The translation product of the VMA1 gene of Saccharomyces cerevisiae undergoes protein splicing, in which the intervening region is autocatalytically excised and the flanking regions are ligated. The splicing reaction is catalyzed essentially by the in-frame insert, VMA1-derived endonuclease (VDE), which is a site-specific endonuclease to mediate gene homing. Previous mutational analysis of the splicing reaction has been concentrated extensively upon the splice junctions. However, it still remains unknown which amino acid residues are crucial for the splicing reaction within the entire region of VDE and its neighboring elements. In this work, a polymerase chain reaction-based random mutagenesis strategy was used to identify such residues throughout the overall intervening sequence of the VMA1 gene. Splicing-defective mutant proteins were initially screened using a bacterial expression system and then analyzed further in yeast cells. Mutations were mapped at the N- and C-terminal splice junctions and around the N-terminal one-third of VDE. We identified four potent mutants that yielded aberrant products with molecular masses of 200, 90, and 80 kDa. We suggest that the conserved His residues (266, 267), newly identified as the essential residue for the splicing reaction, contributes to the first cleavage at the N-terminal junction, whereas His (267) assists the second cleavage by Asn cyclization at the C-terminal junction. Mutations in these regions did not appear to destroy the endonuclease activity of VDE.

Protein splicing is a posttranslational process, in which an intervening protein sequence is autocatalytically removed from a precursor protein and the two flanking sequences are ligated (1). In the budding yeast Saccharomyces cerevisiae, the nascent 120-kDa VMA1 translational product (Vma1 protozyme (1)) catalyzes protein splicing to yield a 70-kDa catalytic subunit of the vacuolar H+-ATPase and a 50-kDa site-specific endonuclease (VMA1-derived endonuclease; VDE) (2, 3). Since the discovery of protein splicing in S. cerevisiae (2, 3), this compelling reaction has been found in a number of protozymes in eucarya (2–4), bacteria (5–8), and archaea (9, 10).

The VDE region in the Vma1 protozyme plays a central role for the protein splicing reaction (11, 12). We have also shown that VDE bracketed by only 6 proximal and 4 distal amino acids is autocatalytically processed in vitro (13). These results support the idea that the VDE sequence and a few external amino acids contain sufficient information for protein splicing. Only short amino acid sequence motifs are conserved among protein splicing elements (7). Thiol- or hydroxyl-containing Cys, Ser, or Thr is found at both splice junctions. A few hydrophobic amino acids are present in front of an invariant His-Asn dipetide at the C-terminal splice junction. These residues around the splice sites have been found to play key roles in the protein splicing reaction, based mostly on site-directed mutagenesis studies (5, 10, 11, 12).

To understand a mechanism and structural integrity for protein splicing in the Vma1 protozyme, we performed a systematic search for splicing-defective mutants. We introduced random mutations throughout the entire VDE region by an error-prone PCR method for the first time and mapped three core regions essential for the splicing reaction.

EXPERIMENTAL PROCEDURES

Strains—Escherichia coli strain SCS1 carrying plasmid pGEX-VMA1 (see below) was used to produce the recombinant GST-Vma1 fusion protein. E. coli strain BL21(DE3) carrying plasmid pET-17bVDE (see below) was used to produce recombinant VDE. Yeast strain NY101, which is a vma1::URA3 derivative of YPH499, containing plasmid pSN001,2 was used to express the full-length VMA1 gene product.

Plasmids—To express a GST-Vma1 fusion protein, an expression plasmid pGEX-VMA1 was created as follows. A 652-bp ScaI-ScaI fragment from plasmid pMVMA1 (11) was ligated into the vector pGEX-5X-3 (Pharmacia Biotech Inc.), which had been digested with EcoRI and blunt-ended by T4 DNA polymerase. The resultant plasmid, which had the insert in the correct orientation, was digested with BgII and SmaI, and then a 1.8-kb BgII-NcoI (blunted) fragment from pMVMA1 was introduced into this gap to generate plasmid pGEX-VMA1. The numbering of the amino acid sequence in this report refers to the numbering of the original VMA1 gene product (2).

To express the full-length VMA1 gene product in yeast cells, the 2.2-kb KpnI-SphI fragment of the pGEX-VMA1 plasmid was ligated into pSNO01 that had been digested with the same enzymes.

To express recombinant VDEs in E. coli, a plasmid pET-17bVDE was constructed as follows. The pET-17b vector (Novagen) was digested with NheI and BamHI, blunt-ended by T4 DNA polymerase, and self-ligated. The resultant plasmid was digested with EcoRV, and a Mr1 linker, dGACGGCTC, (New England Biolabs) was inserted into this gap to create a stop codon, yielding pET-17bΔNE. The BanHI-EcoRI fragment of wild-type or mutant pGEX-VMA1 was introduced into the vector.

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1 The abbreviations used are: VDE, VMA1-derived endonuclease; Vma1p, spliced product of the Vma1 protozyme, the 70-kDa subunit of the yeast vacuolar membrane H+-ATPase; GST-Vma1p, glutathione S-transferase-Vma1 fusion protein; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis.

2 S. Nogami, Y. Satow, Y. Ohya, and Y. Anraku, submitted for publication.

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Protein Splicing of the Yeast Vma1 Protozyme

BamIII-EcoRI gap of pET-17b-NE to create pET-17bVDE. The pET-17bVDE expresses VDE bracketed by 18 proximal (MARIPRNYSNS-DNAIIYVG) and 17 distal (CGERGNEMAEVLMEFCR) amino acids.

VDE cleaves the VMA1 gene, which lacks the VDE coding sequence (VDE) at the precise insertion site (14, 15). A plasmid substrate pBS-JVDE, which contains a VDE cleavage site, was digested with BamHI and EcoRI, to produce pBS-PJ. A VDE cleavage site was generated by a PCR-based deletion mutagenesis using a standard PCR condition and Pfu polymerase (Stratagene). One reaction mixture contained oligonucleotide primer A (5'-ATCTTGGGATCCGACGAAATGATGTTAACTGC-3') (codons 557–577); primer B (5'-ACCTCTTCTCGACGGAGCTATGAAATGCTCA-3'), which has 15 nucleotides (codons 2212–2226) followed by 22 nucleotides (838–849) of the strand; and pMVMA1 as a template. Another reaction mixture contained primer C (5'-ATCTATGTGCGGAGGAAAGTGATGAACT-3') (72° C for 1 min, 55° C for 1 min, and 72° C for 1 min). The PCR product was digested with BamHI and EcoRI, and the resultant 72-bp BamI-EcoRI fragment was amplified and introduced into the 1.4-kb BamHI-EcoRI gap of pBS-PJ to yield pBS-JVDE. The BamI-EcoRI region of pBS-JVDE was confirmed by direct sequencing.

PCR Mutagenesis—Random mutations were introduced throughout the VDE region of pGEX-VMA1 plasmid by error-prone PCR (16). The 5'-primer was 5'-CGTGGGAATCCCGGAAATGATGTTAACTGC-3', which contains a BamHI site (underlined). The 3'-primer was 5'-GGGAATTCCCATCAAGACTTCTGCCATTTC-3', which contains an EcoRI site (underlined). The reaction mixture contained 100 ng of pGEX-VMA1 plasmid, 0.01 OD units of each primer, 0.2 mM dATP, 0.2 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.01% gelatin, 7 mM MgCl2, and 5 units of Taq polymerase (Takara) in a 100-μl volume. To avoid extreme mutagenic effect, MnCl2 was not supplemented. The reaction conditions were as follows: 25 cycles of 94° C for 1 min, 55° C for 1 min, and 72° C for 1 min. The PCR product was digested with BamHI and EcoRI and then ligated to pGEX-VMA1 that had been digested with the same enzymes.

Screening for Protein Splicing-defective GST-Vma1 Mutants in E. coli—E. coli strain SCS1 carrying mutagenized pGEX-VMA1 was cultured in 1 ml of LB medium containing 100 μg/ml ampicillin in 24-well tissue culture plates. The culture was grown at 37 °C to the exponential phase. Then 0.1 ml IPTG was added, and the culture was transferred to room temperature. After an additional 3-h incubation, total cell extracts were prepared by pelleting the cells for 2 min at 10,000 × g followed by lysis in 50 μl of SDS-PAGE sampling buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, 0.01% bromphenol blue) and boiling for 3 min. Samples (5 μl) were subjected to Western blotting analysis, which was done essentially as described previously (2, 13). Expression of Mutant VDEs in Yeast—Yeast strain NY101 carrying pS2001 plasmid was used for the complementation test of mutant VMA1 genes. pY3014 plasmid was used as a control vector. The complementation test was done using YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with 100 mM CaCl2. Wild-type and mutant cell extracts were prepared from cultures grown at 30 °C in YNB medium (0.67% yeast nitrogen base without amino acids and 2% glucose) supplemented with 0.5% casamino acids and buffer with 50 mM succinate/NaOH, pH 5.0. Preparation of cell lysates and Western blotting analysis was done as described previously (2, 13).

Assay of Endonuclease Activity of Mutant VDEs—E. coli BL21(DE3) carrying mutagenized pET-17bVDE plasmid was cultured in M9 minimal media containing 100 μg/ml ampicillin. A 0.1-mл overnight culture was added to 5 ml of fresh medium and grown at 23 °C to the early exponential phase (A600 = 0.07). Protein expression was then induced by the addition of 0.1 mM IPTG for 4 h to an A600 of about 0.2. Cells were pelleted and resuspended in 1 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 20 mM MgCl2) containing 1 mg/ml lysozyme (Sigma). After incubation on ice for 30 min, cells were lysed by three freeze-thaw cycles. The subsequent purification steps were carried out at 4 °C. The lysates were clarified by centrifugation at 10,000 × g for 10 min. Solid ammonium sulfate was added to the supernatant up to 70% saturation. The precipitate was collected by centrifugation (10,000 × g for 10 min) and resuspended in 1 ml of buffer A (10 mM sodium phosphate, pH 7.4, and 0.1 mM EDTA). The resuspended solution was dialyzed against buffer A for 2 h. The dialysate was batch-loaded onto 0.2 ml of CM-Sepharose CL-6B beads (Pharmacia) previously equilibrated with buffer A. The mixture was rotated for 10 min, and beads were pelleted and washed with 1 ml of buffer A. Bound proteins were then eluted with 0.5 ml of buffer A containing 200 mM NaCl. This fraction was dialyzed against buffer B (10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA) and used for endonuclease activity analysis. The protein concentration of the sample was estimated using BCA reagent (Pierce).

Mutants could be classified into roughly five groups. One group of mutants (100 mutants out of the 324 screened mutants) exhibited the excised 50-kDa VDE predominantly near wild-type levels. A second group of mutants (106 mutants) showed a somewhat reduced level of processing, accumulating a comparable amount of the 50-kDa VDE and the 115-kDa precursor. A third group of mutants (33 mutants) exhibited a markedly reduced amount of the 50-kDa VDE and predominant accumulation of the precursor. A fourth group of mutants (44 mutants) failed to show any detectable 50-kDa VDE in a Coomassie Blue-stained gel. A fifth group of mutants (41 mutants) failed to express the full-length precursor, presumably because a stop codon was introduced into the open reading frame.

Mutants of the fourth group identified in the initial screen were then subjected to Western blotting analysis, which was done essentially as described previously (2). Then 17 mutants were found not to produce any immunodetectable splicing products. DNA sequencing of these mutant plasmids was done by using the Sequenase kit (U.S. Biochemical Corp.) with appropriate primers. The number of amino acid substitutions which varied from one to six. In addition, mutants 93, 95, 175, 198, 323, and 330 contained a single silent mutation, and mutant 347 had two silent mutations. Then individual mutant plasmid DNAs were digested by restriction fragment swapping to identify which amino acid changes were responsible for the defect of splicing activity. The VDE-coding region was divided into three segments: segment 1 (530-bp BamHI-BglII), segment 2 (760-bp BglII-SacI), and segment 3 (150-bp SacII-EcoRI). These segments of each mutant DNA were individually ligated into wild-type pGEX-VMA1 plasmid to replace the corresponding regions. As for the mutant 330, which had a mutation upstream of the KpnI site, DNA was dissected to segment 0 (60-bp BamHI-KpnI) and segment 1 (470-bp KpnI-BglII). Mutant 197 could not be dissected, because the mutation abolished the KpnI restriction site. Then splicing activities of the reconstituted mutants (designated by the addition of hyphenated segment numbers) were reexamined by immunoblotting. Nineteen reconstituted mutants did not show any detectable amount of the 50-kDa VDE (among them, the mutant 239-1 showed a very slight amount of the 50-kDa VDE near the background level of detection). The other mutants with splicing activity were excluded.

Expression of GST-Vma1 Fusion Protein in E. coli—A bacterial expression system was used to produce normal and mutated recombinant Vma1 protozymes for monitoring their processing activity. The sequence, which covers the entire VDE region and the C-terminal segment of the VMA1 gene, was fused to the GST gene under control of the tac promoter (Fig. 1A). This construct expresses a chimeric protein (GST-Vma1) that is composed of GST and 11 residues from the Vma1 N-terminal region (~30 kDa), VDE (50 kDa), and the Vma1 C-terminal region (35 kDa). When this authentic GST-Vma1 fusion protein was expressed in E. coli (E. coli strain BL21(DE3) was processed into a 50-kDa VDE and a 60-kDa GST-Vma1 C-terminal fusion protein. The 115-kDa precursor and the excised 50-kDa VDE were easily detected by Coomassie Blue staining (Fig. 1B) and also by anti-VDE polyclonal antibody (Fig. 1C). The 65-kDa spliced product (GST-Vma1C) was hardly detected by Coomassie Blue staining, but it was detected by 5M39 monoclonal antibody (Fig. 1D), which recognizes the C-terminal region of Vma1p (11). We found that
Protein splicing efficiency in *E. coli* was temperature-sensitive. Induction at 37 °C resulted in predominant accumulation of the 115-kDa precursor in an insoluble fraction (data not shown). Induction at lower temperatures yielded a larger amount of the processed 50-kDa VDE, which was recovered in a soluble fraction.

**Screening for Protein Splicing-defective GST-Vma1 Mutants in *E. coli*—**Mutations were introduced randomly throughout the entire VDE region of the GST-VMA1 fusion gene by error-prone PCR. The mutagenized 1.4-kb BamHI-EcoRI fragment covers the entire VDE and external 14 proximal and 13 distal amino acids. After induction at room temperature with 0.1 mM IPTG, total proteins of *E. coli* cell extracts that expressed wild-type (lane 1) or mutagenized GST-Vma1 fusion proteins (lanes 2, 3, and 4) were analyzed by 8% SDS-PAGE followed by Coomassie Blue staining. The splicing activity of mutants was estimated by comparing the density of the 115-kDa precursor band with the density of the 50-kDa band of excised VDE. Defective mutants (44 mutants of the 324 candidate mutants) that accumulated the precursor and did not show detectable splicing products in the Coomassie Blue-stained gels were then subjected to the second level of screening using Western blotting analysis. Of the 44 mutants tested, 17 mutants showed no immunodetectable amount of the excised 50-kDa VDE against the anti-VDE antibody. The plasmid DNAs from these 17 mutants were purified and sequenced within the mutagenized region. The results of the sequencing are shown in Table I.

Mutations were distributed throughout the entire mutagenized region, and the number of amino acid substitutions in each mutant varied from one to six. To identify which amino acid changes are responsible for the defect of splicing activity, individual mutant plasmid DNAs were dissected by restriction fragment swapping (see “Experimental Procedures”). Then the splicing activity of the reconstituted mutants was reexamined by immunoblotting. Nineteen reconstituted mutants did not yield any detectable 50-kDa VDE. These mutations were distributed in their sequences as shown in Fig. 2. Of the 19 plasmids, 7 plasmids contained a single amino acid substitution. Two of them (G283V and C284Y) were at the N-terminal splice junction, and another two (N737S and N737K) were at the C-terminal junction. These four mutations at the splice junctions are in agreement with previous studies (5, 10, 11, 12).

We found three additional new mutants within the VDE region (H362L, V403D, and S639P). As for the conserved His362 (7), it was substituted in two mutants (175-1 and 303-1).

**Protein Splicing Activity of Mutant VDEs in Yeast—**We next tested whether these mutants defective in splicing in *E. coli* show the defect in yeast cells as well. The mutant VDE genes were introduced into a plasmid carrying the entire VMA1 gene to replace the wild-type VDE region. The splicing activity of mutant Vma1 protozymes was tested by their ability to complement the calcium-sensitive growth defect of Δvma1 cells. A loss of protein splicing of the Vma1 protozyme leads to a lack of Vma1p, the 70-kDa catalytic subunit of the vacuolar H⁺-ATPase. Since yeast mutants that lack Vma1p are sensitive to calcium-containing medium (18), the ability to complement calcium sensitivity correlates with the splicing activity in yeast (11). We found that several mutants were capable of complementing the calcium-sensitive growth defect of Δvma1 strain fully or partially (Fig. 3 and Table II), suggesting that the efficiency of protein splicing may be different in yeast and *E. coli* cells.

Of 17 mutants tested, six mutants (89-3, 93-3, 95-3, 175-1, 238-3, and 323-2) were capable of complementing calcium-sensitive growth defect of Δvma1 strain. To identify which amino acid changes are responsible for the defect of splicing activity, individual mutant plasmid DNAs were dissected by restriction fragment swapping (see “Experimental Procedures”). Then the splicing activity of the reconstituted mutants was reexamined by immunoblotting. Nineteen reconstituted mutants did not yield any detectable 50-kDa VDE. These mutations were distributed in their sequences as shown in Fig. 2. Of the 19 plasmids, 7 plasmids contained a single amino acid substitution. Two of them (G283V and C284Y) were at the N-terminal splice junction, and another two (N737S and N737K) were at the C-terminal junction. These four mutations at the splice junctions are in agreement with previous studies (5, 10, 11, 12).

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### Table I

**Mutations that abolish protein splicing of GST-Vma1p in *E. coli***

| Mutant No. | No. of amino acid changes | BamHI-KpnI | KpnI-BglII | BglII-SacII | SacII-EcoRI |
|------------|--------------------------|-------------|-------------|-------------|-------------|
| 89         | 4                        | M311K,N434T |             |             |             |
| 93         | 2                        | R325S       |             |             |             |
| 95         | 3                        | M330K,T361I,M392T | A463T,D607G,A648V |
| 115        | 3                        | T357S,H362L |             |             |             |
| 129        | 3                        | K222F,V365A |             |             |             |
| 175        | 3                        | T357S,H362L |             |             |             |
| 197        | 3                        | V289I,M293T |             |             |             |
| 239        | 3                        | T357S,D399V |             |             |             |
| 285        | 1                        | C284Y       |             |             |             |
| 282        | 5                        | N290K,I298F |             |             |             |
| 303        | 3                        | H362L       |             |             |             |
| 323        | 2                        | V403D       |             |             |             |
| 327        | 3                        |             |             |             |             |
| 330        | 2                        | G283V       |             |             |             |
| 347        | 2                        | T368A       |             |             |             |
| 347        | 2                        |             |             |             |             |

* The D446V mutation of mutant 197 abolished the BglII restriction site.
Mutations that cannot complement the calcium sensitivity of the yeast vma1 cells are circled. Numbers in parentheses indicate the number of amino acid substitutions.

Western blotting analysis with anti-VDE antibody was performed on yeast cell lysates from the mutant strains. The excised 50-kDa VDE was detected in the cell lysates in response to their ability to grow on the calcium-containing medium, showing that protein splicing occurred normally in some strains (Fig. 4A). Interestingly, we could detect several protein species with intermediate sizes between the 120-kDa precursor and excised 50-kDa VDE in four mutants 89-3, 95-3, 175-1, and 303-1 (H362L), an 80-kDa polypeptide was detected with anti-VDE and R70 antibodies, suggesting that the cleavage occurred only at the C-terminal splice site. These observations obtained with new VDE mutants are important for considering the mechanism of protein splicing (see “Discussion”).

Endonuclease Activity of the Splicing-defective VDE Mutants—We then examined whether the defect in protein splicing affects the endonuclease activity of VDE. To produce the wild-type VDE and the splicing-defective VDE in E. coli, the BamHI-EcoRI fragment was connected downstream of the T7 promoter. The resulting construct was expected to express a VDE polypeptide bracketed by 18 proximal and 17 distal amino acids (Fig. 5A). When the wild-type polypeptide was expressed at 23 °C, two protein bands were observed around 50 kDa (Fig. 5B). It is plausible that the minor upper band represents the precursor and that the major lower band represents the excised VDE, since the upper band disappeared after purification (13). None of the mutants produced the excised form, consistent with their inability to splice the external regions. However, protein products of intermediate sizes were seen in several mutants on an SDS-PAGE gel (Fig. 5B), probably resulting from aberrant processing or proteolysis, as observed in yeast cell lysates (Fig. 4).

The majority of the products were recovered in a soluble fraction of E. coli lysate, with the exception of mutants 238-1 and 327-3 (Fig. 5B). These two mutant proteins were highly insoluble and could not be purified. Other mutant VDE proteins could be easily purified by ammonium sulfate precipitation and CM-Sepharose CL-6B chromatography, although heterogeneous polypeptides were copurified together in some cases (Fig. 5B). The endonuclease activity of mutant VDEs was assayed with these samples using a plasmid containing the specific sequence (VMA1exonc) as substrate. Then we found that all of the purified mutant proteins had normal cleavage activity (Fig. 5C). As control, we used a bracketed VDE mutant, 64-2, which contains two mutations, D609V and V643A. The residue Asp609 is in the dodecapeptide motif and known to be essential for endonuclease activity (17). This mutant showed normal splicing activity (Fig. 5B), but could not cleave the substrate DNA (Fig. 5C).

**DISCUSSION**

In this study, we discovered that the His362 residue of the Vma1 proteome is critical for protein splicing, in addition to the previously mentioned residues around the N- and C-terminal splice junctions (5, 10–12). We noticed that the induction at a high temperature (37 °C) decreased the splicing efficiency in E. coli. A lower temperature (~25 °C) seemed to allow productive folding of the precursor for splicing. Interestingly, several mutants that could not splice in E. coli cells were found to splice in yeast cells to some extent. This may be due to differ-
ences in conditions of the two cell systems, such as the rate of precursor synthesis, intracellular pH, ion composition, reducing potentials, and molecular chaperones.

We analyzed six potent mutants in detail, which showed abortive splicing in yeast cells and completely lost normal splicing activity. The amino acid residues altered in these six mutants were found to cluster in three core regions. They were located around the N-terminal splice junction (mutant 238-1), the C-terminal splice junction (mutants 89-3, 93-3, 95-3, and 327-3), and the N-terminal one-sixth portion of VDE (mutant 175-1).

**N-terminal Splice Junction**—Mutant 238-1 had two mutations, V291G and M293T. Cooper et al. (12) previously reported that the changes of the Val291 residue (V291F and V291D) completely blocked splicing in yeast. Although mutants 265-1 (C284Y) and 330-0 (G283V) were not tested in yeast cells, these are already known to be essential (11, 12). To our knowledge, the G283V mutation is the first example (except for the Cys738 residue at the C-terminal splice junction) showing an essential requirement of the residue outside the VDE region for protein splicing. In addition, Nogami et al. have recently demonstrated

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**FIG. 4. Detection of the mutant *vma1* gene products in yeast cell extracts.** Extracts from the yeast cells shown in Fig. 3 were prepared as described under “Experimental Procedures.” A, proteins were resolved in 8% SDS-PAGE and probed by Western blotting using anti-VDE polyclonal antibody. B, wild-type (WT) and mutant cells (89-3, 95-3, 175-1, and 303-1) were analyzed with R70 and 5M39 monoclonal antibodies, which recognize the N-terminal and C-terminal regions of Vma1p, respectively.

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**TABLE II**

Growth phenotypes of yeast cells carrying mutant VDEs

| Mutant number | Mutations | Splicing in *E. coli* | Yeast growth<sup>a</sup> |
|---------------|-----------|-----------------------|-------------------------|
|               |           |                       | 23 °C                   | 30 °C | 37 °C |
| Wild-type     | None      | ++ ++ ++               | ++ ++ ++ ++            |
| 89-3          | H725L,N737I | --                     | --                      | --    | ±    |
| 93-1          | M311K,N434T | --                     | ++ ++ ++                | ++    | ±    |
| 93-3          | N737S     | --                     | --                      | --    | --   |
| 95-3          | N737K     | --                     | --                      | --    | --   |
| 115-1         | M330K,T361I,M392T | --                     | ++                     | ±     | ±    |
| 129-2         | A463T,D607G,A648V | --                     | ++                     | ±     | ±    |
| 175-1         | T357S,H362L | --                     | --                      | --    | --   |
| 197           | K322E,V365A,D446V,G451V,I485F,E524V | --                     | --                      | --    | --   |
| 198-2         | V644G,A682T | --                     | ++ ++ ++                | ++    | +    |
| 238-1         | V291G,M293T | --                     | --                      | --    | --   |
| 239-1         | T357S,D399V | ±                      | ++ ++ ++ +              | +     | +    |
| 265-1         | C284Y     | --                     | NT<sup>b</sup>          | NT    | NT   |
| 282-2         | L449F,Q460L | --                     | ++ ++ ++                | +     | +    |
| 303-1         | H362L     | --                     | ±                       | ±     | ±    |
| 323-1         | V403D     | --                     | ++ ++ ++                | ±     | ±    |
| 323-2         | S639P     | --                     | ++ ++ ++                | ±     | ±    |
| 327-3         | D724V,L729P | --                     | --                      | --    | --   |
| 330-0         | G283V     | --                     | NT                      | NT    | NT   |
| 347-2         | K552R,L602H | --                     | ++                      | --    | ±    |

<sup>a</sup> ++, +, ±, and -- indicate successively weaker growth than that of wild type.

<sup>b</sup> NT, not tested.
that the three hydrophobic residues (Ile279-Ile280-Tyr281) upstream of the N-terminal junction genetically interact with the hydrophobic residues (Val733-Val734-Val735) at the C-terminal junction to fulfill the splicing reaction. Our current results suggest that the Gly283 residue at the N-terminal junction may serve the splicing reaction by conferring conformational flexibility to the splice site, although Cooper et al. (12) reported that the G283R mutation did not affect the splicing efficiency.

**C-terminal Splice Junction—Asn737** at the C-terminal junction plays a critical role in protein splicing to form a succinimide ring (20). Cooper et al. (12) reported that substitutions of Asn737 to seven other amino acids (Lys, Ala, Tyr, Gln, Glu, His, and Asp) all resulted in nonspliced products. In this study, we obtained three mutants in which the Asn737 residue was substituted (mutants 89-3, 93-3, and 95-3), in good agreement with the previous observations (11, 12). Mutant 327-3 had two mutations near the C-terminal junction (D724V and L729P). We found that mutant 89-3 (H725I and N737I) and 95-3 (N737K) accumulated in yeast cells a 90-kDa side product that is supposed to result from cleavage at the N-terminal splice site (Fig. 4B). Hirata and Anraku (1, 11) also showed previously that N737V mutant accumulates a 90-kDa product. Furthermore, mutant 95-3 (N737K) accumulated a species that migrated more slowly (around 200 kDa) than the 120-kDa precursor and reacted with three antibodies (Fig. 4B). Thus, this species is likely to be a branched molecule that had been observed in an in vitro splicing reaction of the precursor from Pyrococcus DNA polymerase (19). Recently, Chong et al. (21) also reported that a branched intermediate accumulated in vitro using the N737A/C738S mutant of VDE. Our results are consistent with the succinimide-mediated cleavage model at the C-terminal junction (20).

**The Region around His362**—The third region crucial for protein splicing was newly identified in this study. The substitution of His362 with Leu caused a serious defect in protein splicing in yeast (mutant 303-1). An additional conservative mutation in the neighborhood (T357S) blocked splicing completely (mutant 175-1). These mutants yielded a 80-kDa C-terminal cleavage product (Fig. 4B). This suggests that His362 is required for the first cleavage at the N-terminal splice site. Cooper et al. (21) investigated the side reaction products accumulated in the VDE mutants that substituted the four key residues at the splice junctions: Cys738, His736, Asn737, and Cys738. They proposed that protein splicing of the yeast Vma1 protozyme occurs via a mechanism similar to that in the thermostable archaea (20, 21). According to their model, splicing proceeds as follows: step 1, N-S acyl rearrangement at the N-terminal splice junction involving Cys738, step 2, transesterification involving Cys738 to yield a branched intermediate; step 3, peptide cleavage at the C terminus by cyclization of Asn737, and step 4, S-N acyl shift of the transient spliced product to yield a normal peptide bond. In our study, the H362L mutant allowed cleavage at the C-terminal splice site, while it prevented the N-terminal scission. Thus, we suggest that the H362L mutant is likely to have a defect in step 1 or 2. The His362 residue may assist the N-S rearrangement of the Cys738 residue (step 1) or be required to activate the Cys738 residue as a nucleophile for the transesterification reaction (step 2) by acting as a proton acceptor for the thiol of either Cys residue.

Pietrokovski (7) pointed out that the His362 residue is invariant among all of the known protein splicing elements (His in motif B). Our results provide the first experimental evidence for the crucial requirement of the His362 residue. Besides the dodecapeptide motifs, there are only three invariable residues among protein splicing elements; in the Vma1 protozyme they are the His736-Asn737 dipetide at the C-terminal splice junction and His362 (7). Like a H736L mutant (12), the H362L mutant does not block splicing completely and allowed a low level of splicing activity (Table II). Chong et al. (21) reported that a His362 mutant (H736L/C738S) as well as Asn737 mutants accumulated the branched intermediate. This suggests that His362 participates in cyclization of Asn737 to resolve the branched intermediate, which is the late step of the splicing reaction. Conversely, His362 may participate in the early step of the reaction.

**Endonuclease Activity of Nonsplicing Mutant VDEs**—The present work also demonstrates the independence of two functions of VDE: protein splicing activity and endonuclease activity. VDE has been shown to be a site-specific endonuclease (14). Cleavage of the VMA1Δvde gene by VDE in a VMA1/VMA1Δvde heterozygote initiates gene homing that converts the VMA1Δvde allele into VMA1 (14). The dodecapeptide motifs in VDE are crucial for the cleavage activity (17). The mutagenesis of the dodecapeptide motif in T7H1 abolished endonuclease activity, whereas protein splicing was unaffected, indicating that the endonuclease activity is not required for splicing (10).

In this study, the splicing-defective VDE mutants were expressed in E. coli and purified to assess semiquantitatively their endonuclease activities in vitro. Then all mutants tested (89-3, 93-3, 95-3, 175-1, 265-1, and 330-0) appeared to possess endonuclease activities near the wild-type level. These results also confirm that these mutations do not cause global conformational changes of the protein.
cleavage step in the splicing reaction. Furthermore, mutations in these regions do not abolish the endonuclease activity of VDE.

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