Nup145p is a component of the nuclear pore complex of *Saccharomyces cerevisiae* and is essential for mRNA export. Nup145p and its apparent vertebrate homologue are the only known nucleoporins to be composed of two functionally independent peptide moieties resulting from the post-translational cleavage of a large precursor molecule. In this study, the proteolytic cleavage site of Nup145p has been mapped upstream of an evolutionary conserved serine residue. Cleavage occurs at the same site when a precursor is artificially expressed in *Escherichia coli*. A hydroxyl-containing residue is critical for the reaction, although a thiol-containing residue offers an acceptable replacement. In vitro kinetics experiments using a purified precursor molecule demonstrate that the cleavage is self-catalyzed and that the catalytic domain lies within the N-terminal moiety. Taken together, our data are consistent with a proteolytic mechanism involving an N>O acyl rearrangement and a subsequent ester intermediate uncovered in other self-processing proteins.

Nuclear pore complexes (NPCs) are large structures through which nucleocytoplasmic exchange of soluble macromolecules occurs in eukaryotic cells. The NPCs of yeast and higher eukaryotes share the same basic architecture, including a characteristic 8-fold symmetry, and are believed to be functionally similar (1–3). Constitutive and regulated import or export through NPCs is generally energy-dependent and requires substrate-specific transporters that are targeted to the NPC via repeat-containing nucleoporins (or nuclear pore proteins) (4–6). In yeast, about 30 nucleoporins have already been identified (7). Yet their precise role in transport reactions and/or in NPC biogenesis is far from being understood.

One of the components of the NPC of *Saccharomyces cerevisiae*, Nup145p, is expressed as a 145-kDa precursor that is rapidly processed to yield two moieties, a N-terminal domain of 65 kDa (N-Nup145p) and a C-terminal domain of 80 kDa (C-Nup145p) (8, 9). Repression of NUP145 (YGL092w) leads to the rapid accumulation of mRNA molecules in the nucleus, concomitant with a nucleolar disorganization and a clustering of NPCs (10–12).

Previous experiments in which the N- and C-Nup145p domains were expressed separately or as a part of a noncleavable precursor have shown that the two moieties perform distinct functions in the cell (9). C-Nup145p is a component of a NPC subcomplex that includes Nup120p, Nup85p, Nup84p, Seh1p, and Sec13p (9, 13). Mutations in most of these nucleoporins, including C-Nup145p, severely affect mRNA export and nuclear architecture (8, 9, 12, 13). The mature N-Nup145p domain does not belong to this complex. Nevertheless, deletion of the corresponding part of the NUP145 gene is lethal in a genetic context where other nucleoporins, such as Nup188p and Nle96p, are mutated (9). Whereas the function of C-Nup145p is not disturbed in mutants of Nup145p where processing is abolished, the function of N-Nup145p is affected as judged from its interaction with Nup188p. In addition, mislocalization of N-Nup145p in the nucleoplasm occurs when it is expressed separately from C-Nup145p (9). Thus, the existence of a precursor molecule, followed by its processing, are a requisite for the function of N-Nup145p for reasons that remain to be elucidated.

Interestingly, the processing of Nup145p is conserved in evolution. It was recently reported that the putative rat homologue of Nup145p is expressed as a Nup98-Nup96 precursor that is also cleaved in vivo to generate two nucleoporins, the previously characterized nucleoporin Nup98 (14–16), which shares similarity with N-Nup145p and a novel nucleoporin, Nup96 (17). Nup98, expressed independently from an alternatively spliced mRNA is also proteolytically processed (17). As in yeast, the correct maturation of the Nup98-Nup96 and Nup98 precursors is essential for the proper localization of the subsequent cleavage products, suggesting that it may be important for NPC assembly (17).

In this paper, we demonstrate the self-catalyzed processing of Nup145p and localize the processing activity in the N-terminal moiety. The data presented suggest a mechanism involving an ester intermediate as has been described for other self-processing proteins.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The *Escherichia coli* expression vector pE151 was constructed by inserting a *Nde*I-*Bam*HI-digested polymerase chain reaction product, obtained using oligonucleotides A and B (Table I) on a *GST-NUP145* fusion gene (10), into pET16b (Novagen). This vector expresses the His<sup>6</sup>NC<sup>691</sup>His construct.

The yeast expression vector pE165 contains the same construct placed under the doxycycline repressible promoter of the plasmid pCM190 (18). This construct was obtained by co-transformation (19) in yeast strain FYB2L-5D (20) of a polymerase chain reaction product (using oligonucleotides C and D on pE151) with the pCM190 linearized DNA (21). Expression was monitored by Western blotting, and the recombinant plasmid was subsequently transformed (22) into the protease-deficient yeast strain BJ2168 (23).

The vectors pE172–175 expressing the His<sup>6</sup>NC<sup>691</sup>HA proteins were constructed as was pE151 except that the polymerase chain reaction...
product was amplified using oligonucleotides E (which introduces the RRASV site) and any of oligonucleotides F, G, H, and I (which introduce the HA tag preceded by the wild type and S606C, S606T, and S606A mutations, respectively). The inserts of these plasmids were completely sequenced by the MWG-Biotech. For cloning manipulations E. coli strains TG1 (Transgene) or XL2-blue (Strategene) were used.

Total Protein Extracts and Western Blotting—Purified tagged proteins or those in total extracts from yeast (9) or E. coli (24) were detected by Western blot using the anti-N-Nup145p polyclonal antibody (10), a monoclonal (HIS-1) anti-polyhistidine-peroxidase conjugate (Sigma), diluted at 1:10,000 and anti-HA-peroxydase conjugate at a dilution 1:500. The peroxidase conjugates were revealed with ECL (Amersham Pharmacia Biotech).

His396NC613HA Purifications—Yeast cells expressing the His396NC613HA construct were grown in 1 liter of minimal medium (25) lacking uracil at an A600 of 7.8, washed, frozen at −70 °C, and resuspended in 25 ml of buffer A (150 mM NaCl, 0.1% Triton X-100, 20 mM Tris, pH 8) containing 20 mM imidazole. Cells were broken with a French press at 20,000 p.s.i., and the homogenate was clarified by centrifugation at 20,000 g for 30 min. Supernatant was injected into a 1-ml Hitrap Chelating column (Amersham Pharmacia Biotech) previously loaded with NiSO4. The column was washed with 5 volumes of buffer A containing 20 mM imidazole and eluted with a linear imidazole gradient from 20 to 300 mM in buffer A. The His396NC613HA and C891His cleavage products eluted at the highest concentration of imidazole.

E. coli cells B2/1(D3/lys) (Novagen) expressing the His396NC613HAHis construct were induced overnight in 800 ml of LB medium containing 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, and 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were washed, pelleted, resuspended in buffer B (150 mM NaCl, 0.1% Triton X-100, 20 mM Hepes, 8 mM urea, pH 8) containing 20 mM imidazole, and sonicated. The homogenate was centrifuged at 40,000 × g for 30 min, and the supernatant was loaded onto a 1-ml HiTrap Chelating column (Amersham Pharmacia Biotech) in an Akta purifier system (Amersham Pharmacia Biotech). The column was washed with buffer B containing 20 mM imidazole and eluted with a linear gradient of imidazole from 20 to 500 mM in buffer B. The tagged products eluted at 200 mM imidazole. Fractions were pooled, diluted 1:8 in buffer C (20 mM Hepes, 8 mM urea, pH 8) containing 20 mM NaCl, injected in a Hitrap G column (Amersham Pharmacia Biotech), and eluted with a linear gradient of NaCl from 20 to 600 mM in buffer C. Two major peaks containing, respectively, the His396NC613HAHis (100 mM NaCl, fraction Ec2; see Fig. 2) and the C891His (350 mM NaCl, fraction Ec1; see Fig. 2) were kept for analysis.

Microsequencing and Mass Spectrometry Determination—N-terminal sequences of the His396NC613HAHis fractions purified in yeast and E. coli (see Fig. 2) were determined by Edman degradation. In the Ec1 fraction (see Fig. 2), two minor bands migrating just above the major band corresponding to C891His (with an N terminus Ser-Ile-Trp) were also sequenced and revealed N termini Met-Lys-Asp and Met-Arg-Glu, corresponding to internal sequences of Nup145p at positions 564 and 583, respectively. We suggest that these forms correspond to products of internal initiations of translation that could not be processed. For the mass-spectrometry determination, 150 µg of purified His396NC613HA from E. coli (fraction Ec2; see Fig. 2) was dialyzed against 50 mM ammonium bicarbonate and lyophilized. An aliquot of the pellet was dissolved in 100 µl of water/methanol/formic acid (50:50:10) and introduced to a API 365 triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Canada).

RESULTS

Analysis of the Cleavage Products of Nup145p—Previous results suggested that cleavage of Nup145p (Fig. 1) occurs around residues 606–609 (9), but the cleavage site was not located precisely. Therefore, as a first step toward characterization of the processing mechanism, we decided to characterize the cleavage products of Nup145p. To do so, a truncated version of Nup145p (10) containing the expected cleavage region and fused at its N and C termini with several His residues (His396NC613His; Fig. 1) was overexpressed in yeast and purified by affinity chromatography using a Nickel column. A Coo-massie Blue staining of the purified products (Fig. 2; lane Sc) shows a major band corresponding in size to the His396NC613His (around 30 kDa) and a minor band corresponding in size to the C891His (around 40 kDa), demonstrating that this truncated construct retains the capacity to be cleaved in vivo. The difficult solubilization of C891His under the native conditions used for this experiment might account for the nonstoichiometry of these two cleavage products. Despite its low abundance, the C891His moiety was gel purified and subjected to Edman degradation. The sequence Ser-Ile-Trp was found at the N terminus of C891His. This sequence corresponds to a unique internal sequence of Nup145p at positions 606–609 (Fig. 1) and thus demarcates cleavage of the peptide bond immediately upstream of the serine 606.

Nup145p Cleavage Occurs in Heterologous System—Auto-proteolysis at internal sites preceding serine, cysteine, or threonine positions is now established for several proteins (28). It demonstrates cleavage of the peptide bond immediately up-stream of the serine 606.
contain, respectively, the His396N (Ec2) and the C891His (Ec1). The major peaks recovered after the anion exchange chromatography and followed by Coomassie Blue staining.

E. coli (His396NC891His precursor products purified from S. cerevisiae) that is present in E. coli itself. Thus, Nup145p cleavage either requires a trans-acting factor generated by in vivo degradation. Analyzed fractions contained mainly the His396N or the C891His form (Fig. 2, lane Ec1 and Ec2) correspond to the two major peaks recovered after the anion exchange chromatography and contain, respectively, the His396N (Ec2) and the C891His (Ec1). The weak band corresponding to mature C891His in yeast is marked with a dot. STD stands for protein standards.

Fig. 2. Cleavage products of Nup145p in yeast and E. coli. His396NC891His precursor products purified from S. cerevisiae (Sc) and E. coli (Ec) were analyzed by SDS-PAGE (8–16% precast gel, Bio-Rad) followed by Coomassie Blue staining. Ec1 and Ec2 correspond to the two major peaks recovered after the anion exchange chromatography and contain, respectively, the His396N (Ec2) and the C891His (Ec1). The weak band corresponding to mature C891His in yeast is marked with a dot. STD stands for protein standards.

Under denaturing conditions on a Nickel column followed by an anion exchange chromatography. Analyzed fractions contained mainly the His396N or the C891His form (Fig. 2, lane Ec1 and Ec2, respectively), confirming that the cleavage of this precursor also takes place in E. coli. Note that the His396N produced in E. coli has a slightly slower electrophoretic mobility in SDS-PAGE than the His396N produced in yeast. This might be due to post-translational modifications in S. cerevisiae (see “Discussion”).

N-terminal microsequencing of the C891His gel purified band (Fig. 2, lane Ec1) revealed an N terminus beginning with the sequence Ser-Ile-Trp, showing that the N terminus of C891His generated by in vivo cleavage is the same in yeast and E. coli. Thus, Nup145p cleavage either requires a trans-acting factor that is present in E. coli or is catalyzed by the Nup145p protein itself.

The results presented above show that cleavage occurs at the peptide bond preceding the serine 606 generating a free amino group. To characterize the C-terminal generated by the proteolytic reaction, we analyzed the purified His396N produced in E. coli (Fig. 2, lane Ec2) by mass spectrometry. One peak at 26,428 ± 83 Da was obtained that corresponds to the theoretical mass expected for the His396N lacking the first methionine (26,427.41 Da). N-terminal microsequencing of the gel purified band (Fig. 2, lane Ec2) indeed confirms that His396N starts with the glycine immediately following the methionine.

From these measurements, we conclude that proteolysis occurs between phenylalanine 605 and serine 606 and that the last residue of His396N generated by cleavage is not additionally modified. Cleavage in E. coli is therefore the result of hydrolysis of the 605–606 peptide bond, generating two peptides with free C and N termini.

Role of the Hydroxyl Side Chain of the Serine 606—Self-proteolysis reactions preceding serines, cysteines, or threonines involve a nucleophilic attack by the hydroxyl or thiol group of the respective amino acids on the preceding peptide bond, resulting in the replacement of the peptide bond by an ester or a thioester bond (28). These bonds are more reactive than the peptide bonds and can then be attacked by a second nucleophile and broken. This model implies that any of the three residues serine, cysteine, or threonine is essential for the reaction and can be replaced by one another with only limited effects on catalytic activity (29, 30).

Because Nup145p cleavage takes place before a serine, we tested the importance of the hydroxyl residue in the reaction. Therefore, serine 606 was replaced by a cysteine, a threonine, or an alanine by directed mutagenesis. For experimental purposes, we used an even shorter truncated version of Nup145p (His398NC613HA; Fig. 1), where the C-Nup145p is almost entirely substituted by the HA tag. Each construct was then transformed into E. coli, and expression of the encoded protein was induced. The processing capacity of the different precursors was analyzed at different times after induction (Fig. 3).

For the wild type form His398NC613HA(WT), a major band that corresponds in size to the mature N-terminal moiety is revealed by Western blot using the antibody directed against the His tag (Fig. 3, α-His). This band is nearly constant in intensity for all time points analyzed after induction. The antibody directed against the HA tag only detects a faint band migrating about 2 kDa above the mature form as expected for the precursor molecule (Fig. 3, α-HA). This experiment indicates that the truncated version of Nup145p retains the capacity to undergo cleavage in vivo and that the processing is already efficient even at early stages after induction. Thus, deletion of most C-Nup145p does not affect the cleavage capacity.

![Fig. 1. A schematic representation of the wild type Nup145p and its truncated versions.](image-url)
Self-catalyzed process, we undertook the purification of a precursor definitively that the cleavage of the Nup145p precursor is a Coomassie Blue staining (Fig. 4). The purification was assessed by SDS-PAGE followed by (45 min) at 30 °C (data not shown). Protein products were then sor form accumulates. The best yield of uncleaved precursor be very difficult because of the high efficiency of the in vivo processing (see above). We therefore made use of the mutants S606C and S606T to define conditions under which the precursor form. Expression of the S606C mutant produced two anti-His reacting forms. They correspond respectively to the precursor form, which also reacts with the anti-HA antibody and with the mature N-terminal form (Fig. 3), showing that proteolytic cleavage can take place in this mutant. The precursor form is the major product at early stages after induction (Fig. 3, α-His, 1.5 h and decreases at later stages (Fig. 3, α-His, 6 h). This shows that the cleavage reaction is delayed in this mutant compared with the results obtained for the wild type. The situation is similar for the S606T mutant, with the difference that the ratio of precursor to product observed along the time course is slightly lower, suggesting that this mutation is less deleterious to the cleavage reaction in vivo. Overall, these results suggest that a hydroxyl- or thiol-containing residue at the cleavage junction is required for the reaction.

In Vitro Cleavage of a Purified Precursor—To demonstrate definitively that the cleavage of the Nup145p precursor is a self-catalyzed process, we undertook the purification of a precursor form and tested its ability to undergo self-cleavage in vitro. Precursor purification from the wild type construct would be very difficult because of the high efficiency of the in vivo processing (see above). We therefore made use of the mutants S606C and S606T to define conditions under which the precursor form accumulates. The best yield of uncleaved precursor was obtained using the S606C mutant induced for a short time (45 min) at 30 °C (data not shown). Protein products were then extracted and purified on a nickel column under native conditions. The purification was assessed by SDS-PAGE followed by Coomassie Blue staining (Fig. 4A). Two major bands corresponding respectively to the His<sup>398NC613HA</sup>(S606C) precursor and to the His<sup>398N</sup> product were obtained, indicating that partial cleavage of the precursor does occur during extraction but that the proportion of precursor molecules is sufficiently high to allow subsequent analysis in in vitro reactions.

To quantify the cleavage reactions, we chose to follow radio-labeled products. For this purpose, we introduced the artificial pentapeptide phosphorylation site RRASV (26, 27) following the His tag in the His<sup>398NC613HA</sup> construct. Using [γ<sup>32</sup>P]ATP as substrate, the purified mixture His<sup>398NC613HA</sup>(S606C): His<sup>398N</sup> was labeled by phosphorylation using a commercial bovine kinase. Analysis of proteins by SDS-PAGE followed by gel scanning shows that both His<sup>398NC613HA</sup>(S606C) and His<sup>398N</sup> were successfully labeled (Fig. 4B, 0 min).

The next step was to determine adequate conditions for the in vitro cleavage. To do so, we hypothesized that the requirement for a hydroxyl (or thiol) group at the cleavage site of Nup145p precursor reflects the involvement of an ester (or a thioester) intermediate in the mechanism. Because thioesters are particularly susceptible to nucleophiles (31), we decided to test for the reactivity of His<sup>398NC613HA</sup>(S606C) in the presence of DTT known to accelerate this type of reaction (30, 32, 33). The in vitro cleavage was then performed with or without 50 mM DTT at 30 °C, and aliquots of the reaction were analyzed at different times (Fig. 4B). In the presence of DTT, the band corresponding to the precursor decreases concomitantly with the increase of the band corresponding to the mature product. This precursor-product kinetic relationship demonstrates that His<sup>398NC613HA</sup>(S606C) has the capacity to self-process in vitro into His<sup>398N</sup> in the presence of DTT. In the absence of DTT, the ratio His<sup>398NC613HA</sup>(S606C):His<sup>398N</sup> is maintained during the 2 h of the reaction (Fig. 4B). Because DTT-induced cleavage could involve the thiol as a nucleophile or as a reductant, we made use of a nonthiol disulfide reductant, the tri(2-carboxyethyl)phosphine (34). We found that the half-life of the precursor in the presence of 50 mM tri(2-carboxyethyl)phosphine was increased 15 times compared with the reaction in the presence of 50 mM DTT in a 2 h reaction (see below and data not shown). This suggests that the accelerating effect of DTT on the in vitro cleavage reaction is due to its ability to carry out nucleophilic attack. Longer times of incubation were attempted, but results were obscured by unspecified secondary reactions, which might be due to partial denaturation of the precursor. Consistent with this hypothesis is the observation that reaction is less efficient at higher concentrations of protein, resulting in its precipitation (data not shown).

Quantification of the reaction was done from three independent experiments (Fig. 4C). At time 0 of the experiments, the ratio His<sup>398NC613HA</sup>(S606C):His<sup>398N</sup> was ~60:40. In the presence of DTT, this ratio decreased to about 30:70 in 2 h, whereas in the absence of DTT, it remained unchanged. The points obtained for the incubation in the presence of DTT suggest a (pseudo) first order reaction, and the precursor half-life time is comprised between 8.6 and 11.5 min. Under the conditions used, approximately half of the purified precursor was able to cleave into the mature form. The incomplete character of the reaction might be due to partial denaturation of the molecules as mentioned above. However, we cannot exclude the possibility that the independent secondary reactions may become non-negligible at late times of incubation, either because of the purification process or because of other uncharacterized properties of the precursor.
DISCUSSION

Post-translational cleavage of the yeast nucleoporin Nup145p generates two functionally distinct proteins. The different lines of evidence presented here support the notion that this reaction is self-catalyzed. First, Nup145p cleavage is observed in heterologous expression systems including *E. coli* and a mammalian *in vitro* transcription/translation system (data not shown). Second, a purified precursor form of Nup145p is cleaved *in vitro* following a (pseudo) first order reaction kinetics (at least for the early times of the reaction) and cleavage occurs in highly diluted samples, free of detectable contaminants. The participation of a hypothetical specific trans-acting factor, conserved in both prokaryotes and eukaryotes, is therefore highly improbable. Third, all our results are consistent with the mechanism described for autoproteolysis taking place in peptide bonds preceding hydroxyl- or thiol-containing residues; in particular, the *in vitro* reaction studied here is dependent upon the presence of DTT.

It has been known since the 1960s that peptide bonds involving hydroxyl-containing residues can shift to ester bonds (N-O acyl rearrangement) under laboratory conditions (35). There is now formal evidence that these reactions exist in several protein maturation pathways, including protein splicing (36), *hedgehog* protein maturation (32), pyruvoyl enzymes (37), and N-terminal nucleophile hydrolases (29, 38) activations. In these cases, the hydroxy- or thiol-containing residues, serine, threonine, or cysteine, undergo a N-O acyl-shift resulting in an ester or thioester bond much more reactive than the peptide bond. The ester or thioester bond is then resolved by an attack by a second nucleophile that can be for example water (N-terminal nucleophile hydrolases) (39) or cholesterol (*hedgehog*) (40). Our results do not provide direct evidence for the exact reaction mechanism involved but are consistent with previous mechanisms since they include (i) the cleavage site before a serine, (ii) requirement for a hydroxyl or thiol group at the cleavage position, (iii) the sensitivity of *in vitro* cleavage of the S606C mutant to DTT, and (iv) the incapacity of tris(2-carboxyethyl)phosphine to promote such a cleavage. In addition, cleavage of Nup145p in *E. coli* results from the hydrolysis of peptide bond 605–606, indicating that water might account for the second nucleophilic attack. We cannot exclude, however, that the proteolysis in yeast may involve other modifications, as suggested by the difference in electrophoretic mobility observed for the His396N produced in yeast and in bacteria. This situation may be reminiscent of *hedgehog* processing, where the covalent addition of cholesterol during the self-catalyzed processing results in an increased mobility on SDS-PAGE of the mature protein (40, 41).

The catalytic domain for the proteolysis of Nup145p must be included between amino acids 398 and 613 because the minimal protein used in this study, His398NC613HA, is still able to undergo self-processing. Taken together with other independent experiments (8), the catalytic site can be narrowed down to the regions containing amino acids 398–523 and 593–613. Furthermore, deletion of the region comprised between residues 470 and 551 abolishes cleavage (10). This latter region

**FIG. 4.** In *vitro* cleavage of Nup145p in the mutated version His398NC613HA(S606C). A, *E. coli* His398NC613HA(S606C):His398N purified mixture resolved by SDS-PAGE (13% gel) and stained with Coomassie Blue. STD stands for protein standards. B, the purified mixture was radiolabeled and diluted in the reaction buffer with (+DTT) or without (−DTT) 50 mM DTT. The samples taken at different times were analyzed by SDS-PAGE (13% gel) and gel scanning. Total amounts of radioactivity are not significantly different between lanes. C, quantification of three independent experiments as in B.
contains a RNP-I-like octapeptide motif (Fig. 1) and was proposed to be responsible for RNA affinity of Nup145p in vitro (10). Preliminary experiments show that point mutations in this octapeptide also affect cleavage (data not shown), suggesting that this region may be part of the catalytic site or necessary for its correct folding.

Nup145p has a putative homologue in vertebrates, Nup98-Nup96, that is also cleaved in vitro at the serine of the conserved cleavage site (17). Sequence similarity between Nup98-Nup96 and Nup145p (9, 17) is particularly extensive in the region that we have determined as necessary for the self-processing of Nup145p. It can thus be proposed that the mechanism of processing of the Nup98-Nup96 precursor is also self-catalyzed. Self-processing systems offer the advantage that the specificity is built up into a single molecule (42) and limit the control of the cell over the reaction unless specific interactions may affect the reaction in vitro. Interestingly, maturation of either yeast Nup145p or rat Nup98-Nup96 has a direct role in the localization of the precursor and the subsequent cleavage products (9, 17). Thus, self-cleavage of these nucleoporin precursors may have been conserved in eukaryotes for the specific targeting to the NPC.

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Note Added in Proof—Another report of self-catalysis of rat Nup98 precursor appeared while this article was in press (Rosenblum, J. S., and Blobel, G. (1999) Proc Natl Acad Sci U S A 96, 11370–11373).

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