

# Ski7p G protein interacts with the exosome and the Ski complex for 3'-to-5' mRNA decay in yeast

Yasuhiro Araki, Shinya Takahashi,  
Tetsuo Kobayashi, Hiroaki Kajiho,  
Shin-ichi Hoshino<sup>1</sup> and Toshiaki Katada<sup>1</sup>

Department of Physiological Chemistry, Graduate School of  
Pharmaceutical Sciences, University of Tokyo, Tokyo 113-0033, Japan

<sup>1</sup>Corresponding authors

e-mail: hoshino@mol.f.u-tokyp.ac.jp or katada@mol.f.u-tokyp.ac.jp

**Two cytoplasmic mRNA-decay pathways have been characterized in yeast, and both are initiated by shortening of the 3'-poly(A) tail. In the major 5'-to-3' decay pathway, the deadenylation triggers removal of the 5'-cap, exposing the transcript body for 5'-to-3' degradation. An alternative 3'-to-5' decay pathway also follows the deadenylation and requires two multi-complexes: the exosome containing various 3'-exonucleases and the Ski complex consisting of the RNA helicase Ski2p, Ski3p and Ski8p. In addition, Ski7p, which has an N-terminal domain and a C-terminal elongation factor 1 $\alpha$ -like GTP-binding domain, is involved in the 3'-to-5' decay. However, physical interaction between the exosome and the Ski complex, together with the function of Ski7p, has remained unknown. Here we report that the N domain of Ski7p is required and sufficient for the 3'-to-5' decay. Furthermore, the exosome and the Ski complex interact with the different regions of Ski7p N domain, and both interactions are required for the 3'-to-5' decay. Thus, Ski7p G protein appears to function as a signal-coupling factor between the two multi-complexes operating in the 3'-to-5' mRNA-decay pathway.**

**Keywords:** exosome/G protein/mRNA decay/SKI genes/yeast

## Introduction

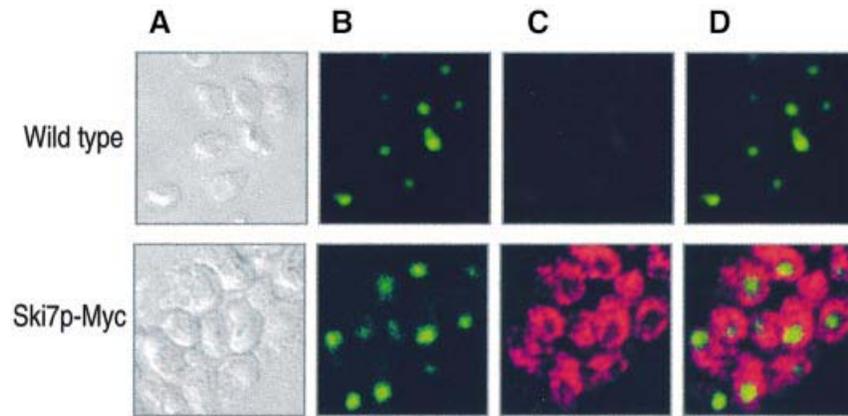
In addition to transcriptional regulation, mRNA decay plays an important role in the quality and quantity control of gene expression. mRNA degradation is carried out in the nucleus and the cytoplasm of budding yeast *Saccharomyces cerevisiae* (Bousquet-Antonelli *et al.*, 2000). Two cytoplasmic mRNA-decay pathways have been characterized in yeast (for reviews see Caponigro and Parker, 1996; Mitchell and Tollervey, 2000a). In both, the initial step is shortening of the 3'-poly(A) tail of mRNAs to A<sub>10</sub> or less. This step appears to involve the removal of a poly(A)-binding protein (PABP) from the poly(A) tail, to be degraded by a deadenylating enzyme (Coller *et al.*, 1998). In the major pathway, the poly(A) shortening triggers the removal of the 5'-cap structure by a decapping enzyme, Dcp1p, exposing the transcript body to an exonuclease, Xrn1p, for the rapid 5'-to-3' digestion (Muhlrad and Parker, 1992; Decker and Parker, 1993; Hsu and Stevens,

1993; Muhlrad *et al.*, 1994, 1995; Beelman *et al.*, 1996). An alternative 3'-to-5' degradation pathway also follows the deadenylation and requires at least two multi-complex machineries: the exosome and a ternary complex consisting of Ski2p, Ski3p and Ski8p (we refer to the complex as the Ski complex) (Muhlrad *et al.*, 1995; Jacobs Anderson and Parker, 1998; Brown *et al.*, 2000).

The yeast exosome is a protein complex comprising at least 10 essential components. Among them, Rrp4p, Ski6p (Rrp41p) and Rrp44p (Dis3p) have been demonstrated to have 3'-to-5' exoribonuclease activity *in vitro*, and the other members (Rrp40p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p) except Csl4p (Ski4p) have high sequence homology to *Escherichia coli* 3'-to-5' exoribonucleases, RNase PH and PNPase (for reviews see van Hoof and Parker, 1999; Mitchell and Tollervey, 2000b). The yeast exosome and its human counterpart, the PM-Scl complex, are present in both the cytoplasm and the nucleus (Mitchell *et al.*, 1997; Allmang *et al.*, 1999a). The cytoplasmic exosome degrades mRNAs, whereas the nuclear exosome processes small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs) and rRNAs, in addition to the degradation of pre-mRNA and pre-rRNA spacer (Mitchell *et al.*, 1997; Jacobs Anderson and Parker, 1998; Allmang *et al.*, 1999a, 2000; van Hoof *et al.*, 2000a). The nuclear exosome has an additional subunit, Rrp6p, which also possesses 3'-to-5' exoribonuclease activity (Briggs *et al.*, 1998; Allmang *et al.*, 1999b; Burkard and Butler, 2000). Consistent with its nuclear localization, *RRP6* mutant strains do not exhibit defects in cytoplasmic mRNA turnover (van Hoof *et al.*, 2000a).

On the other hand, the superkiller (*SKI*) genes encoded in yeast nuclear genome were initially identified from mutations that cause overexpression of a killer toxin encoded by the endogenous double-stranded RNA (Toh-e *et al.*, 1978). Subsequent studies demonstrated that, in addition to the exosome, the products of *SKI2*, *SKI3* and *SKI8* genes are required for the 3'-mRNA degradation (Jacobs Anderson and Parker, 1998). Ski2p, Ski3p and Ski8p are a putative RNA helicase, a tetratricopeptide-repeat protein and a protein containing WD motif, respectively (Rhee *et al.*, 1989; Matsumoto *et al.*, 1993; Widner and Wickner, 1993). The three Ski proteins form a stable complex, and the complex is localized in the cytoplasm (Brown *et al.*, 2000). Mutations in the three genes inhibit 3'-to-5' mRNA decay, but do not affect the other function of the exosome (Jacobs Anderson and Parker, 1998; van Hoof *et al.*, 2000a). Therefore, the Ski complex appears to be a cofactor for the exosome to degrade yeast mRNAs. However, direct interaction between the exosome and the Ski complex has not been observed (Brown *et al.*, 2000).

More recently, another member of the Ski proteins, Ski7p, has been reported to be responsible for the 3'-to-5'



**Fig. 1.** Intracellular localization of Ski7p in yeast. A yeast strain expressing Myc-tagged SKI7 (MC028, top panels) and the wild type (W303a, bottom panels) were analyzed by confocal microscopy as described in Materials and methods. (A) Nomarski images of the cells. (B) DNA of the cells was stained with PicoGreen. (C) Ski7p-Myc was stained with anti-Myc (primary) and anti-mouse IgG coupled to Alexa-568 (secondary) antibodies. (D) Overlay of the two signals shown in (B) and (C).

mRNA decay (van Hoof *et al.*, 2000b). The *SKI7* gene was initially identified as one of the *SKI* gene family (Benard *et al.*, 1999). van Hoof *et al.* (2000b) reported that Ski7p is also required for 3'-to-5' mRNA degradation and acts in the same pathway as Ski2p, Ski3p and Ski8p, because deletion of the *SKI7* gene caused impaired 3'-mRNA decay similar to *SKI2*, *SKI3* or *SKI8* deletion. However, formation of the Ski complex, which had been impaired in *ski2*, *ski3* or *ski8* mutants, was still intact in the *ski7* mutant, suggesting that Ski7p is not a member of the Ski complex and is not required for the formation of the Ski complex (Brown *et al.*, 2000). Thus, the roles of Ski7p and the Ski complex members appear to be different in 3'-mRNA degradation.

Ski7p is a GTP-binding protein consisting of two separate domains; one is the C-terminal region homologous to the GTP-binding elongation factor 1 $\alpha$  (EF1 $\alpha$ ), and the other is an extra N-terminal domain that was not present in EF1 $\alpha$ . This domain structure is quite similar to that of the GTP-binding eukaryotic releasing factor 3 (eRF3). We previously indicated that eRF3 functions not only in translation termination, but also as an initiator of poly(A) shortening by interacting with PABP (Hoshino *et al.*, 1998, 1999b). The N domain of eRF3 associated with PABP to unmask the poly(A) tail of mRNAs, presumably for their degradation (Hoshino *et al.*, 1999b).

In order to understand the molecular details of the 3'-mRNA decay pathway, we investigated the role of Ski7p and its interacting molecules. Here we report that the N domain of Ski7p is required and sufficient, and its EF1 $\alpha$ -like C domain is dispensable, for the 3'-to-5' mRNA degradation. Furthermore, the Ski7p N domain was capable of interacting with the exosome and the Ski complex physically and functionally. Thus, the N domains of this G protein family appear to function in common as regulatory factors in mRNA-decay pathways.

## Results

### ***Ski7p is involved in the cytoplasmic 3'-to-5' mRNA-decay pathway***

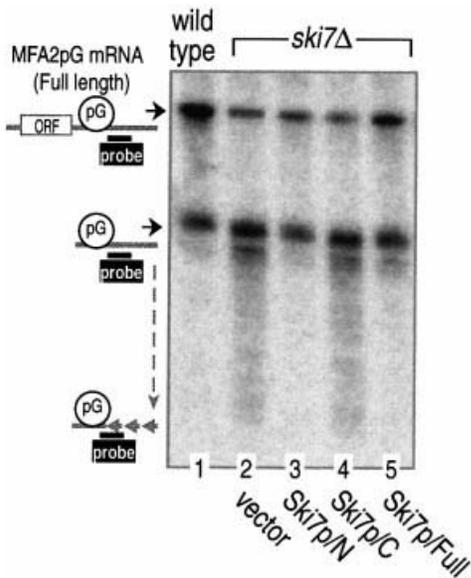
A recent study has revealed that Ski7p is required for 3'-to-5' degradation of yeast mRNAs (van Hoof *et al.*, 2000b).

We first confirmed the previous findings using an MFA2pG mRNA that contained a poly(G) tract inserted into its 3'-untranslated region. Since the poly(G) tract forms a stable structure that inhibits 5'-exoribonuclease activity (Decker and Parker, 1993), 5'-to-3' degradation of the mRNA produces an intermediate that stretches from the poly(G) tract to the 3' end of the mRNA. The resultant intermediate is normally degraded by the 3'-to-5' mRNA-degradation pathway (Jacobs Anderson and Parker, 1998). Thus, mutants having a defect in 3'-to-5' decay show a slow digestion of the 3'-end fragment, resulting in the accumulation of its fragments upon northern blot analysis. In accordance with the previous report (van Hoof *et al.*, 2000b), deletion of the *SKI7* gene (*ski7* $\Delta$ ) caused accumulation of the 3'-end fragments of MFA2pG mRNA (data not shown, but see Figure 2).

If Ski7p is directly involved in the cytoplasmic 3'-mRNA-decay pathway, at least a portion of this protein should be present in the cytoplasm. The chromosomal copy of *SKI7* was tagged with nine Myc epitopes (Ski7p-Myc), and the Myc-tagged protein was produced under native transcriptional conditions. Figure 1 shows the cellular localization of Ski7p-Myc observed by indirect immunofluorescence, together with nuclear DNA stained with PicoGreen. Ski7p-Myc localized primarily in the cytoplasm and did not overlap with the nuclear signals. These observations suggested that Ski7p is directly involved in the 3'-to-5' mRNA-decay pathway in yeast cytoplasm.

### ***Ski7p N domain is required and sufficient for the 3'-to-5' mRNA degradation***

Ski7p consists of two separated domains, the extra N-terminal domain (amino acid sequence 1–264) and the C-terminal EF1 $\alpha$ -like domain (sequence 265–747). To identify which domain is responsible for the 3'-mRNA decay, each of the two domains was expressed in the *ski7* $\Delta$  strain. As shown in Figure 2, the N domain of Ski7p (Ski7p/N), together with the full-length form (Ski7p/Full), rescued the *ski7* $\Delta$  phenotype, which was characterized by the accumulation of 3'-end fragments of MFA2pG mRNA. However, such recovery was not observed with the EF1 $\alpha$ -like C domain (Ski7p/C). This indicated that Ski7p/N is

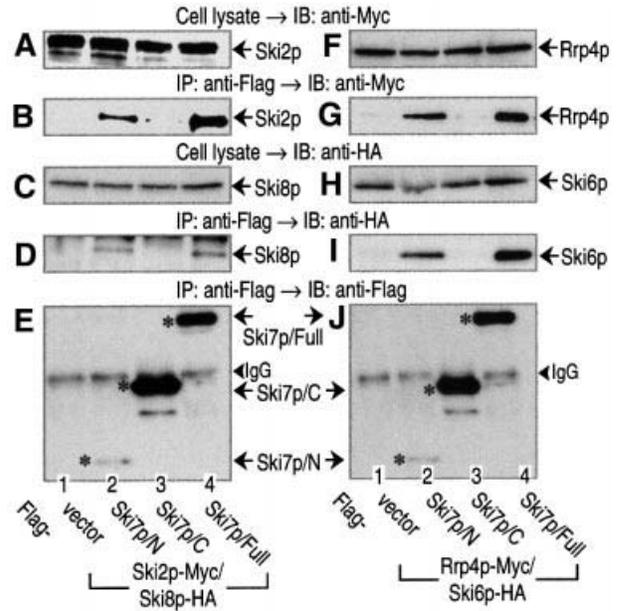


**Fig. 2.** The N domain of Ski7p is necessary and sufficient for the 3'-to-5' degradation of mRNA. Yeast strains MC046 (vector), MC047 (Ski7p/N), MC048 (Ski7p/C) and MC049 (Ski7p/Full), carrying the *GAL1:MFA2pG* reporter, were grown in a galactose-containing medium and harvested at the mid-log phase ( $OD_{600} = 0.4-0.5$ ). RNA extracted from the cells was subjected to polyacrylamide gel northern blot analysis as described in Materials and methods. Positions of the full-length and poly(G) (pG) to 3'-end fragments of MFA2pG mRNA are indicated on the left. ORF, open reading frame.

required and sufficient, and Ski7p/C is dispensable, for the 3'-mRNA decay.

### **Ski7p interacts physically with both the exosome and the Ski complex**

It has been reported that 3'-to-5' mRNA degradation in yeast requires at least two multi-complexes: the exosome and the Ski complex consisting of Ski2p, Ski3p and Ski8p (Jacobs Anderson and Parker, 1998). Therefore, we investigated whether Ski7p was capable of interacting with these components (Figure 3). The chromosomal copies of *SKI2* and *SKI8* were tagged with Myc and hemagglutinin (HA) epitopes, respectively, and vectors expressing Ski7p-deletion variants under the control of the *GAL1* promoter were introduced into the same yeast strain with a Flag epitope. Extracts were prepared from these strains and subjected to immunoprecipitation assay with an anti-Flag antibody. As shown in Figure 3B and D, both members of the Ski complex, Ski2p-Myc and Ski8p-HA, were co-precipitated with Ski7p/N and Ski7p/Full, but not with Ski7p/C. We also tagged the chromosomal copies of *RRP4* and *SKI6*, the components of the exosome, with Myc and HA epitopes. As shown in Figure 3G and I, not only Rrp4p-Myc, but also Ski6p-HA, could be detected in the fractions precipitated with Ski7p/N and Ski7p/Full. These experiments were performed with the constructs overexpressing Ski7p variants. However, we found that the expression constructs rescued the phenotype of the *SKI7*-disruption strain (see Figure 2). Therefore, these interactions could be detected with a normal level of Ski7p. When yeast cell extract was fractionated on a gel-filtration column, Ski7p behaved as a complex (>300 kDa



**Fig. 3.** The N domain of Ski7p interacts with the components of the Ski complex and the exosome. Cell extracts were obtained from the indicated yeast strains expressing Ski2p-Myc and Ski8p-HA (A-E) or Rrp4p-Myc and Ski6p-HA (F-J). The yeast strains also expressed the indicated forms of Flag-tagged Ski7p (A-E, lanes 1-4 for MC146-MC149; F-J, lanes 1-4 for MC151-MC154). The cell extracts were immunoprecipitated (IP) with an anti-Flag antibody. The precipitated proteins (B, D, E, G, I and J), together with the whole-cell extracts (A, C, F and H), were immunoblotted (IB) with anti-Myc (9E10), anti-HA (12CA5), and anti-Flag (M2) antibodies to detect Ski2p (or Rrp4p), Ski8p (or Ski6p) and Ski7p. The asterisks in (E) and (J) indicate Flag-tagged Ski7p mutants expressed in the various strains. The arrowheads indicate the positions of IgG bands.

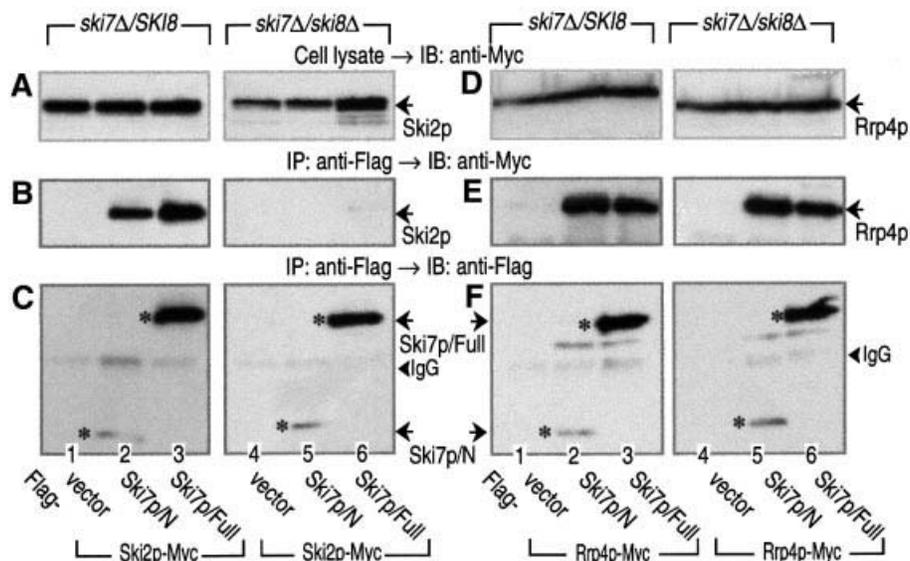
protein) larger than its predicted 85 kDa monomer, and it appeared to associate with the components of the Ski complex and the exosome (data not shown). Thus, the N domain of Ski7p is capable of interacting physically with the components of both the Ski complex and the exosome.

### **Ski7p interacts with the exosome independently of its interaction with the Ski complex**

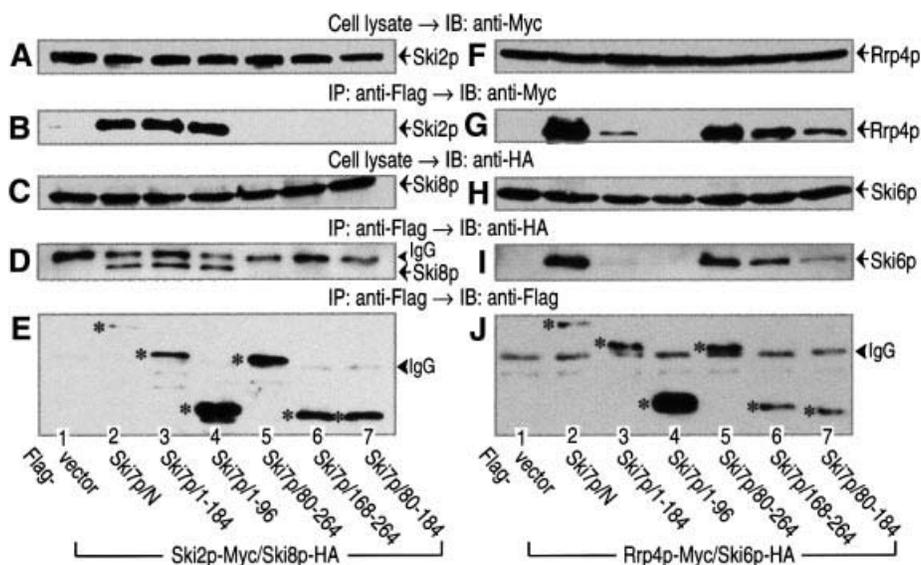
Disruption of *SKI8* has been shown to abolish the association between Ski2p and Ski3p (Brown *et al.*, 2000). Therefore, we tested whether the deletion of *SKI8* could exert its influence on the Ski7p-Ski2p and/or Ski7p-Rrp4p interaction. As shown in Figure 4B, the association of Ski7p/N (or Ski7p/Full) with Ski2p observed in Ski8p-expressing strains was completely abolished in *SKI8*-disruption strains (*ski8Δ*). However, the interaction between Ski7p/N (or Ski7p/Full) and Rrp4p was still present in the *ski8Δ* strains (Figure 4E). These results indicated that an intact form of the Ski complex is required for the association between Ski7p and the Ski complex, and that the interaction between Ski7p and the exosome is unaffected by the presence or absence of the Ski complex.

### **The Ski complex and the exosome interact with the different regions of the Ski7p N domain**

Since Ski7p appeared to interact with the Ski complex and the exosome independently through the N domain, we



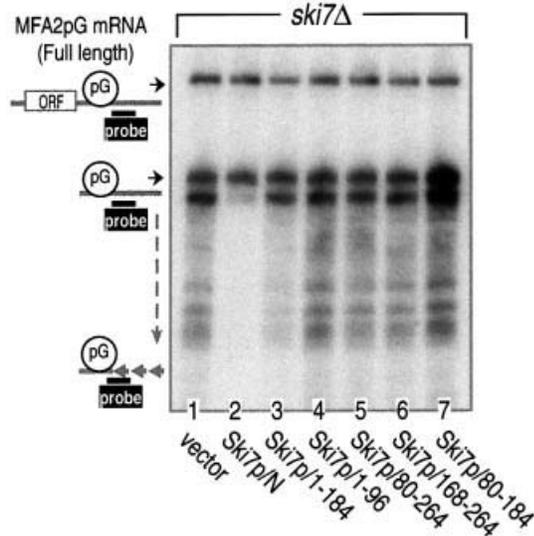
**Fig. 4.** Interaction of the N domain of Ski7p with Rrp4p is maintained in the Ski complex-disrupted yeast strains. Cell extracts were prepared from wild-type *SKI8* or *ski8Δ* strains carrying Ski2p-Myc (A–C, lanes 1–6 for MC052, MC053, MC055, MC130, MC131 and MC133) or Rrp4p-Myc (D–F, lanes 1–6 for MC126, MC127, MC129, MC134, MC135 and MC137). The yeast strains also expressed the indicated forms of Flag-tagged Ski7p. The cell extracts were immunoprecipitated (IP) with the anti-Flag antibody. The precipitated proteins (B, C, E and F), together with the whole-cell extracts (A and D), were immunoblotted (IB) with anti-Myc (9E10) and anti-Flag (M2) antibodies to detect Ski2p (or Rrp4p) and Ski7p, respectively. The asterisks in (C) and (F) indicate Flag-tagged Ski7p mutants expressed in the various strains. The arrowheads indicate the positions of IgG bands.



**Fig. 5.** The Ski complex and the exosome interact with the different regions of the Ski7p N domain. Cell extracts were obtained from the indicated yeast strains expressing Ski2p-Myc and Ski6p-HA (A–E) or Rrp4p-Myc and Ski2p-HA (F–J). The yeast strains (MC146, MC147, MC151, MC152, MC155–164) also expressed the various deletion mutants of Ski7p N domain with a Flag tag (lanes 1–7). The cell extracts were immunoprecipitated (IP) with the anti-Flag antibody. The precipitated proteins (B, D, E, G, I and J), together with the whole-cell extracts (A, C, F and H), were immunoblotted (IB) with anti-Myc (9E10), anti-HA (12CA5) and anti-Flag (M2) antibodies to detect Ski2p (or Rrp4p), Ski8p (or Ski6p) and Ski7p. The asterisks in (E) and (J) indicate Flag-tagged Ski7p mutants expressed in the various strains. The arrowheads indicate the positions of IgG bands.

mapped binding regions for the two multi-complexes on the N domain. Yeast strains expressing five different Flag-tagged variants of Ski7p/N were prepared (see Figure 8), and the association with the Ski complex and the exosome was investigated by immunoprecipitation assay with the anti-Flag antibody. As shown in Figure 5B and D, the Ski complex-binding region, which had been measured by Ski2p-Myc and Ski8p-HA binding, was located in the amino acid sequence 1–96 of Ski7p/N. On the other

hand, either of the two sequences, 80–184 or 168–264 of Ski7p, was sufficient for the association with the exosome measured by Rrp4p-Myc and Ski6p-HA binding (Figure 5G and I). It is unlikely that the exosome-binding site was present in only the overlapped region 168–184 between the above independent two sequences, since the more narrow sequences 80–176 and 177–264 were both capable of binding to the exosome (data not shown). Taken together, these results indicated that different regions on



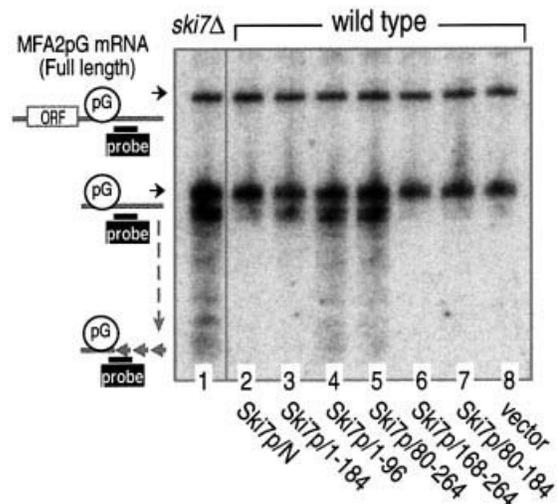
**Fig. 6.** Both the Ski complex- and the exosome-binding regions of Ski7p N domain are required for the 3'-to-5' degradation of mRNA. The *ski7Δ* strain was transformed with plasmids carrying the indicated deletion mutants of Ski7p N domain (lanes 1–7 for MC046, MC047 and MC138–MC142). The transfected cells were grown in the galactose-containing medium and harvested at mid-log phase. RNA extracted from the cells was subjected to polyacrylamide gel northern blot analysis as described in Figure 2. Positions of the full-length and poly(G) to 3'-end fragments of MFA2pG mRNA are indicated on the left.

Ski7p/N are involved in the interaction with the exosome and the Ski complex.

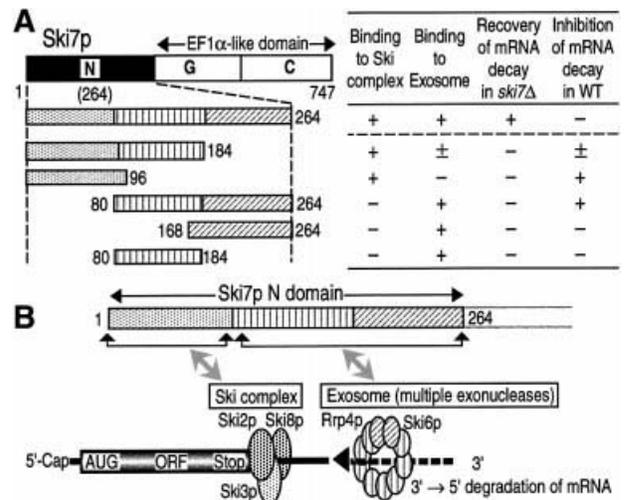
**Interaction of Ski7p/N with both the exosome and the Ski complex is required for 3'-to-5' mRNA decay**

We investigated further which regions on Ski7p/N were responsible for the 3'-to-5' mRNA decay. The five Ski7p/N-deletion variants used in Figure 5 were expressed in the *ski7Δ* strain, and their effects on the MFA2pG mRNA decay were monitored by northern blot analysis. As shown in Figure 6, all five deletion variants failed to rescue the *ski7Δ* phenotype; the accumulation of 3'-end fragments of MFA2pG mRNA was only abolished by the presence of the whole Ski7p/N sequence (lane 2). These results are consistent with the theory that Ski7p functions in 3'-to-5' mRNA decay through its interaction with both the Ski complex and the exosome.

This theory predicted that interfering with either the interaction of Ski7p with the Ski complex or the exosome would stabilize 3'-to-5' mRNA degradation. To test this prediction, we overexpressed the deletion variants of Ski7p/N in a wild-type strain containing MFA2pG mRNA. As shown in Figure 7, expression of the two mutants induced significant inhibition of 3'-to-5' mRNA decay. One mutant was the sequence 1–96, identified as the Ski complex-binding region (see Figure 5B and D), and the other mutant was the sequence 80–264, which includes both exosome-binding regions (see Figure 5G and I). In addition, the sequence 1–184, which contains the Ski complex-binding region and one of the two exosome-binding regions, also inhibited the 3'-mRNA decay, but to a lesser extent. The lesser effect of the sequence 1–184 might be due to its low-level expression under the present



**Fig. 7.** Inhibition of 3'-to-5' mRNA degradation by overexpressing either the Ski complex- or exosome-binding region of Ski7p N domain. The yeast strain MC035 (*SKI7*; wild type) was transformed with plasmids carrying the indicated deletion mutants of Ski7p N domain (lanes 1–8 for MC046, MC043, MC118–MC122 and MC042). The transfected cells were grown in the galactose-containing medium and harvested at mid-log phase. RNA extracted from the cells was subjected to polyacrylamide gel northern blot analysis as described in Figure 2. Positions of the full-length and poly(G) to 3'-end fragments of MFA2pG mRNA are indicated on the left.



**Fig. 8.** The N domain of Ski7p interacting with the Ski complex and the exosome. (A) Deletion mutants of Ski7p used in this study and the summary of the present results obtained in Figures 5, 6 and 7. (B) A proposed model for the Ski complex- and the exosome-interacting sites of the Ski7p N domain.

experimental conditions (data not shown, but see Figure 5E and J). We thus concluded that the interaction between Ski7p/N and the two multi-complexes, the Ski complex and the exosome, is required for 3'-to-5' mRNA degradation.

**Discussion**

A recent study has revealed that Ski7p is a new member involved in the 3'-to-5' mRNA-decay pathway in yeast cytoplasm, and that its role in mRNA decay may be

different from those of the other members of the *SKI* gene family (van Hoof *et al.*, 2000b). This idea was confirmed and extended further by our present findings. First, Ski7p did indeed exhibit cytoplasmic distribution in yeast (Figure 1). Secondly, the N-terminal domain of Ski7p is required and sufficient for 3'-to-5' mRNA degradation. Its EF1 $\alpha$ -like C domain was dispensable for the function of Ski7p (Figure 2). Thirdly, the exosome and the Ski complex, which are known to be multi-complex machineries involved in 3'-mRNA decay, interacted physically with the different regions of Ski7p/N (Figures 3, 4 and 5). Fourthly, interaction of Ski7p/N with the two complexes appeared to be required for 3'-to-5' mRNA decay (Figures 6 and 7). We thus concluded that the N domain of Ski7p mediates 3'-to-5' mRNA degradation through its interaction with the exosome and the Ski complex (Figure 8).

#### **Possible roles of Ski7p in the cytoplasmic mRNA decay of yeast**

In the present study, we indicated that both the Ski complex and the exosome are capable of interacting with Ski7p/N, although direct binding of their constituent components to Ski7p/N has not been determined yet. The interacting sites of the two complexes were mapped on the different regions of Ski7p/N. Moreover, Ski7p/N was essential and sufficient for cytoplasmic 3'-to-5' mRNA decay. These findings gave rise to an important question of how Ski7p/N regulates mRNA decay. There may be at least two independent mechanisms in Ski7p-induced mRNA degradation. One is that Ski7p/N binds to the Ski complex and the exosome in order to stimulate their intrinsic enzyme activities, such as RNA helicase of Ski2p and exoribonucleases in the exosome. Although the effects of Ski7p/N on the enzymatic activities have not been investigated in the present study, the above idea would be consistent with the following observations. Two RNA helicases, RhlB and eIF4A, associated with RNase E and eIF4B, respectively, and these interactions appeared to result in stimulation of their ATPase activities (Rozen *et al.*, 1990; Vanzo *et al.*, 1998).

Alternatively, Ski7p/N may function as a coupling factor between the Ski complex and the exosome for mRNA decay. In *E. coli*, the degradosome, a complex of RNase E, a polynucleotide phosphorylase and the RNA helicase RhlB, is required for 3'-to-5' degradation of mRNA (Carpousis *et al.*, 1994; Py *et al.*, 1996). The C-terminal half of RNase E constitutes binding sites for each of the other two degradosomal components (Kaberlin *et al.*, 1998; Vanzo *et al.*, 1998). *In vitro* analysis of the *E. coli* degradosome indicates that RhlB is required for degradation of structured RNA substrates in a manner dependent on ATP (Py *et al.*, 1996; Coburn *et al.*, 1999). Similarly, Suv3p, a yeast mitochondrial RNA helicase, is a component of the mtEXO complex, which also contains the 3'-exoribonuclease Dss1p (Msu1p) and is required for intron degradation (Margossian *et al.*, 1996). These findings may suggest that the RNA helicase (Ski2p of the Ski complex) and the exoribonucleases in the exosome might form a multi-complex via Ski7p/N for 3'-mRNA degradation. However, the Ski7p-Ski complex and Ski7p-exosome complex appeared to be present separately in yeast cytoplasm, since Ski7p was, but Ski2p (the

component of the Ski complex) was not, co-immunoprecipitated with Rrp4p of the exosome (data not shown). These observations imply that a ternary complex consisting of Ski7p, the Ski complex and the exosome may not exist at the same time. This is consistent with a previous report showing that there is no direct interaction between the Ski complex and the exosome (Brown *et al.*, 2000). Thus, it is very likely that the two complexes associating with Ski7p are formed separately, probably in sequential steps of the mRNA-decay pathway. Although further experiments are required to elucidate the molecular mechanism, our present report is the first indication that Ski7p may function as a signal-coupling factor between the RNA helicase and the exoribonucleases.

#### **Comparison between cytoplasmic and nuclear mRNA degradation in yeast**

The degradation of yeast mRNAs takes place in the nucleus as well as in the cytoplasm (Bousquet-Antonelli *et al.*, 2000). There are many similarities between the nuclear pre-mRNA-degradation and cytoplasmic mRNA-decay pathways. Both pathways involve 3'-to-5' degradation by their exosomes, while 5'-to-3' degradation is carried out by two homologous exoribonucleases: Rat1p in the nucleus and Xrn1p in the cytoplasm (Hsu and Stevens, 1993; Muhrad *et al.*, 1994, 1995; Johnson, 1997; Jacobs Anderson and Parker, 1998; Bousquet-Antonelli *et al.*, 2000). Therefore, we predict that the two 3'-to-5' decay pathways may be carried out in a similar fashion. Both processing and degradative activities in their exosomes have been shown to require substrate-specific cofactor(s) (van Hoof and Parker, 1999). The Ski complex and Ski7p are thus cofactors for the cytoplasmic mRNA decay induced by the exosome, although factors for nuclear pre-mRNA degradation have not yet been determined. It is unlikely that the Ski complex and Ski7p also function in pre-mRNA degradation because of their cytoplasmic localization.

An interesting model proposed previously was that the role of Mtr4p (Dob1p) could be analogous to that of Ski2p (de la Cruz *et al.*, 1998). This prediction was based on the following observations. First, Mtr4p, a putative ATP-dependent RNA helicase, is highly homologous to Ski2p. Secondly, this molecule functions as an accessory factor for the nuclear form of the exosome, which is involved in 3'-to-5' exonucleolytic processing of 5.8S rRNA, snoRNAs and U4 snRNA in the nucleus (de la Cruz *et al.*, 1998; Allmang *et al.*, 1999a; van Hoof *et al.*, 2000a). Thirdly, mutations in *MTR4* lead to the nucleolar accumulation of poly(A)<sup>+</sup> RNAs (Liang *et al.*, 1996). Finally, Mtr4p is localized throughout the nucleus (Liang *et al.*, 1996). According to this model and our assumption, an additional cofactor equivalent to the cytoplasmic Ski7p G protein would be required for nuclear pre-mRNA degradation. Such a candidate may be Hbs1p, the only GTP-binding protein, structurally related to Ski7p and eRF3 in the genome of *S. cerevisiae*. *HBS1* was identified as a suppressor in the slow growth of the *ssb1* and *ssb2* double mutant (Nelson *et al.*, 1992). Hbs1p is highly conserved in mammals, as well as Mtr4p and the exosome, but is not required for the exosome-mediated RNA processing (van Hoof *et al.*, 2000b). Further experiments

should allow us to provide evidence for the above possibility.

### ***Ski7p as a member of the eRF3 GTP-binding protein family***

*Ski7p* appears to belong to the eRF3/GSPT family of GTP-binding proteins, since it has a two-domain structure with an extra N-terminal domain (~260 amino acids) and the C-terminal (GC) domain homologous to the GTP-binding EF1 $\alpha$  (Figure 8). We previously indicated that eRF3 functions not only in translation termination as an eRF1-carrying protein, but also in 3'-poly(A) shortening by interacting with PABP (Hoshino *et al.*, 1998, 1999a,b). The two functions of eRF3 were mediated separately through the N and C domains: the N domain was capable of binding to PABP to inhibit its multimerization on the poly(A) tail, and only the EF1 $\alpha$ -like C domain was required for the association with eRF1 (Hoshino *et al.*, 1999b). This separated domain-function architecture would be quite comparable to the characteristics of *Ski7p* observed in the present study. The N domain of *Ski7p* was required and sufficient for 3'-to-5' mRNA degradation. Moreover, Hbs1p, which was supposed to be involved in the nuclear pre-mRNA degradation (see above), may belong to this family. Thus, the eRF3 G protein family appear to function in common as regulators in mRNA-decay pathways through their N domains.

There was no marked change in the interaction between *Ski7p*/N and the exosome (or the Ski complex) when a non-hydrolyzable GTP analogue or GDP was added to the immunoprecipitation assay shown in Figure 3 (data not shown). Thus, the interaction appears not to be regulated by the GTP- or GDP-form of *Ski7p* G protein. This is consistent with our findings that the interaction between the N domain of eRF3 and PABP is insensitive to the nucleotide-bound forms of the G protein. Instead, the association between the C domain of eRF3 and eRF1 required GTP rather than GDP (T.Kobayashi, S.Hoshino and T.Katada, unpublished data). There are still many unresolved issues in the structure and function of *Ski7p*. For example, what is the role of *Ski7p*/C in mRNA decay? In this regard, it is very likely that *Ski7p*/C may interact with a component(s) responsible for poly(A) shortening, since the 3'-to-5' mRNA decay follows deadenylation (Muhlrad *et al.*, 1995). Furthermore, it will be very interesting to determine how GDP-GTP exchange and GTPase reactions are involved in the regulation of *Ski7p* function(s). These important issues are currently under investigation in our laboratory.

## **Materials and methods**

### ***Yeast strains and growth conditions***

All yeast strains used in this study were derived from W303a, and are listed in Table I. The yeast cells were grown in standard culture media (Adams *et al.*, 1997) and transformed with DNA by the lithium acetate method (Ito *et al.*, 1983). Disruption of *SKI7* and *SKI8* genes was achieved using a PCR-based method with the genomic DNA and *LEU2* marker gene of *Candida glabrata*, generous gifts from Y.Ohya (Baudin *et al.*, 1993). Proofreading polymerases were used in all the PCR applications. Gene disruption was confirmed by the phenotypic analysis and/or PCR reactions with primers specific for the genes.

Epitope tagging of *SKI2*, *SKI6*, *SKI7*, *SKI8* and *RRP4* with nine Myc epitopes or three HA epitopes was performed by the one-step tagging method described by Knop *et al.* (1999). Transformants were checked by

PCRs for correct integration. All epitope-tagged proteins expressed in this study were fully functional. The sequences of the oligonucleotides are as follows:

*SKI2*, TTGATTAAGAGAGATATTGTTTTTCGCCGCAAGTTTGTA-TTTACGTACGCTGCAGGTCGAC and ACTTTTATAAACATGACT-CACATTGAGAATAAATGAGCTCTATCGATGAATTCGAGCTCG;

*SKI6*, AGGAAACATGCTCAGAAAAGAGTCAGTAACGCCTGCTAGGCGTACGCTGCAGGTCGAC and ACTTTTATAAACAG-TGGCAATTAATGGCGTTTTTTATTATCGATGAATTCGAGCTCG;

*SKI7*, TAGATCATAAGGTTATCGCAGTTGGCAGAATTGCATG-CCAGCGTACGCTGCAGGTCGAC and AATAAGTATGAATGCCT-AGTATAATTTCTTAGTTGTAGGAATCGATGAATTCGAGCTCG;

*SKI8*, TTAGATAGAAGCATCAGGTGTTTAGAGAAGCTGGCG-GTAAACGTACGCTGCAGGTCGAC and GATGTAAGGTTACATGC-AATATATCAAGATATTACTAGAGAATCGATGAATTCGAGCTCG;

*RRP4*, AGTGACATTCTTACC GCCGAAAAAATGAGAGGTAACG-GCAACCGTACGCTGCAGGTCGAC and TGTAGTTTATAAATGTG-TAAACAGCGGCTTTTGTATTCTAATCGATGAATTCGAGCTCG.

### ***Plasmid constructions***

*pURAGALI* and *pTRPGALI* were derived from pRS316 and pRS314, respectively (Sikorski and Hieter, 1989). The *NdeI* site of *URA3* was altered by site-directed mutagenesis as described previously (Kunkel *et al.*, 1991) using the oligonucleotide GTATATATACGCATACGT-AGTGTGAAGAAA (the mutation position is underlined). These plasmids contained the promoter fragment of *GALI* gene and the transcription terminator of the *CMK1* gene. To obtain *pURAGALI*-His<sub>6</sub>-Flag and *pTRPGALI*-His<sub>6</sub>-Flag, a DNA fragment was made by annealing eight oligonucleotides (AATTCATATAATGA, GGTCATACCAT-CACCATCACGGT, GACTACAAGGATGACGATGACAAGGGT, CATATGGCTCGAGTTCGACTTGGATCCGTAG, ATGTGTAG-ACCAGTCATTATAGT, CATCCTTGTAGTACCCTGTATGGTG, GACTCGAGCCATATGACCCTGTGCATCGT and AGCTTCTAC-GGATCCAAGTC). The fragment was then inserted into the region between the *EcoRI* and *HindIII* sites of *pURAGALI* and *pTRPGALI*. *pURAGALI*-His<sub>6</sub>-Flag and *pTRPGALI*-His<sub>6</sub>-Flag can overexpress His<sub>6</sub>-Flag tagged proteins under the control of the *GALI* promoter.

Plasmids for expressing the full-length and truncated proteins of *Ski7p* were constructed as follows. These genes were amplified from the genomic DNA by PCR using the following oligonucleotides: full-length, CCGGATCCTTCATATGTCGTTATTAGAGCAATTAG and CCGT-CGACTTACTGGCATGCAATTCTGCCAAC; N domain, CCGGAT-CCTTCATATGTCGTTATTAGAGCAATTAG and CCGTGCAT-TAATGGGTGGCAATGAATGAATG; C domain, CGTCCGACAT-ATGCCTCTGAATTTGACATGTTG and CCGTGCACTTACTGG-CATGCAATTCTGCCAAC; 1-184, CCGGATCCTTCATATGTCG-TTATTAGAGCAATTAG and GGGTCGACTTAATGTTATGCT-TTTTTAAGC; 1-96, CCGGATCCTTCATATGTCGTTATTAGAG-CAATTAG and GGGTCGACTTATCCTTGTCTCCAAATCAC; 80-264, CCGGATCCTTCATATGTTAAAGTTGTCTGCCTTAAAG and GGGTCGACTTAATGGGTGGCAATGAATGAATG; 168-264, CCGGATCCTTCATATGCCGCTATGTCGAGAAATTC and GGG-TCGACTTAAATGGGTGGCAATGAATGAATG; 80-184, CCGGAT-CCTTCATATGTTAAAGTTGTCTGCCTTAAAG and GGGTCGACT-TAATGTTATGCTTTTTTAAGC. These fragments were then inserted into the region between the *NdeI* and *SalI* sites of *pURAGALI*-His<sub>6</sub>-FLAG.

*pGAL-MFA2pG* was constructed essentially as described previously (Decker and Parker, 1993), with some modifications. To generate *pGEM-MFA2*, a DNA fragment containing *MFA2* and its 3' untranslated region (UTR) was amplified from the genomic DNA by PCR using two primers (CCGAATTCTCGAGCCATGCAACCGATCACCAGTGC and GGG-GTACCAAGCTTTCCGTGACTGGTTG), and the fragment was subcloned into *pGEM-T* Easy vector (Promega). *pGEM-MFA2/B178* was constructed by the one-day mutagenesis method (Imai *et al.*, 1991) with two oligonucleotides (AATATCTACCCTTTCATTTATTACG and GATTAGATCTCTTGGTTGTCGTC) to create a new *BglIII* site at B178 in the 3'-UTR of *MFA2* (Muhlrad and Parker, 1992). To obtain *pGEM-MFA2pG*, two oligonucleotides were annealed and inserted into *pGEM-MFA2/B178* previously digested with *BglIII* (GATCTAGGA-ATTTGGGGGGGGGGGGGGGGAATTCCT and GATCAGGAA-TTCCCCCCCCCCCCCAATTCCTA). *pGAL-MFA2pG* was constructed by inserting *MFA2pG* into *pTRPGALI*-His<sub>6</sub>-Flag. *MFA2pG* was isolated from *pGEM-MFA2pG* as a *XhoI*-*KpnI* fragment and inserted into *pTRPGALI*-His<sub>6</sub>-Flag previously digested with *XhoI* and *KpnI*.

**Table I.** Yeast strains used in this study

Name	Relevant genotype
MC028	<i>SKI7-9Myc</i>
MC035	<i>pGAL-MFA2pG</i>
MC042	<i>pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-FLAG</i>
MC043	<i>pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC046	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC047	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC048	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/C</i>
MC049	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC052	<i>ski7::cgHIS3, SKI2-9myc, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC053	<i>ski7::cgHIS3, SKI2-9myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC055	<i>ski7::cgHIS3, SKI2-9myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC118	<i>pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-184</i>
MC119	<i>pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-96</i>
MC120	<i>pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-264</i>
MC121	<i>pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/168-264</i>
MC122	<i>pTRPGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-184</i>
MC126	<i>ski7::cgHIS3, RRP4-9myc, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC127	<i>ski7::cgHIS3, RRP4-9myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC129	<i>ski7::cgHIS3, RRP4-9myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC130	<i>ski7::cgHIS3, ski8::cgLEU2, SKI2-9Myc, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC131	<i>ski7::cgHIS3, ski8::cgLEU2, SKI2-9Myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC133	<i>ski7::cgHIS3, ski8::cgLEU2, SKI2-9Myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC134	<i>ski7::cgHIS3, ski8::cgLEU2, RRP4-9Myc, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC135	<i>ski7::cgHIS3, ski8::cgLEU2, RRP4-9Myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC137	<i>ski7::cgHIS3, ski8::cgLEU2, RRP4-9Myc, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC138	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-184</i>
MC139	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-96</i>
MC140	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-264</i>
MC141	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/168-264</i>
MC142	<i>ski7::cgHIS3, pGAL-MFA2pG, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-184</i>
MC146	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC147	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC148	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/C</i>
MC149	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC151	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG</i>
MC152	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/N</i>
MC153	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/C</i>
MC154	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/Full</i>
MC155	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-184</i>
MC156	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-96</i>
MC157	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-264</i>
MC158	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/168-264</i>
MC159	<i>ski7::cgHIS3, SKI2-9Myc, SKI8-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-184</i>
MC160	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-184</i>
MC161	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/1-96</i>
MC162	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-264</i>
MC163	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/168-264</i>
MC164	<i>ski7::cgHIS3, RRP4-9Myc, SKI6-3HA, pURAGAL1-His<sub>6</sub>-FLAG-SKI7/80-184</i>

All strains are derivatives of W303a with the following full genotype: *MATa, ade2-1, trp1-1, can1-100, leu2-3 112, his-11,15, ura3, GAL, psi<sup>+</sup>*.

### Immunofluorescence microscopy

Immunofluorescence analysis of Ski7p-Myc localization was carried out essentially as described previously (Heyer *et al.*, 1995), with some modifications. The wild-type yeast strain (W303a) and *SKI7-9Myc* (MC028) were grown in a selective medium to mid-exponential phase. Yeast cells were fixed with 4% formaldehyde at room temperature for 40 min. The Myc epitope was detected by a mouse monoclonal antibody, 9E10 (diluted 1:300). The secondary antibody was a goat anti-mouse IgG coupled to Alexa-568 (diluted 1:1000). DNA was stained with PicoGreen (Molecular Probes). Immunofluorescence images were obtained with a Zeiss Laser Scanning Microscope 510.

### Immunoprecipitation of epitope-tagged proteins

Immunoprecipitation of Flag-tagged proteins and subsequent detection of co-precipitated proteins were performed as follows. Logarithmically growing cells ( $5 \times 10^8$ ) in the selective medium containing 2% galactose were resuspended in 250  $\mu$ l of a lysis buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40 and protease inhibitors. The cells were mixed with glass beads (1 g) and disrupted by 12 cycles of vortexing for 30 s followed by incubation on

ice for 1 min. The cell extracts were obtained by two consecutive centrifugations (14 000 g for 5 min). After addition of anti-Flag antibody-conjugated beads (M2-AGAROSE-AFFINITY; Sigma), the extracts were incubated on a rotator at 4°C for 2 h. Beads were pelleted and washed extensively with the lysis buffer without protease inhibitors. Proteins binding to the beads were eluted with the SDS-PAGE sample buffer by boiling for 5 min. Equivalent amounts of the cell extract and the eluted proteins were separated by SDS-PAGE and immunoblotted with anti-Myc (9E10), anti-HA (12CA5) and anti-Flag (M2) monoclonal antibodies.

### RNA analysis

The *pGAL-MFA2pG* plasmid was transformed in the *ski7 $\Delta$*  strain as well as in the isogenic wild-type strain. The degradation of this reporter was assayed as described (Boeck *et al.*, 1998). The *ski7 $\Delta$*  strain as well as the wild-type strain were grown at 30°C to mid-log phase ( $OD_{600} = 0.4-0.5$ ) in the galactose-containing selective medium. RNA that had been extracted from the frozen cell pellets by the hot phenol procedure (Boeck *et al.*, 1998) was separated by PAGE and transferred to a Hybond N (Amersham Pharmacia) by electroblotting. The reporter was detected by

northern blotting using the oligonucleotide oPR140, whose sequence is: MFA2pG, oPR140 (ATATTGATTAGATCAGGAATTCC) (Caponigro and Parker, 1995).

All experiments were performed at least three times with different samples of the yeast strains, and the results were fully reproducible. Hence, most of the data shown are representative of several independent experiments.

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