Nascent Peptide-dependent Translation Arrest Leads to Not4p-mediated Protein Degradation by the Proteasome*[S]

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The potentially deleterious effects of aberrant mRNA lacking a termination codon (nonstop mRNA) are ameliorated by translation arrest, proteasome-mediated protein destabilization, and rapid mRNA degradation. Because polylysine synthesis via translation of the poly(A) mRNA tail leads to translation arrest and protein degradation by the proteasome, we examined the effects of other amino acid sequences. Insertion of 12 consecutive basic amino acids between *GFP* and *HIS3* reporter genes, but not a stem-loop structure, resulted in degradation of the truncated green fluorescent protein (GFP) products by the proteasome. Translation arrest products derived from *GFP-R12-FLAG-HIS3* or *GFP-K12-FLAG-HIS3* mRNA were detected in a *not4Δ* mutant, and MG132 treatment did not affect the levels of the truncated arrest products. Deletion of other components of the Ccr4-Not complex did not increase the levels of the translation arrest products or reporter mRNAs. A L35A substitution in the Not4p RING finger domain, which disrupted its interaction with the Ubc4/Ubc5 E2 enzyme and its activity as an ubiquitin-protein ligase, also abrogated the degradation of arrest products. These results suggest that Not4p, a component of the Ccr4-Not complex, may act as an E3 ubiquitin-protein ligase for translation arrest products. The results let us propose that the interaction between basic amino acid residues and the negatively charged exit tunnel of the ribosome leads to translation arrest followed by Not4p-mediated ubiquitination and protein degradation by the proteasome.

Cells have surveillance systems that recognize and eliminate aberrant mRNAs to prevent the production of potentially harmful protein products. In eukaryotes, an aberrant mRNA lacking a termination codon (nonstop mRNA) may be recognized and eliminated by the quality control system referred to as nonstop decay (1, 2). It has been shown that nonstop mRNA is rapidly degraded by a 3′-to-5′ degradation pathway in yeast (1). We have shown that translation of nonstop mRNA is repressed after the initiation step (3, 4); the level of the product of nonstop mRNA containing a poly(A) tail was reduced by ∼100-fold, and this reduction was due to rapid mRNA degradation, translation repression, and protein destabilization, at least in part, by the proteasome (3, 4). Interestingly, insertion of a poly(A) tract upstream of a termination codon resulted in translation repression and protein destabilization but not rapid mRNA decay (3, 4). Recently, it has been shown that translation of nonstop mRNA also is repressed after initiation in mammalian cells and that the ribosome stalls on poly(A) sequences, a common modification of eukaryotic mRNA that is not normally translated (5). Therefore, translation repression following polylysine synthesis may be conserved among eukaryotes. We propose that ribosomes recognize the translation of poly(A) tails as an aberrant process, resulting in translation arrest and degradation of the protein product by the proteasome.

Translation elongation is an important step of gene regulation; the expression levels of several genes are known to be regulated by translation arrest. In prokaryotes, the regulation of the *secM* gene, for example, has been extensively analyzed. The interaction between the SecM nascent peptide and the wall of the ribosomal exit tunnel functions as a discriminating gate during translation (6, 7). Several eukaryotic examples of translation arrest also have been described (8, 9). Moreover, the synthesis of polylysine caused by translation of a poly(A) sequence leads to translation arrest (3, 4). Recent measurement of the electrostatic potential of the ribosome exit tunnel using chemical modification methods revealed that the wall of the tunnel is negatively charged in eukaryotes (10, 11). These results suggest that consecutive positively charged amino acid residues in a nascent protein may have a high affinity for the negatively charged ribosomal exit tunnel. A more recent report demonstrated that positively charged residues cause pausing in mammalian ribosomes *in vitro* and showed how far the positively charged side chains move into the tunnel (12).

The evolutionarily conserved Ccr4-Not complex regulates mRNA biosynthesis at various steps. First, Ccr4p/Caf1p has a deadenylase activity that functions in mRNA deadenylation (13–15). Second, Ccr4p/Caf1p regulates histone H3K4 trimethylation (H3K4me3), which plays a significant role in chromatin organization, gene transcription, and epigenetic regulation via interactions with the proteasome to regulate its recruitment to genes (16). In yeast, the Ccr4-Not core complex is composed of nine components. The Not1−5 proteins are critical for transcriptional repression. It recently was shown that *NOT1, NOT2, NOT4,* and *NOT5* are required for trimethylation of H3K4 (16, 17). Third, the Not4p subunit of the Ccr4-Not complex has an E3[3] ubiquitin-protein ligase activity for the conserved ribosome-associated het-
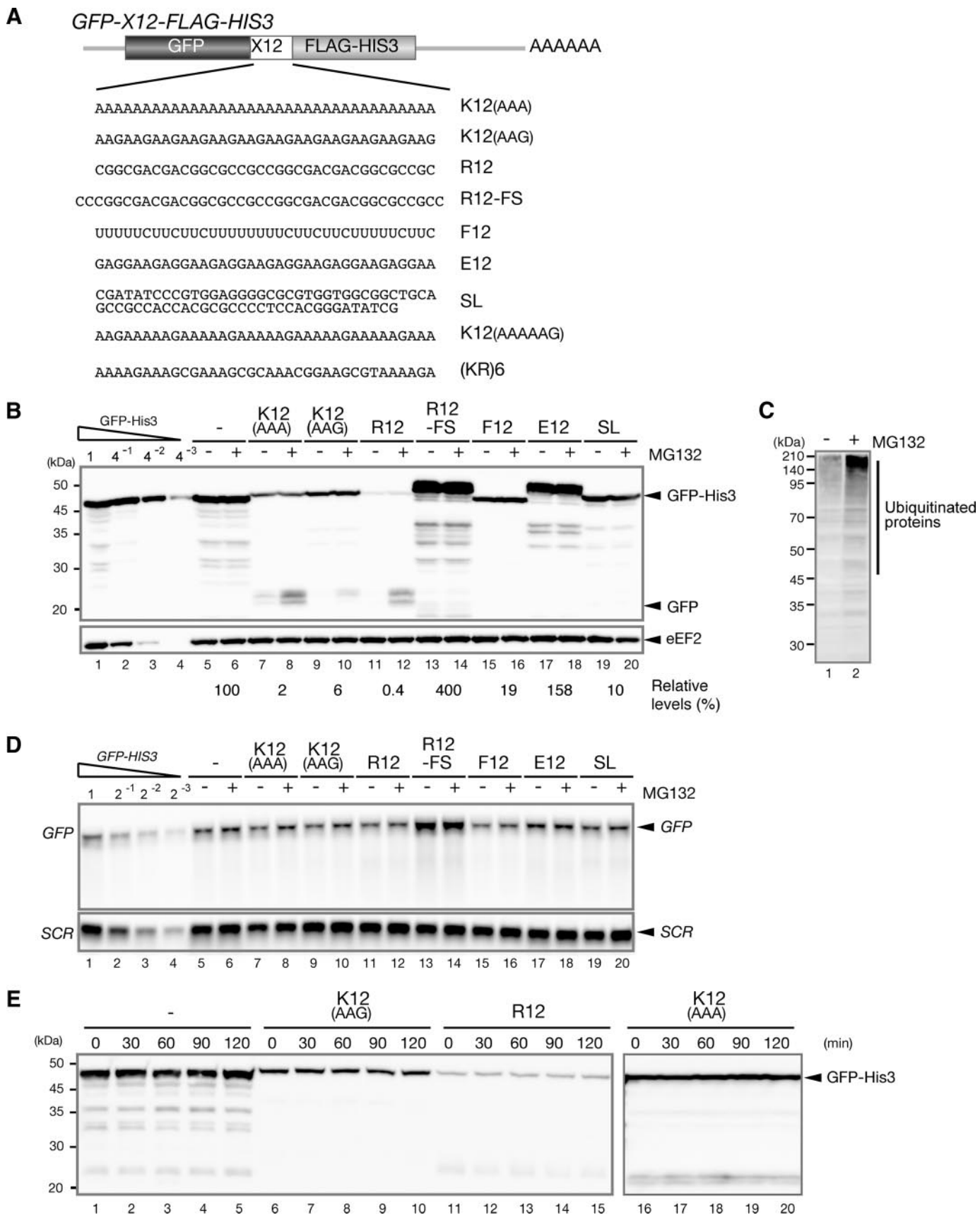

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Fig. S1.

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[3] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin carrier protein; GFP, green fluorescent protein; DIG, digoxigenin.

A

GFP-X12-FLAG-HIS3

GFP | X12 | FLAG-HIS3 — AAAAAA

Sequence	Label
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	K12(AAA)
AAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAGAAG	K12(AAG)
CGGCGACGACGGCGCCGCCGGCGACGACGGCGCCGC	R12
CCCGGCGACGACGGCGCCGCCGGCGACGACGGCGCCGCC	R12-FS
UUUUUCUUCUUCUUUUUUUUUCUUCUUCUUUUUCUUC	F12
GAGGAAGAGGAAGAGGAAGAGGAAGAGGAAGAGGAA	E12
CGATATCCCGTGGAGGGGCGCGTGGTGGCGGCTGCA GCCGCCACCACGCGCCCCTCCACGGGATATCG	SL
AAGAAAAAGAAAAAGAAAAAGAAAAAGAAAAAGAAA	K12(AAAAAG)
AAAAGAAAGCGAAAGCGCAAACGGAAGCGTAAAAGA	(KR)6

B

100	2	6	0.4	400	19	158	10												Relative levels (%)

C

D

E

erodimeric EGD complex. This complex consists of the Egd1p and Egd2p subunits in yeast and is named NAC (nascent polypeptide-associated complex) in mammals. Finally, the Not4p displays an Ubc4p-dependent ubiquitination activity *in vitro* (18, 19), which depends on the RING finger domain of Not4p (18).

In this study, we show that 12 consecutive basic amino acid residues cause translation arrest followed by cotranslational protein degradation by the proteasome. In contrast, a stem-loop structure induces only translation arrest but not degradation of the arrested product. Not4p is required for the degradation of translation arrest products, although Not3p and Not5p of the Ccr4-Not complex are not. In addition, translation arrest products are not degraded by the proteasome in the *not4L35A* mutant, in which ubiquitination of the EGD complex and the interaction with E2 enzymes are defective. These results strongly suggest that nascent peptide sequences play crucial roles in translation elongation arrest, leading to Not4p-mediated protein degradation by the proteasome. More general roles of endogenous nascent peptides in the regulation of translation and protein degradation also are discussed.

EXPERIMENTAL PROCEDURES

Strains and General Methods—Standard procedures were used to manipulate yeast cells (20). The yeast strains and plasmids used in this study are described in supplemental Table S1. Deletions of *NOT3, NOT4*, and *NOT5* were constructed using a PCR-based method (21). Recombinant DNA procedures were carried out as described previously (22).

Plasmids—Two oligonucleotides (supplemental Table S2) were annealed and inserted into the SpeI site of pSA144 (p*GPDp-GFP-FLAG-HIS3-CYC1ter*) to create the p*GPD-GFP-X-FLAG-HIS3* reporters, which contained sequences encoding various 12-amino acid stretches or a stem-loop structure between *GFP* and *HIS3*. A *NOT4* XbaI-XhoI fragment was amplified in a PCR using two primers (5′-GGTCTAGAC-GTATATAATCCAGTCATAATGATG-3′ and 5′-CCCTCGA-GGAAAAATATTTAGAGTCGGATTAATTACCGGCGA-3′) and inserted into p415*ADH1p* to generate p415*ADH1p-NOT4*. To construct p415*ADH1p-NOT4L35A*, a mutation was introduced using site-directed mutagenesis. Two oligonucleotides (5′-CTAGAATGGACTACAAGGACGACGATGACAAGG-GTCTGGTA-3′ and 5′-CTAGTACCAGACCCTTGTCAT-CGTCGTCCTTGTAGTCCATT-3′) were annealed and inserted into the XbaI site of p415*ADH1p-NOT4* to generate p415*ADH1p-FLAG-NOT4*.

Determination of the Relative Product Levels Using Western Blotting—Yeast cells were grown on SC-Ura medium, and proteins from cell extracts equivalent to 0.2 A_{600} were prepared.

The products derived from the reporter genes were detected using Western blotting with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). The primary antibodies used for Western blotting were anti-FLAG antibodies (F1804; Sigma), anti-GFP antibodies (sc-9996; Santa Cruz Biotechnology), and anti-FK2 antibodies (PW8810; BIOMOL). The anti-FK2 antibodies recognize the multiubiquitin chains of polyubiquitinated proteins but not free ubiquitin or the protein moieties (23). The intensities of the bands were quantified with the LAS3000 mini system using Multi-Gauge version 3.0 (Fuji Film, Japan), and the relative levels of the products were determined based on a comparison with a standard curve. When the intensity of the band was outside the range of the standard curve, a series of sample dilutions was prepared. The intensities of the bands for the diluted samples were compared with those from the standard curve, and the relative levels of the products in the samples compared with the control products were determined.

Northern Blotting—Total RNA was separated using 1.2% agarose gel electrophoresis in the presence of formaldehyde and blotted onto Hybond-N$^+$ membrane (Amersham Biosciences). mRNA was visualized using digoxigenin (DIG) reagents and kits for PCR-based nonradioactive nucleic acid labeling detection (Roche Applied Science) according to the manufacturer's instructions. DIG-labeled probes were prepared with the following oligonucleotides: *HIS3* (5′-GCTCTAGATGACAG-AGCAGAAAGCCCTAG-3′ and 5′-CGGGATCCCATAAG-AACACCTTTGGTGGAGG-3′) and *GFP* (5′-GCTCTAGAG-GCCTATGCGGCCGCAGTAAAGGAG-3′ and 5′-CGGGA-TCCTTTGTATAGTTCATCCATGCC-3′). The band intensities were quantified using the LAS3000 mini system and Multi-Gauge version 3.0 (Fuji Film).

Yeast Extract and Sucrose Gradient Separation—Yeast cells were grown exponentially at 30 °C and harvested by centrifugation. The cells were washed once with lysis buffer, and extracts were prepared as described previously (24). The equivalent of 50 A_{260} units was then layered onto linear 10–50% sucrose density gradients (10–50% sucrose in 10 mM Tris acetate, pH 7.4, 70 mM ammonium acetate, 4 mM magnesium acetate), prepared in 25 × 89-mm polyallomer tubes (Beckman Coulter) using a gradient master. Crude extracts were layered on top of the sucrose gradients and centrifuged at 27,000 rpm in a P28S rotor (Hitachi Koki) for 3 h at 4 °C. The gradients were then fractionated (TOWA lab, Tsukuba). Polysome profiles were generated with continuous absorbance measurement at 254 nm using a single path UV-1 optical unit (ATTO Biomini UV monitor) connected to a chart recorder (ATTO digital mini-re-

FIGURE 1. **Protein degradation by the proteasome in response to translation arrest caused by consecutive basic amino acid residues.** *A*, construction of the p*GPDp-GFP-X-FLAG-HIS3* reporter plasmids, which contain various sequences encoding 12 consecutive amino acids inserted between *GFP* and *HIS3*. *B*, consecutive basic amino acid sequences induce translation arrest coupled with protein degradation by the proteasome. Cells harboring the indicated p*GPDp-GFP-X-FLAG-HIS3* plasmid were grown on SC-Ura medium, and the samples were analyzed using Western blotting with anti-GFP (*top panel*) or anti-eEF2 (*bottom panel*) antibodies. When indicated (+), cell extracts were prepared 2 h after the addition of 0.2 mM MG132. When determining the relative expression levels, we did not include shorter (degraded) products. *C*, proteasome activity is inhibited in the presence of MG132. W303 cells were grown on SC medium, and cell extracts were prepared 2 h after the addition of 0.2 mM MG132 when indicated (+). The samples were analyzed using Western blotting with anti-FK2 antibodies, which recognize monoubiquitin and polyubiquitin but not free ubiquitin. *D*, truncated mRNA is not detected in the presence of MG132. W303 cells harboring the indicated p*GPDp-GFP-X-FLAG-HIS3* plasmid were grown on SC-Ura medium, and RNA samples were analyzed using Northern blotting with DIG-labeled *GFP* or *SCR* probes. *E*, the stabilities of GFP-K12(AAG)-FLAG-His3, GFP-R12-FLAG-His3, and GFP-K12(AAA)-FLAG-His3 were analyzed using Western blotting. Samples of W303 cells harboring the indicated plasmid were prepared at the indicated time points after the addition of cycloheximide (0.1 mg/ml). The levels of the remaining proteins were determined by Western blotting with anti-GFP antibodies.

corder). Equal volume fractions were collected and processed for Western blotting as described above.

RESULTS

Consecutive Basic Amino Acid Residues Induce Protein Degradation by the Proteasome—It was reported that insertion of a poly(A) tract upstream of a termination codon results in translation repression and protein destabilization but not rapid mRNA decay (3, 4). To determine whether translation is arrested by other amino acid sequences and whether the translation arrest product is degraded by the proteasome, we inserted fragments encoding various amino acid sequences between the *GFP* and *HIS3* genes (Fig. 1*A*). We hypothesized that translation may be arrested when the ribosome synthesizes polylysine, and the translation arrest product could be detected as a truncated GFP product in the presence of MG132, a proteasome inhibitor. As expected, the level of the full-length product was significantly lower (2–6% of the control product), and in the presence of MG132, a translation arrest product was detected as a truncated GFP product (Fig. 1*B*, *lanes 8* and *10*). We also found that insertion of 12 consecutive arginine residues strongly reduced the level of the full-length GFP-R12-FLAG-His3 product (less than 1% of the control sample; Fig. 1*B*, *lanes 11* and *12*). The p*GPDp-GFP-R12-FS-FLAG-HIS3* construct is identical to the p*GPDp-GFP-R12-FLAG-HIS3* construct except for an additional nucleotide just before the sequence encoding polyarginine and two more nucleotides following the sequence. This frameshift mutation completely eliminated translation repression (Fig. 1*B*, *lanes 13* and *14*), indicating that the basic amino acid sequence plays a crucial role for translation repression. There was a difference between the translation arrest efficiencies of K12(AAA) and K12(AAG) (Fig. 1*B*, *lanes 7–10*). The mRNA levels of *GFP-K12(AAA)-FLAG-HIS3* and *GFP-K12(AAG)-FLAG-HIS3* were similar, indicating that the nucleotide sequence could play a role in translation repression. One possibility is that Pab1p may bind to poly(A) sequences and inhibit translation elongation. To confirm that MG132 indeed inhibited proteasomes under the test conditions, we performed Western blotting with anti-FK2 antibodies, which recognize monoubiquitin and polyubiquitin but not free ubiquitin. As shown in Fig. 1*C*, the levels of ubiquitinated proteins were significantly higher in the presence of MG132. In addition, we confirmed that the levels of the truncated products derived from reporters increased in the presence of MG132 in *erg6Δ* mutant cells, which show an increased uptake of MG132 (supplemental Fig. S1) (25).

We also confirmed that the levels and sizes of the mRNAs derived from *GFP-K12(AAA)-FLAG-HIS3*, *GFP-K12(AAG)-FLAG-HIS3* or *GFP-R12-FLAG-HIS3* were not affected by MG132 (Fig. 1*D, lanes 7–12*), indicating that the truncated proteins were not produced from truncated mRNAs. We found that the levels of the mRNA and protein derived from *GFP-R12-FS-FLAG-HIS3* were 2-fold higher than those derived from the control reporter (Fig. 1, *B* and *C, lane 13*). MG132, however, did not affect level of mRNA and protein derived from *GFP-R12-FS-FLAG-HIS3* (Fig. 1, *B* and *D, lanes 13* and *14*). Insertion of polyphenylalanine but not polyglutamic acid significantly reduced the levels of the full-length products, mimicking the

effects of polylysine (AAG) (Fig. 1*B*, *lanes 15–18*). Truncated product derived from the construct expressing both polyglutamic acid and polyphenylalanine was not detected, however, even in the presence of MG132. These results suggest that sequential basic amino acid residues may induce proteasome-dependent degradation of arrest products.

We also examined whether nascent peptides are degraded by the proteasome when translation is arrested by a stem-loop structure. The level of the intact product expressed from *GFP-SL-FLAG-HIS3* mRNA was reduced significantly (10% of the control product) (Fig. 1*B*, *lanes 5* and *19*), whereas the mRNA level was not significantly reduced (Fig. 1*C*, *lanes 5* and *19*). These results clearly indicate that translation is physically arrested by stable stem-loop structures, as previously described (26). Importantly, MG132 treatment did not affect the expression of the putative small products from translation arrest caused by the stem-loop structure (Fig. 1*B*, *lanes 19* and *20*). This strongly suggests that specific nascent peptides, not translation arrest induced by a physical barrier, result in cotranslational protein degradation by the proteasome. To determine whether the truncated products derived from *GFP-K12-FLAG-HIS3* or *GFP-R12-FLAG-HIS3* may result from protease-mediated cleavage after translation is completed, the proteins remaining after inhibition of translation were examined using Western blotting. The levels of the remaining products were not markedly changed as a function of time after the addition of cycloheximide, indicating that the full-length products were as stable as GFP-His3 (Fig. 1*E*). Consistently, MG132 treatment did not increase the levels of full-length GFP-K12(AAA)-FLAG-His3, GFP-K12(AAG)-FLAG-His3, or GFP-R12-FLAG-His3 (Fig. 1*B*, *lanes 7–12*). These results suggest that the truncated products derived from *GFP-FLAG-HIS3*, *GFP-K12-FLAG-HIS3*, and *GFP-R12-FLAG-HIS3* were not produced by degradation or the cleavage of full-length products and are consistent with a model in which the truncated product detected in the presence of MG132 is produced by translation arrest. Thus, we propose that specific nascent peptides induce translation arrest, which in turn leads to cotranslational protein degradation by the proteasome in yeast.

Rare Codons Are Not Required for Translation Arrest and Subsequent Protein Degradation—To address the specificity of the amino acid sequences required to induce translation arrest and cotranslational protein degradation, we examined the expression of *GFP-X-FLAG-HIS3* reporters containing sequences that encode other 12-amino acid sequences between *GFP* and *HIS3*, in the presence or absence of a proteasome inhibitor. We observed significant reductions in the levels of the full-length proteins containing 12 sequential hydrophobic residues, (valine, phenylalanine), although truncated GFP products derived from these reporter genes were not detected, even after MG132 treatment (Fig. 2*A*, *lanes 19, 20, 29*, and *30*). In contrast, truncated products derived from *GFP-W12-FLAG-HIS3* were detected only upon the addition of MG132 (Fig. 2*A*, *lanes 27* and *28*), although the observed levels were not as high as those produced by 12 consecutive Lys or Arg residues. Introduction of a frameshift mutation abrogated the arrest product in the presence of MG132 (Fig. 2*B*, *lanes 7* and *8*). These results suggest that translation of consecutive 12 basic or Trp residues
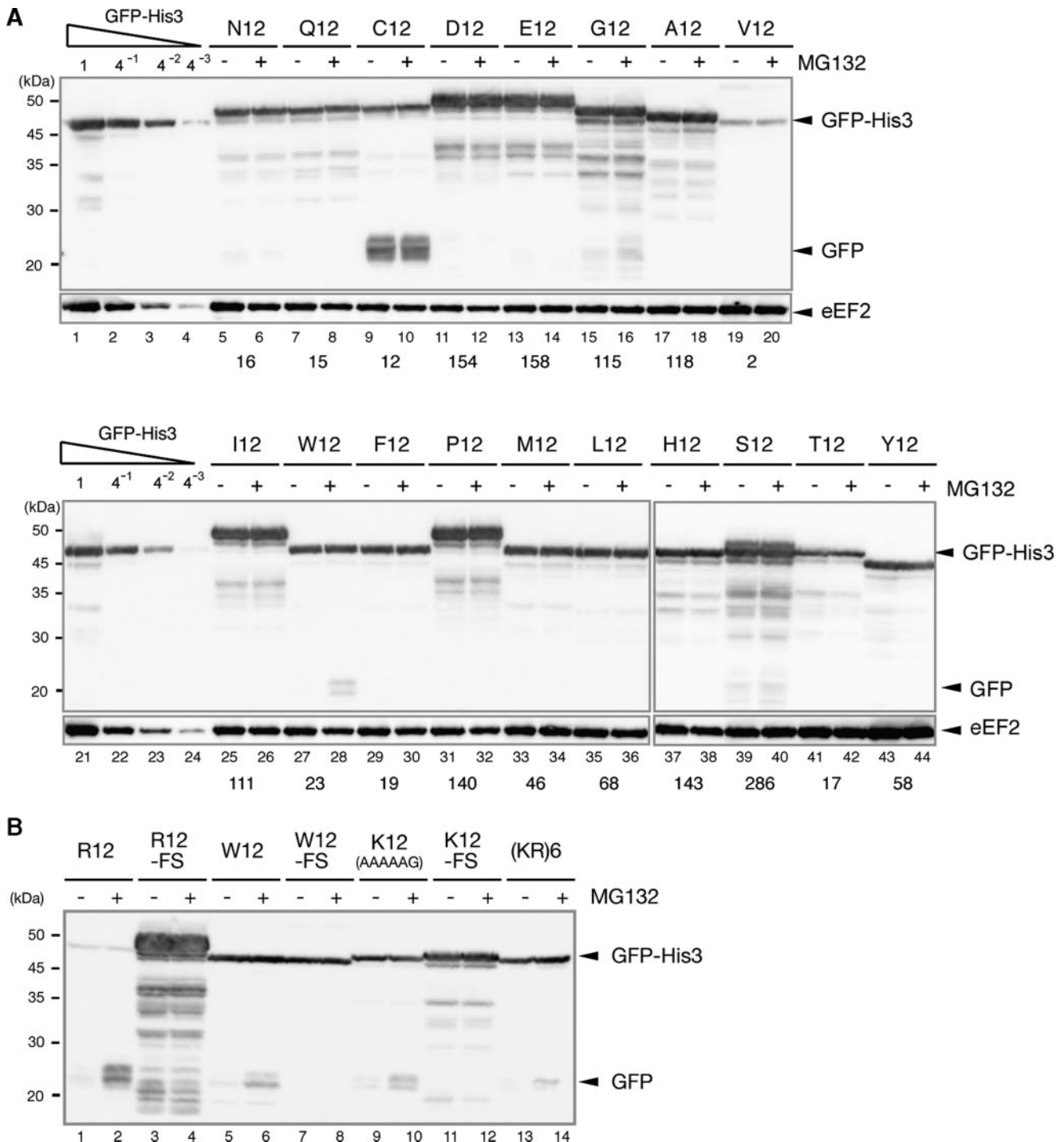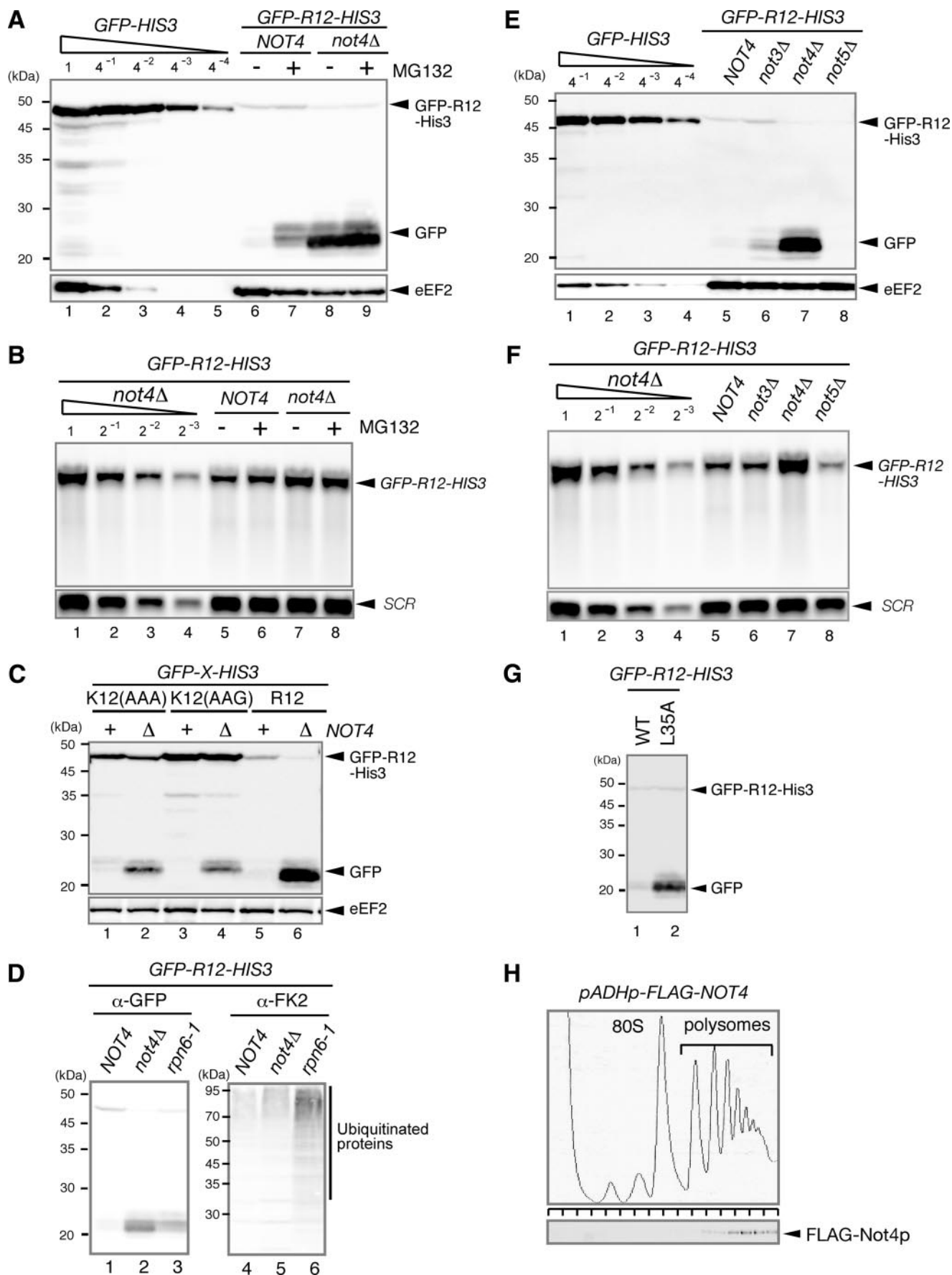
FIGURE 2. **Consecutive basic amino acid residues rather than rare codons induce translation arrest coupled with protein degradation.** *A*, sequence specificity of the translation arrest-induced protein degradation process mediated by the proteasome. W303 cells harboring the indicated p*GPDp-GFP-X-FLAG-HIS3* plasmids were grown on SC-Ura medium, and cell extracts were prepared. When indicated (+), cell extracts were prepared 2 h after the addition of 0.2 mM MG132. The relative levels of the reporter proteins were determined using Western blotting with anti-GFP or anti-eEF2 antibodies. The levels of the products derived from *GFP-S12-HIS3*, *GFP-D12-HIS3*, *GFP-E12-HIS3*, *GFP-G12-HIS3*, *GFP-I12-HIS3*, and *GFP-P12-HIS3* were higher than that of the product derived from *GFP-HIS3*. Additionally, the relative levels of those products were determined using a standard curve of samples from cell harboring p*GPDp-GFP-S12-HIS3* or p*GPDp-GFP-HIS3*. *B*, W303 cells harboring the indicated p*GPDp-GFP-X-FLAG-HIS3* plasmid were grown on SC-Ura medium, and the samples were analyzed using Western blotting. When indicated (+), cell extracts were prepared 2 h after the addition of 0.2 mM MG132.

results in translation arrest, which leads to nascent protein degradation by the proteasome. We also identified truncated products expressed from the *GFP-K12(AAAAAG)-FLAG-HIS3* reporter, which contains six repeats of the AAAAAG sequence and encodes polylysine (Fig. 2*B*, *lanes 11* and *12*). The levels of

truncated products increased in the presence of MG132 and a frameshift mutation again inhibited production of truncated products (Fig. 2*B*, *lanes 13* and *14*). These results indicate that polylysine, rather than sequential AAA codons, are required for the translation arrest coupled with protein degradation as pre-

viously described (4). We also detected several different truncated GFP products derived from *GFP-G12-FLAG-HIS3*. The level of full-length GFP-G12-FLAG-His3, however, was similar to that of control GFP-FLAG-His3 product (Fig. 2*A*, *lanes 1* and *15*). In addition, the levels of truncated products did not increase in the presence of MG132 (Fig. 2*A*, *lanes 15* and *16*). These results strongly suggest that the truncated GFP products are not translation arrest products. We also detected a truncated GFP product derived from *GFP-C12-FLAG-HIS3* without MG132 treatment (Fig. 2*A*, *lanes 9* and *10*). A truncated His3 product was detected using Western blot analysis with anti-FLAG antibodies, and its level relative to that of the intact GFP-C12-FLAG-His3 product was similar to the results observed for truncated GFP (data not shown). These findings suggest that GFP-C12-FLAG-His3 is cleaved post-translationally independently of the proteasome because MG132 had no effect on the levels of either truncated product (Fig. 2*A*, *lanes 9* and *10*).

To confirm that 12 consecutive basic amino acid residues induce translation arrest, we constructed a *GFP-(KR)6-FLAG-HIS3* reporter that contains a sequence encoding six Lys-Arg repeats between *GFP* and *HIS3.* Importantly, the inserted sequence did not contain any rare codons. The truncated product was identified only in the presence of MG132 (Fig. 2*B*, *lanes 9* and *10*). These results strongly suggest that consecutive basic amino acids rather than rare codons or pure polylysine or polyarginine sequences are required for translation arrest coupled with protein degradation.

Not4p Is Required for Proteasome-induced Degradation of Translation Arrest Products—Our results indicated that nascent peptide-dependent protein degradation is cotranslational, suggesting that an E3 ubiquitin-protein ligase may be associated with the ribosome. Not4p, a component of the Ccr4-Not complex, has been identified as an E3 ubiquitin-protein ligase for EGD complexes associated with nascent polypeptides (19). Therefore, we examine the expression from *GFP-R12-FLAG-HIS3* in a *not4Δ* mutant. In the *not4Δ* mutant, the level of the truncated product derived from *GFP-R12-FLAG-HIS3* was markedly higher, and, along with the level of *GFP-R12-FLAG-HIS3* mRNA, was not affected by the addition of MG132 (Fig. 3, *A*, *lanes 6–9*, and *B*, *lanes 5–8*). A corresponding truncated mRNA was not detected, even in the presence of MG132 (Fig. 3*B*, *lanes 5–8*). We also found that the levels of truncated products derived from *GFP-K12(AAA)-FLAG-HIS3* or *GFP-K12(AAG)-FLAG-HIS3*

also were higher in the *not4Δ* mutant (Fig. 3*C*, *lanes 2* and *4*). These results strongly suggest that Not4p is required for the degradation of translation arrest products by the proteasome. The level of *GFP-R12-FLAG-HIS3* mRNA also increased significantly in the *not4Δ* mutant (Fig. 3*B*, *lanes 5* and *7*), although the level of the GFP-R12-FLAG-His3 protein was slightly lower (Fig. 3*A*, *lanes 6* and *8*), suggesting that translation arrest of *GFP-R12-FLAG-HIS3* mRNA may be enhanced in the *not4Δ* mutant.

Because Not4p is involved in transcriptional regulation via H3K4 trimethylation, the proteasomes in the *not4Δ* mutant may be generally impaired. To address this possibility, we determined the levels of ubiquitinated proteins using Western blotting with anti-FK2 antibodies. It was shown previously that Rpn6p is a component of the lid of the proteasome and that the activity of the proteasome is impaired in the *rpn6−2* temperature-sensitive mutant (27). We found that the levels of ubiquitinated proteins were markedly higher in the *rpn6−2* mutant compared with the *not4Δ* mutant (Fig. 3*D*, *lanes 4–6*), indicating that the activity of the proteasome in the *rpn6−2* mutant was more severely impaired than in the *not4Δ* mutant. The increase in the truncated GFP product in the *rpn6−2* mutant, however, was smaller than in the *not4Δ* mutant (Fig. 3*D*, *lanes 1–3*). The truncated product in the *not3Δ* and *not5Δ* mutants also was not augmented (Fig. 3*E*, *lanes 6* and *8*). We obtained similar results for the mRNA levels (Fig. 3*F*, *lanes 6* and *8*). Because Not4p and Not5p, rather than Not3p, are required for trimethylation of H3K4 (16, 17), these results suggest that the H3K4me3-mediated defect in transcriptional regulation in the *not4Δ* mutant may not account for the increased levels of the truncated product. These results are consistent with the idea that Not4p is required for the degradation of truncated products by the proteasome.

The N-terminal domain of Not4p contains a RING finger domain, which mediates its interaction with the E2 ligase Ubc4p/Ubc5p; the L35A mutation disrupted the ubiquitin-protein ligase activity of Not4p (18). The level of the truncated product derived from *GFP-R12-HIS3* was markedly higher in the *not4Δ* mutant harboring the p*NOT4*-L35A plasmid (L35A) but not in the *not4Δ* mutant harboring the p*NOT4* plasmid (wild type) (Fig. 3*G*). Interestingly, FLAG-Not4p was distributed mainly in the heavy polysome fractions of sucrose gradients (Fig. 3*H*), suggesting that Not4p may be associated with ribosome. Thus, Not4p appears to

FIGURE 3. **Not4p is required for the degradation of translation arrest products by the proteasome.** *A*, Not4p is required for the proteasome-mediated degradation of the truncated product. W303 or W303*not4Δ* cells were transformed with the p*GPDp-GFP-R12-FLAG-HIS3* plasmid. The cells were grown in SC-Ura, and samples were analyzed using Western blotting with anti-GFP antibodies. When indicated (+), cell extracts were prepared 2 h (W303) or 5 h (W303*not4Δ*) after the addition of 0.2 mM MG132. *B*, RNA samples were prepared from cells shown in *A* and analyzed using Northern blotting with DIG-labeled *GFP* or *SCR* probes. *C*, the level of truncated product derived from *GFP-K12-FLAG-HIS3* was higher in the *not4Δ* mutant. W303 or W303*not4Δ* cells were transformed with the indicated p*GPDp-GFP-X-FLAG-HIS3* plasmid. The cells were grown in SC-Ura, and samples were analyzed using Western blotting. *D*, W303, W303*not4Δ*, or W303*rpn6−2* (YNK7) cells harboring p*GPDp-GFP-R12-FLAG-HIS3* were grown in SC medium at 30 °C. The samples were prepared and analyzed using Western blotting with anti-GFP antibodies (*lanes 1–3*) or anti-FK2 antibodies that recognize monoubiquitin and polyubiquitin but not free ubiquitin (*lanes 4–6*). *E*, the level of the truncated product derived from *GFP-R12-FLAG-HIS3* was higher in the *not4Δ* mutant. W303, W303*not3Δ*, W303*not4Δ*, or W303*not5Δ* cells were transformed with the p*GPDp-GFP-R12-FLAG-HIS3* plasmid. The cells were grown in SC-Ura, and the samples were analyzed using Western blotting. *F*, RNA samples were prepared from the cells shown in *D* and analyzed using Northern blotting with DIG-labeled *GFP* or *SCR* probes. *G*, the level of truncated product derived from *GFP-R12-FLAG-HIS3* was higher in the *not4Δ* mutant expressing the Not4L35A mutant (mutation in the RING finger domain). W303*not4Δ* cells containing p*GPDp-GFP-R12-FLAG-HIS3* were transformed with p*ADHp-NOT4* or p*ADHp-not4L35A*. The cells were grown in SC-UraLeu medium, and the samples were analyzed using Western blotting. *H*, FLAG-Not4p protein was distributed in the polysome fractions. W303 cells were transformed with p*ADHp-FLAG-NOT4*. Cell extracts were prepared, and polysome analysis was performed as described previously (3). Protein samples prepared from each fraction were analyzed using Western blotting with anti-FLAG antibodies. *WT*, wild type.
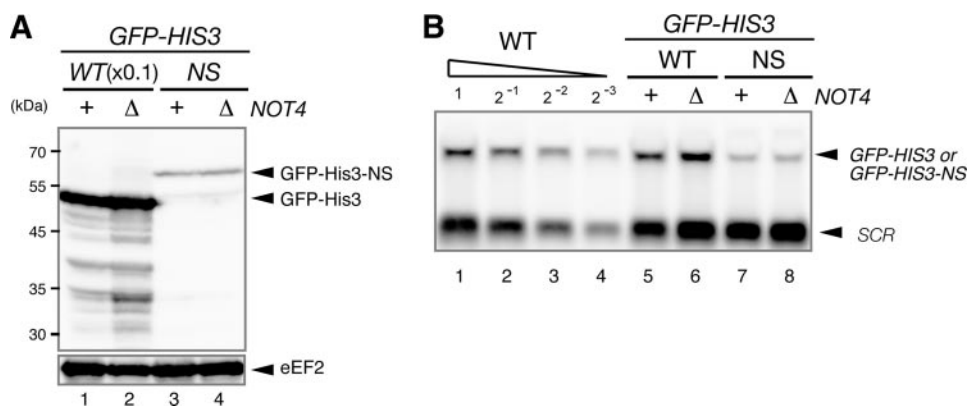
FIGURE 4. **Not4p is not involved in the degradation of nonstop products.** W303 or W303*not4Δ* cells were transformed with the indicated p*GPDp-GFP-FLAG-HIS3* (pSA156) or p*GPDp-GFP-FLAG-HIS3-NS* (pSA157) plasmids (4). *A*, the cells were grown in SC-Ura, and the samples were analyzed using Western blotting with anti-GFP antibodies. *B*, the cells were grown in SC-Ura, and the samples were analyzed using Northern blotting with DIG-labeled *GFP* and *SCR* probes. *WT*, wild type.

function as an E3 ubiquitin-protein ligase for translation arrest products, which is followed by their proteasome-mediated degradation.

Degradation of Nonstop mRNA Products Is Not4p-independent—We previously demonstrated that translation of a poly(A) tail from nonstop mRNA results in translation arrest and post-translational protein degradation of the nonstop products (3, 4). To investigate the role of Not4p in the degradation of nonstop products, we examined the expression of nonstop products in a *not4Δ* mutant. The level of the nonstop product derived from *GFP-FLAG-HIS3-NS* did not increase in the *not4Δ* mutant (Fig. 4*A*, *lanes 3* and *4*), suggesting that Not4p is not involved in the degradation of nonstop products. We also confirmed that the levels of *GFP-FLAG-HIS3* and *GFP-FLAG-HIS3-NS* mRNA were not changed in the *not4Δ* mutant (Fig. 4*B*, *lanes 5–8*), although the level of *GFP-FLAG-HIS3-NS* mRNA was 4-fold lower than that of *GFP-FLAG-HIS3* mRNA as described previously (4). This result is consistent with the role of Not4p as an E3 ubiquitin-protein ligase for translation arrest products but not for aberrant proteins released from the ribosome.

Endogenous mRNA Sequences Induce Translation Arrest Coupled with Protein Degradation—Our results suggest that translation arrest induced by sequences encoding consecutive basic amino acid residues leads to Not4p-mediated protein degradation by the proteasome. To evaluate a possible role for translation arrest in gene regulation, we screened genomic sequences for areas encoding consecutive basic amino acid sequences and identified five genes (Fig. 5*A*). The five endogenous sequences were inserted between *GFP* and *HIS3* to determine whether they induced translation arrest and associated protein degradation (Fig. 5*A*). Truncated products derived from the reporter genes were detected in the presence of MG132. The strongest induction of translation arrest and protein degradation was observed with the CKKKKKRKKKNK sequence derived from Rmp1p (Fig. 5*B*, *lanes 9* and *10*). Truncated products derived from the reporter genes were also detected in the *not4Δ* mutant, and the same CKKKKKRKKKNK sequence showed the translation arrest activity (Fig. 5*C*, *lane 5*). A possible role

for these sequences in gene regulation is currently being investigated.

DISCUSSION

Translation Arrest and Protein Degradation Induced by Consecutive Basic Amino Acids—Gene regulation mediated by the synthesis of specific amino acid sequences and associated translation arrest has been observed in prokaryotes and eukaryotes. Translation arrest of *secM* mRNA is coupled with protein secretion and plays a crucial role in the autoregulation of SecA protein expression (7, 28). The SecM nascent peptide interacts with the exit tunnel of the ribosome at the discrimination gate, thereby regulating the translation elongation rate of *secM* mRNA (6, 7). We found that 12 consecutive basic residues caused translation arrest, suggesting that positively charged side chains interact with the ribosome tunnel formed primarily by ribosomal RNA (29). The exit tunnel wall of the mammalian ribosome has a negative electrostatic potential (10) and may interact with peptides that contain positively charged residues (12). The interaction partner for the consecutive positively charged side chains has yet to be determined, although the phosphate rRNA backbone is a good candidate. Recently, it was shown that translation of nonstop mRNA is repressed after initiation and that the ribosome stalls on poly(A) sequences in mammalian cells (5). In addition, translation of poly(A) mRNA was less efficient in an *in vitro* system using rabbit reticulocyte lysates (5). Therefore, translation repression by consecutive basic amino acids may be conserved among eukaryotes.

Degradation of Translation Arrest Products Depends on Not4p—We found that Not4p is required for the degradation of translation arrest products. Not4p is also involved in transcriptional regulation via trimethylation of H3K4 (16, 17). Therefore, one could argue that Ccr4-Not4 may play a crucial role in the transcription of proteasomal subunits, an activity that may be impaired in the *not4Δ* mutant, leading to the stabilization of translation arrest products. Two lines of evidence do not support this possibility, however. First, we found that the levels of ubiquitinated proteins were markedly higher in the *rpn6–2* mutant compared with the *not4Δ* mutant, although the increased level of the truncated GFP product was less than that in the *not4Δ* mutant (Fig. 3*D*). Second, translation of nonstop mRNA was not enhanced in the *not4Δ* mutant (Fig. 4*A*), although the nonstop product was degraded by the proteasome (3, 4). These findings suggest that the reduced degradation of translation arrest products in the *not4Δ* mutant is not due to a general defect in proteasomal activity.

We found that translation arrest induced by a stable stem-loop does not lead to arrest product degradation by the proteasome (Fig. 1*B*). This result is consistent with the idea that specific nascent peptide sequences are required for cotranslational degradation of arrest products by the proteasome. What is the
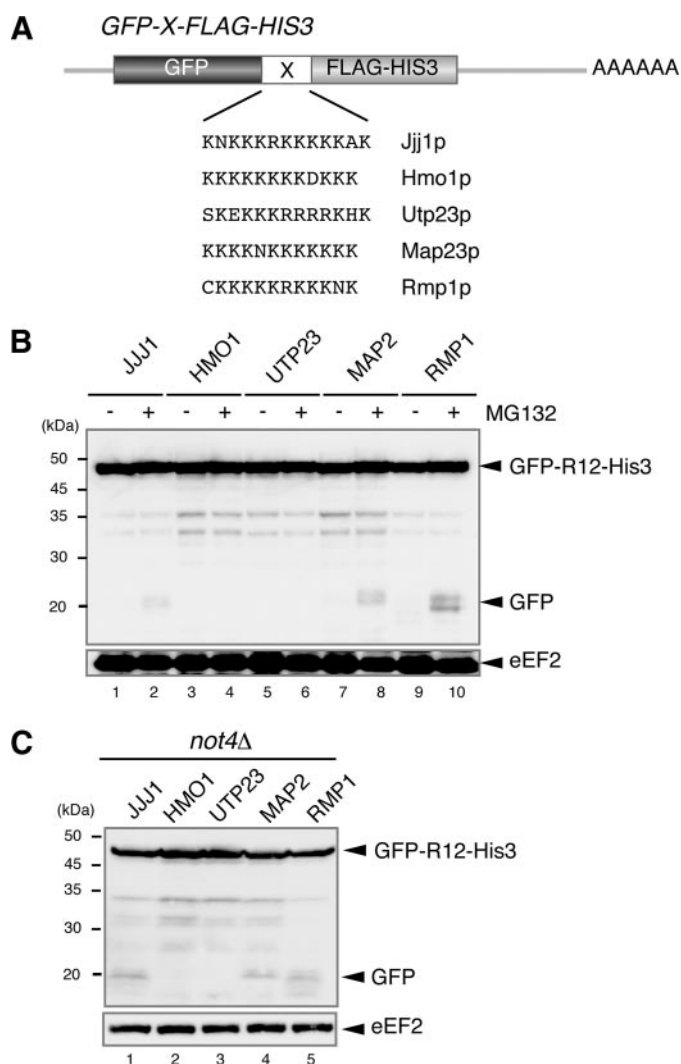
FIGURE 5. **Endogenous sequences induce translation arrest coupled with protein degradation.** *A*, construction of the p*GPDp-GFP-X-FLAG-HIS3* reporter plasmids. These plasmids contain various sequences inserted between *GFP* and *HIS3*. The inserted amino acid sequences and original gene names are shown. *B*, endogenous sequence elements induce translation arrest coupled with protein degradation by the proteasome. W303 cells were transformed with p*GPDp-GFP-X-FLAG-HIS3* plasmids that contained sequences from the indicated genes. The cells were grown in SC-Ura, and the samples were analyzed using Western blotting with anti-GFP antibodies. When indicated (+), cell extracts were prepared 2 h after the addition of 0.2 mM MG132. *C*, translation arrest products in the *not4Δ* mutant. W303*not4Δ* cells were transformed with p*GPDp-GFP-X-FLAG-HIS3* plasmids that contained sequences from the indicated genes. The cells were grown in SC-Ura, and the samples were analyzed using Western blotting with anti-GFP antibodies.

mechanism underlying cotranslational degradation of the specific translation arrest products? One possibility is that the stalled ribosome may have a high affinity for Not4p, leading to efficient ubiquitination of the nascent peptides. It has been shown that a SecM nascent peptide modulates the conformation of the ribosome (30), indicating that the interaction between a nascent peptide and the exit tunnel wall can regulate translation by modulating the conformation of the ribosome. We suspect that interactions between certain nascent peptides and the exit tunnel wall lead to changes in the conformation of the ribosome and increase binding between the ribosome and Not4p, thereby regulating protein degradation.

It was shown previously that the interaction between Not4p and Ubc4p, which is dependent on the Not4p RING finger domain, is required for the ubiquitin-protein ligase activity for the EGD complex (18, 19). Ubc4p has recently been shown to interact with the 26 S proteasome in response to hygromycin B-induced translational misreading (18). Results from this study suggest that translation arrest induced by consecutive basic amino acids leads to Not4p-mediated protein degradation by the proteasome. Further, we propose a more general regulatory process in which translation arrest caused by specific nascent peptides leads to Not4p-dependent cotranslational protein degradation by the proteasome. Not4p is associated with ribosomes (Fig. 3*H*) and may be involved in the ubiquitination of aberrant nascent polypeptides on ribosomes by recruiting the E2 enzymes Ubc4p and Ubc5p as well as the proteasome.

Translation Arrest by Endogenous Basic Amino Acid Sequences—The results of this study led us to hypothesize that nascent peptides may play more general roles in translation elongation, protein stability, and protein expression, probably by modulating the ribosome structure. We found that the CKKKKKRKKKNK sequence from Rmp1p can induce translation arrest (Fig. 5). Rmp1p is a cytoplasmic component of the RNase MRP (31), an endonuclease that contributes to cell cycle-regulated degradation of daughter cell-specific mRNA (32). Because translation arrest leads to endonucleolytic cleavage of mRNA in prokaryotes and eukaryotes (8, 9, 33–36), translation arrest induced by consecutive basic amino acid sequences may lead to an endonucleolytic cleavage of mRNA, and RNase MRP may be responsible for this cleavage activity. It is likely that the cleavage products are degraded from the 3′ end by an exosome or from the 5′ end by Xrn1p and would only be detected in mutant cells. Experiments to investigate this possibility are ongoing.

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