulation of the ER in *anu2-1* cells is that the transport from the ER is blocked. Hence, we

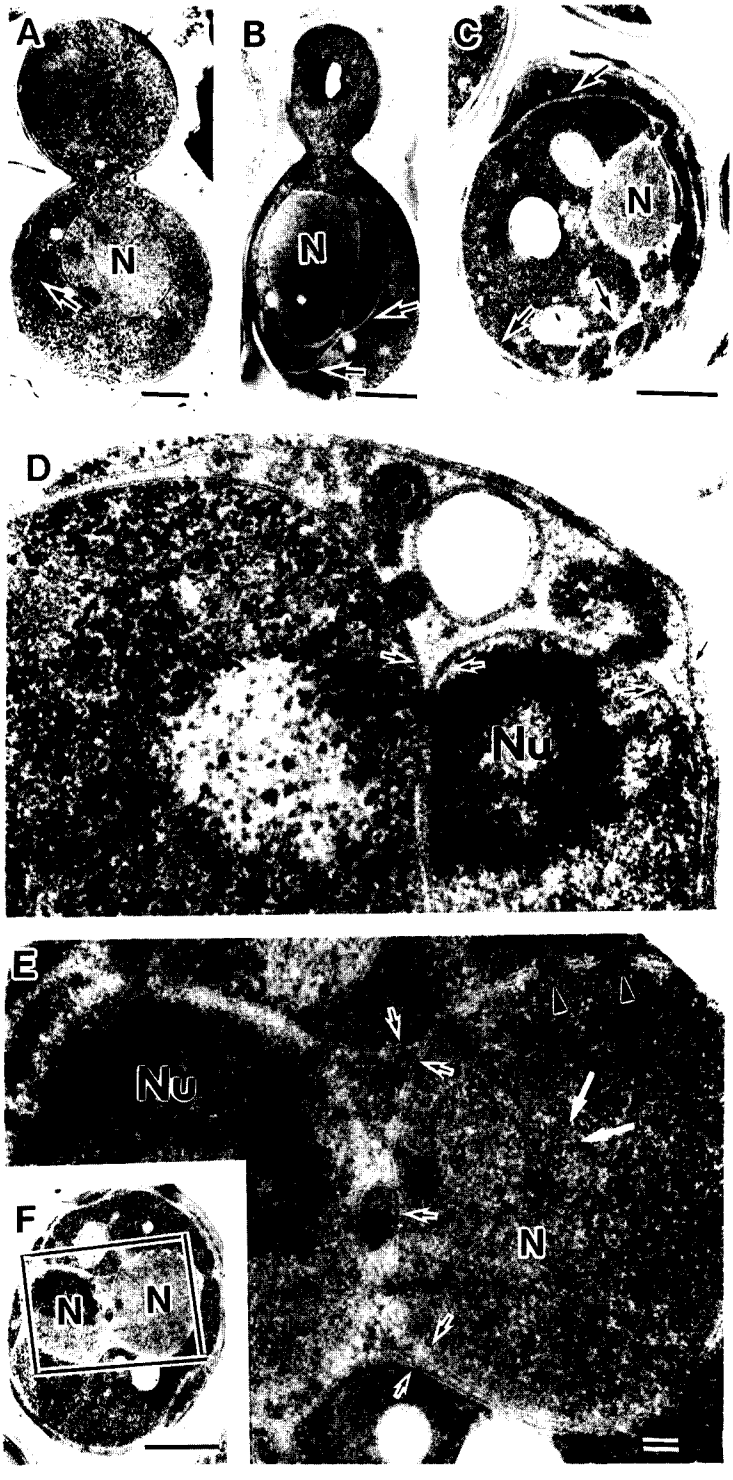

Fig. 3. Electron micrographs of wild-type and *anu2-1* cells. Wild-type (TKO1; A) and *anu2-1* (TKE77; B–F) cells were grown at 23°C to early logarithmic phase in YPD and then shifted to 37°C for 2 h (A, B and D) or 4 h (C, E and F). Samples were subjected to ultrathin sectioning and subsequent electron microscopic analysis. Nuclei (N) and Nucleoli (Nu) are indicated. Arrows in A–C indicate ER. As indicated by small arrows (D and E), separation of outer and inner nuclear membranes is found in *anu2-1* cells. In E, white arrows and arrowheads indicate intranuclear microtubules (cross-sectioned) and nuclear pores, respectively. A region of the cell in F is enlarged in E. Bars: (A–C, and F) 1 μm, (E) 0.1 μm; the same magnification in D and E.

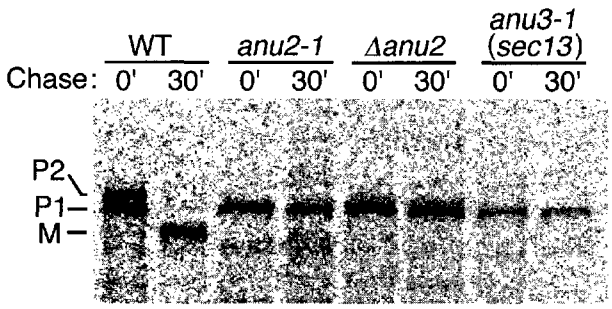

Fig. 4. CPY transport assays in *anu* mutant cells. Wild-type (WT; TKO1), *anu2-1* (TKE77), Δ*anu2* (YNAR1) and *anu3-1* (*sec13*; TKD76) cells were precultured at 37°C for 2 h and pulse labeled at 37°C with [^{35}S]methionine for 10 min. Next, excess amounts of cold methionine and cysteine were added, and cells were further incubated at 37°C for 0 or 30 min (chase). After the pulse-chase, cell lysates were prepared and subjected to immunoprecipitation with anti-CPY antibody. The immunoprecipitates were resolved by SDS-PAGE (8%). The positions of ER-modified (P1), Golgi-modified (P2) and mature (M) forms of CPY are indicated.

checked the involvement of Anu2p in protein transport in the secretory pathway by carrying out CPY transport assay. CPY is known to traverse the secretory pathway, progressing from a core-glycosylated ER form (P1) to an outer chain-glycosylated *medial*-Golgi form (P2) and eventually to a proteolytically processed mature vacuolar form (M) (47). To examine the transport of newly synthesized CPY in wild-type, *anu2-1*, Δ*anu2* and *anu3-1* (*sec13*) cells, pulse-chase experiments were performed at 37°C (Fig. 4). In the wild-type cells, the P1- and P2- forms of CPY were rapidly processed to the M-form, indicating normal vesicular transport. In contrast, the P1-form accumulated in *anu2-1* and Δ*anu2* as well as in *anu3-1* (*sec13*) cells. This result strongly suggests that ER-to-Golgi vesicular transport is severely blocked in cells carrying a mutation or disruption of the *ANU2* gene.

Anu2p is a 34 kD polypeptide with homology to mammalian ε-COP

As described above, the *ANU2* gene contains a single ORF designated YIL076w. If this ORF is fully translated, the product would be a 41 kDa polypeptide consisting of 359 amino acids. We designated this hypothetical polypeptide Long P (Fig. 5). The N-terminal region (amino acid residues 1–34) of Long P contains a cluster of hydrophobic amino acid residues that renders the polypeptide insoluble. Hence, a truncated version of Long P (amino acids 35–359) was bacterially expressed, and this soluble product was injected into rabbits for production of antisera against Anu2p.

Figure 6 shows Western blot analysis of cell lysates from wild-type, *anu2-1* and Δ*anu2* cells using this anti-

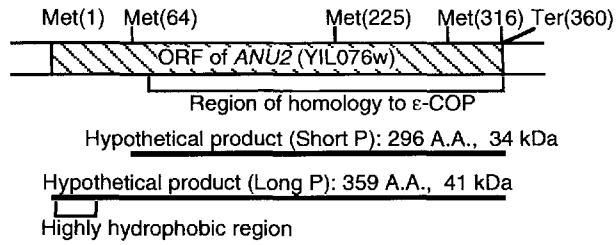

Fig. 5. Schematic representation of the *ANU2* gene and its hypothetical products. All methionine codons (Met) and the termination codon (Ter) in ORF YIL076w are indicated. Short P and Long P are hypothetical *ANU2* products whose initiation codons are Met(64) and Met(1), respectively.

Anu2p antiserum. The antiserum detected a polypeptide with an apparent molecular mass of 35 kDa in wild-type cells. We believe that this polypeptide is Anu2p, as it was not detected from Δ*anu2* cells. Similarly, Anu2p was not detected from *anu2-1* cells, presumably because the *anu2-1* mutation is a nonsense mutation or the resulting protein is very unstable. As shown in Figure 6, Anu2p was detected as a protein with an apparent molecular mass of 35 kDa, a size that is smaller than the deduced size of Long P, 41 kDa. One possible explanation for this is that Long P is proteolytically processed. Indeed, by PSORT program ([http://psort.nibb.ac.jp/]; 30), the N-terminal hydrophobic region of Long P is predicted to act as a cleavable signal sequence for protein translocation through the ER membrane, and the resulting product is calculated to be 37 kDa. However, we considered this explanation unlikely because staining cells overexpressing *ANU2* with anti-Anu2p antiserum showed that Anu2p is localized in the cytoplasm, not in the lumen (data not shown).

Another possible explanation for the smaller size of polypeptide is that instead of the first Met, Met(1) of YIL076W, the actual initiation codon is the next one, Met(64). If this is true, the *ANU2* product is predicted to be a 34 kDa cytoplasmic polypeptide by the PSORT program. We therefore designated this second hypothetical *ANU2* product as Short P (Fig. 5). To demonstrate that the actual *ANU2* product is not Long P but Short P, each polypeptide was expressed from the *GAL1* promoter in Δ*anu2* cells and further characterized (Y_{GAL1p}-Short: Δ*anu2* strain containing a fusion of the *GAL1* promoter and the Short P gene; Y_{GAL1p}-Long: Δ*anu2* strain containing a fusion of the *GAL1* promoter and the Long P gene). The transcription from the *GAL1* promoter is repressed in the presence of glucose, but strongly induced in the presence of solely galactose.

Yeast cells were cultured in the presence of either glucose or galactose, and their extracts were subjected to

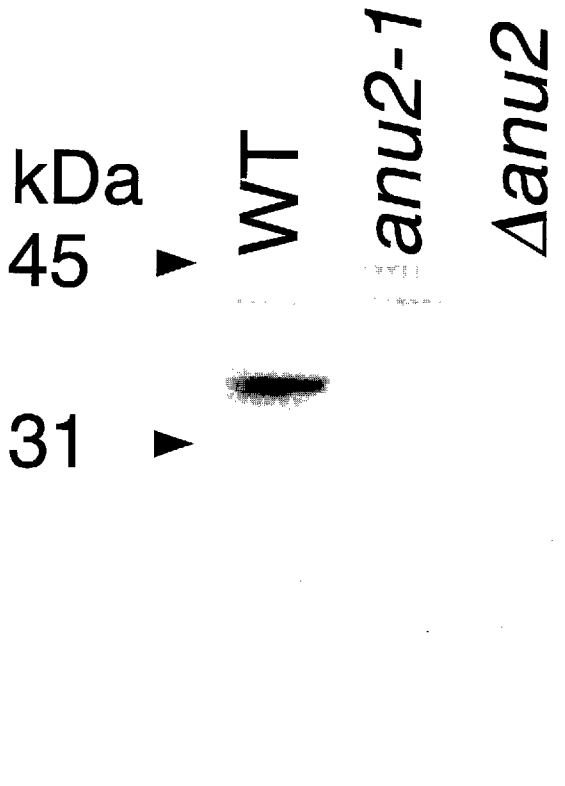

Fig. 6. Detection of Anu2p by Western blot analysis. Lysates of wild-type (WT; TKO1), *anu2-1* (TKE77) and Δ*anu2* (YNAR1) cells were resolved by SDS-PAGE (12.5%) and Western blotted with anti-Anu2p antiserum.

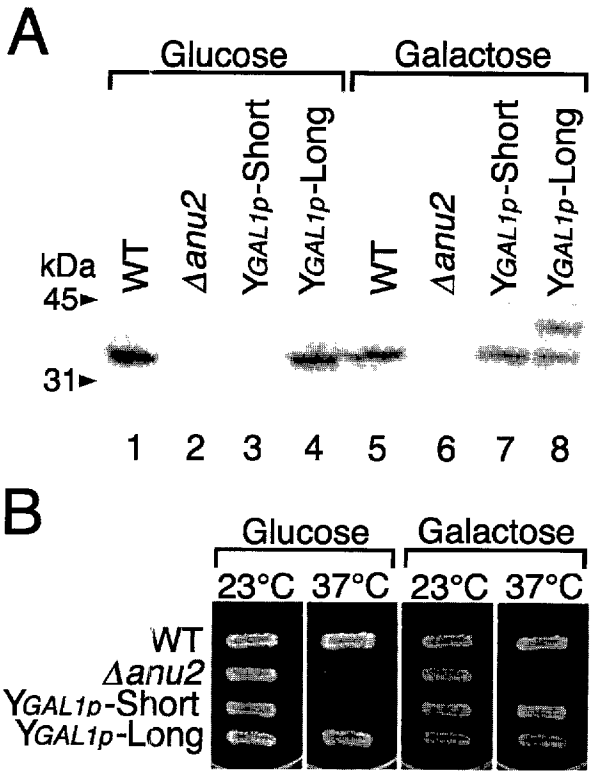

Fig. 7. Expression of hypothetical *ANU2* products in Δ*anu2* cells. Strains Y_{GAL1p}-Short (containing a fusion of the *GAL1* promoter and the Short P gene) and Y_{GAL1p}-Long (containing a fusion of the *GAL1* promoter and the Long P gene) were generated from the Δ*anu2* strain (YNAR1) as described in Materials and Methods and further characterized alongside with wild-type (WT; TKO1) and Δ*anu2* (YNAR1) strains. (A) Those cells were cultured at 23°C in media containing glucose (YPD) or galactose (YPG), and their lysates were resolved by SDS-PAGE (12.5%) and Western blotted with anti-Anu2p antiserum. (B) Cells were replica-plated onto YPD or YPG plates, and these plates were incubated at the indicated temperatures for 2 days and subsequently photographed.

Western blot analysis with anti-Anu2p antiserum (Fig. 7A). In Y_{GAL1p}-Short cells, a 35 kDa polypeptide was produced from the *GAL1* promoter (Fig. 7A, lanes 3 and 7). On the other hand, a 40 kDa polypeptide was produced from the *GAL1* promoter in Y_{GAL1p}-Long cells (Fig. 7A, lanes 4 and 8). A comparison of the molecular mass of these polypeptides with the authentic *ANU2* product (Fig. 7A, lanes 1 and 5) strongly suggests that the actual *ANU2* product is Short P. In Y_{GAL1p}-Long cells, the 35 kDa polypeptide, which is probably Short P, was produced in both glucose and galactose media. We presumed that this is due to the presence of *ANU2* promoter elements located between the Long P and Short P translational start sites that express the Short P product constitutively.

In parallel with the above experiment, we examined whether expression of Short P and/or Long P rescues the temperature-sensitivity of Δ*anu2* cells. As shown in Figure 7B, Y_{GAL1p}-Short and Y_{GAL1p}-Long strains were inoculated onto agar plates containing glucose (YPD) or galactose (YPG) and cultured at 23°C or 37°C. When cultured at 37°C on YPD, Y_{GAL1p}-Short cells, which express neither Short P nor Long P, did not grow. On the other hand, cells expressing Short P only (Y_{GAL1p}-Short on YPG and Y_{GAL1p}-Long on YPD) could grow at 37°C, suggesting that Short P is functional. It is uncertain whether Long P is functional, as we could not obtain cells expressing exclusively Long P, due to the simultaneous constitutive expression of Short P.

By searching the Genome Net databases ([http://www.genome.ad.jp/]) we found out that Short P is homologous to mammalian ε-COP (20% identity). The sequence alignment of Short-P and hamster ε-COP (15) is shown in Figure 8.

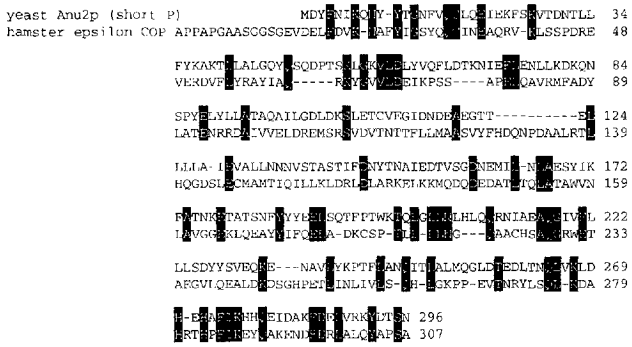

Fig. 8. Sequence alignment of Short-P and hamster ε-COP. Gene Works program (Oxford Molecular Group, Campbell, CA, USA) was used for the alignment. Matched amino acid residues were shaded.

Golgi-to-ER retrograde transport in Δanu2 cells

A double-lysine motif (KKXX) at the C-terminus of some type I transmembrane proteins is required for proper localization within the ER (12, 49). In yeast cells the ER retention of KKXX-tagged proteins is achieved by a KKXX signal-mediated Golgi-to-ER retrieval system. Yeast strains harboring mutations in genes encoding α-, β′-, γ-, δ- and ζ-COP were unable to localize KKXX-tagged proteins within the ER (1, 7, 25, 53). We therefore examined the ER retention of a KKXX-tagged membrane protein in *Δanu2* cells. Wbp1p is a type I ER resident membrane protein which contains the KKXX motif in its C-terminus. A chimeric protein, inv-KK, in which a region of Wbp1p including the transmembrane domain and cytoplasmic tail was fused to the C-terminus of invertase, was used (53). As a control, we used a mutated version, inv-SS, in which the lysine residues of inv-KK (-KKTN-COOH) were changed to serines (-SSTN-COOH). In the backround of known COPI mutants, a large portion of inv-KK fails to be retained within the ER, and like inv-SS, it is transported to the vacuole, where it is proteolytically processed to an approximately 56 kDa fragment (53). Therefore the degree of processing inv-KK is a good indicator of retrieving efficiency from the Golgi to the ER.

Wild-type and *Δanu2* cells expressing these chimeric proteins or containing a control vector plasmid (pRS426) were precultured at the semipermissive temperature of 32°C for 4 h and subjected to a 10 min pulse label with [^{35}S]methionine at 32°C, followed by a 60 min chase, and immunoprecipitation with an anti-invertase antibody. The immunoprecipitates were treated with endoglycosidase H and then subjected to SDS-PAGE (Fig. 9). Inv-SS was cleaved in both wild-type and *Δanu2* cells, confirming that anterograde transport was not blocked under this culturing condition. On the other hand, no cleavage of inv-KK was observed, indicating that this protein was efficiently retrieved from the Golgi and transported back to the ER in both wild-type and *Δanu2* cells. We could not determine the deficiency in ER-retrieval in *Δanu2* cells cultured at temperatures higher than 32°C, because neither inv-SS nor inv-KK was cleaved under these culturing conditions (data not shown).

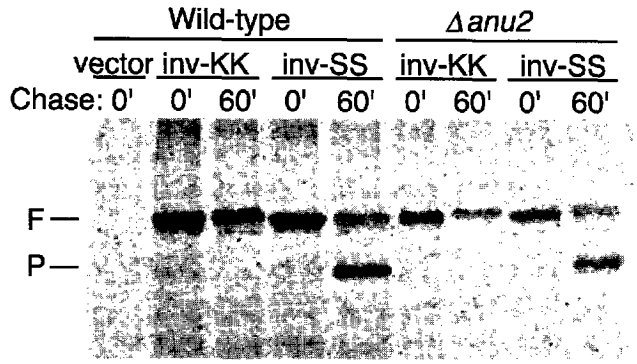

Fig. 9. Invertase-Wbp1p transport assay in *Δanu2* cells. Wild-type (TKO1) and *Δanu2* (YNAR1) cells expressing inv-KK or inv-SS were precultured at 32°C for 4 h and pulse labeled at 32°C with [^{35}S]methionine for 10 min. Next, excess amounts of cold methionine and cysteine were added, and cells were further incubated at 32°C for 0 or 60 min (chase). After the pulse-chase, cell lysates were prepared and subjected to immunoprecipitation with anti-invertase antibody. The immunoprecipitates were treated with endoglycosidase H and resolved by SDS-PAGE (8%). The positions of ER-form (F) and proteolytically processed form (P) of inv-KK or inv-SS are indicated.

Discussion

Nuclear morphology of yeast cells are visualized in detailed outline by expression of GFP-tagged nucleoplasmin. In this study, a collection of temperature-sensitive mutants expressing GFP-tagged nucleoplasmin was screened for abnormal nuclear morphology. Four candidates were obtained and we called then *anu* for abnormal nuclear morphology mutants. Unexpectedly, the products of *ANU1, ANU3* and *ANU4* were found to be involved in the formation or fusion of intracellular membrane vesicles. *ANU1* and *ANU3* were found to be identical to *SEC24* and *SEC13*, respectively, both of which encode components of COPII (19, 44). *ANU4* is identical to *SEC18*, which encodes the yeast NSF, a factor essential for various vesicle fusion events (14, 16, 51).

In this study, the function of *ANU2* gene was investigated. It was isolated by screening of a yeast genomic library for complementation of the temperature-sensitivity of the *anu2-1* strain and further genetic analysis confirmed that it corresponds to YIL076W, as designated in the Saccharomyces Genome Database. Fur-

thermore, we found out that the actual initiation codon is not Met(1) but Met(64) of YIL076w (Fig. 7). Thus the *ANU2* product is deduced to be a 34 kDa soluble polypeptide (Fig. 5). Several lines of evidence support that *ANU2* is indeed the *ε-COP* gene. First, in the electron microscopic analysis, *anu2-1* cells exhibited accumulation of ER membrane, as did various *sec* mutants defective in the vesicular transport between the ER and the Golgi (Fig. 3). Second, the result from the CPY transport assay indicated that the early secretory pathway of *anu2* mutant cells was blocked (Fig. 4). Third, there is no other gene which shares a homology as significant as *ANU2* with the mammalian ε-COP gene in the Saccharomyces Genome Database (Fig. 8). Therefore, we came to the same conclusion as R. Duden's group (see discussion below) that *ANU2* is indeed the yeast *ε-COP* gene.

Disruption of the *ANU2* gene confers temperature-sensitivity growth but is not lethal (Fig. 2B). This is in contrast to all of the other coatomer genes (α-, β-, β′-, γ-, δ- and ζ-COP), which are essential for cell viability (1, 7, 25, 26, 53). Furthermore, alignment of sequences using the Gene Works program (Oxford Molecular Group, Campbell, CA, USA) showed that the sequence identity between mammalian and yeast ε-COP is 20%, whereas other yeast coatomer subunits exhibit a 35–45% identity with their mammalian counterparts. These observations may imply that ε-COP plays a less well-conserved or a supplementary role in coatomer function.

Two sets of experiments supported a role of coatomer in Golgi-to-ER retrograde transport. First, several coatomer subunits were shown to physically interact *in vitro* with the double-lysine (KKXX) ER-retrieval motifs (15). Second, yeast cells with mutations in various coatomer subunits are defective for the retrieval of certain classes of proteins from the Golgi to the ER (1, 7, 25, 26, 53). Hence, we tested whether the retrieval of a KKXX-tagged membrane protein, invertase-Wbp1p, to the ER is affected by the *anu2* mutation. In the invertase-Wbp1p transport assay shown (Fig. 9), we could not detect the escape of this protein from the ER-retrieval system in Δ*anu2* cells cultured at the semipermissive temperature 32°C. As ε-COP is a component of coatomer, we believe that it is involved in the ER-retrieval pathway, and its inactivation in Δ*anu2* cells may cause a block in this pathway when cells are cultured under a more stringent experimental condition, such as incubation at restrictive rather than semipermissive temperatures. However, the defect in the anterograde secretory pathway observed in Δ*anu2* cells cultured at temperatures higher than 32°C (Fig. 4) precludes estimation of defect in the ER-retrieval of invertase-WBP1. Therefore, a new transport assay has to be developed in order to estimate the defect in Golgi-to-ER retrograde transport in Δ*anu2* cells.

In contrast to Golgi-to-ER retrograde transport, where formation of transport vesicles is promoted by COPI, it is widely believed that in ER-to-Golgi anterograde transport this function is managed by COPII. However, ER-to-Golgi anterograde transport (e.g. of CPY) is blocked in *anu2* mutants as well as in mutants of other coatomer genes (8, 20, 53), although COPI is not likely to act directly in the anterograde secretory pathway (12). To determine whether the ER membrane can form COPI-coated vesicles *in vivo*, the method of quantitative immunoelectron microscopic studies used to investigate Golgi-derived COPI-coated vesicles might be helpful (39).

While conducting this study, R. Duden's group independently identified YIL076w as yeast ε-COP gene, and named it *SEC28* (9). They cloned this gene as a high copy suppressor of temperature-sensitive growth defect of an α-COP mutant (*ret1-3*), which produced an unstable α-COP at the restrictive temperature 37°C. They also showed that wild-type α-COP was unstable in the disruptant mutant (*sec28*) at a restrictive temperature. Therefore, they concluded that one of the functions of ε-COP is to stabilize α-COP and hence the overall coatomer complex (9).

Since we isolated *anu2* mutant via abnormal nuclear morphology phenotype at the restrictive temperature, we could study the fine subcellular structure of *anu2* mutant by electron microscopy. The freeze-substituted fixation method was preferred as it causes less distortion to the samples, especially to the roundness of the nucleus as compared to the standard fixation method using osmium tetroxide. The abnormal nuclear morphology of *anu2-1* revealed by this electron microscopic analysis, was also observed in various mutant strains bearing defects in vesicular transport between the ER and the Golgi, such as *sec24/anu1, sar1, sec12, sec16, sec18/anu4* and *uso1* (31; unpublished observations by A. Hirata). In addition, accumulation of misfolded proteins in the ER, such as a prosequence-deleted derivative of an aspartic proteinase, also resulted in irregularly proliferated ER membranes and aberrant nuclei (50). In contrast, such a phenotype was not observed in *sec1, sec7* and *sec14* strains which are defective in the later stage of the secretory pathway (traffic through the Golgi and subsequently to the cell surface) (36–38; unpublished observations of A. Hirata). This difference could be explained as follows. In the eukaryotic cells, the outer nuclear membrane forms an extension to the ER membranes and the inter-nuclear membrane space is equivalent to the ER lumen. Accumulation of membrane proteins and soluble secretory proteins in the ER due to defects in the early secretory pathway triggers propagation of ER membranes and a change in location of ER components such as BiP (35).

These abnormally proliferated and exaggerated ER membranes could perturb the nuclear structure by a yet unknown mechanism. On the other hand, the blockage in the later stage of the secretory pathway does not lead to the accumulation of ER membranes, hence leaving the nuclear structure virtually unaffected. Therefore, as we have shown here, at least, in yeast cells, the content and the physical state of the ER are closely linked to the nuclear structure. In fact, some factors required for the early secretory pathway may be directly participating in the construction of nuclei. There is evidence showing that Sec13p existed not only in COPII but also in a subcomplex of the NPC (46). This subcomplex is composed of six different proteins including Nup84p, Nup85p, Nup120p, Sec13p and a Sec13p homologue (Seh1p), where disruption of any of the genes encoding Nup84p, Nup85p or Nup120p causes defects in the organization of the nuclear membranes and the NPC. Further studies are necessary to address the exact role of vesicular transport factors in the construction or maintenance of nuclear structures.

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