Modulation of Protein Splicing of the *Saccharomyces cerevisiae* Vacuolar Membrane ATPase Intein*

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Protein splicing of the *Saccharomyces cerevisiae* vacuolar membrane ATPase intein involves four highly coordinated reactions that result in precise cleavage and formation of peptide bonds. In this study, we investigated the roles of the last N-extein residue (−1 residue) and the intsein penultimate residue in modulating splicing reactions. Most of the 20 amino acid substitutions at the −1 position had no effect on overall protein splicing but could lead to significant accumulation of thioester intermediates when splicing was blocked by mutation. A subset of −1 substitutions attenuated the initiation of protein splicing and enabled us to demonstrate in *vitro* splicing of a mesophilic intein containing all wild-type catalytic residues. Substitutions involving the intsein penultimate residue allowed modulation of the branch resolution and C-terminal cleavage reaction. Our data suggest that the N-S acyl rearrangement, which initiates splicing, may also serve as the rate-limiting step. Through appropriate amino acid substitutions, we were able to modulate splicing reactions in *vitro* by change in pH or temperature or addition of thiol reagents. Both insertion and deletion were tolerated in the central region of the intsein although splicing or structure of the intein may have been affected.

Protein splicing involves a precise excision of an internal segment, the intsein, from a protein precursor and a concomitant ligation of the flanking regions, the exteins, resulting in the production of two proteins (1). Since the initial discovery of protein splicing, more than 40 inteins have been identified (2). The chemical mechanism of protein splicing of the inteins from the thermophilic archaea and mesophilic eukaryotes (3–5). Protein splicing of the *Sce* VMA intein consists of the following multistep reactions: step 1, an N-S rearrangement at the intsein N terminus (Cys1) to form a thioester bond between Cys1 and the last N-extein residue (−1 residue); step 2, a trans-esterification reaction by the first C-extein residue Cys455 to form a branched intermediate; step 3, succinimide formation at the intsein C terminus (Asn454) to resolve the branched intermediate; step 4, a final S-N rearrangement to form a peptide bond linkage between the ligated exteins (Fig. 1A) (5).

The recently solved crystal structure of the *Sce* VMA intein demonstrates that both intsein terminal residues, Cys1 and Asn454, are in close proximity, forming a structure consistent with their proposed roles in the splicing pathway (6). Since the crystal structure represents the excised intsein, the structure of the extein-intsein precursor that involves at least one extein residue (i.e. Cys455) at the splicing active site is still unknown. Although the crystal structure supports our proposed splicing pathway, many mechanistic details of the splicing reactions have not been elucidated.

Previously, we examined the *Sce* VMA intein in an *in vitro* MYT splicing system (5). The rapid splicing of the *Sce* VMA intein *in vivo* precluded the isolation of the precursor and/or intermediates (5, 7, 8). Our studies utilized amino acid substitutions to arrest or attenuate the splicing process (5). A single substitution, Asn454 to Ala, was shown to block both splicing and C-terminal cleavage but not N-S acyl arrangement (5). Thiols such as dithiothreitol and cysteine were able to shift the N-S equilibrium by attacking the thioester bond and initiating N-terminal cleavage (5, 9). Previous studies have shown that the *Sce* VMA intein penultimate residue, His455, although conserved in almost all inteins, was not essential for protein splicing (5, 7, 8). A double substitution, H453L and C455S, was shown to attenuate protein splicing *in vivo* and allow *in vitro* splicing of the purified precursor (5). Although our data on the *in vitro* splicing reaction support our proposed splicing pathway (5), direct examination of *in vitro* splicing of the *Sce* VMA intein containing unaltered catalytic residues has been a challenge.

The *Sce* VMA intein also functions as a site-specific homing endonuclease which mediates gene mobility (10–12). The central conserved dodecapeptide motifs are directly involved in DNA recognition and cleavage (13, 14). The crystal structure of the *Sce* VMA intein suggests that the splicing and endonuclease functions may reside in two separate domains (6). We made large in-frame deletions which removed the domain containing the dodecapeptide motifs, demonstrating the remaining splicing domain was sufficient for efficient splicing (15).

This paper extends our previous *in vitro* studies to focus on residues that are in close proximity to the reaction center but not directly involved in the splicing reactions, *i.e.* the last...
Experimental Procedures

Numbering of Residues in the Intein Fusion Constructs—Amino acid numbers refer to the position in the S. cerevisiae VMA intein, essentially the same as described previously (Fig. 1B) (5).

Constructions of pMYB130 Containing Different -1 Residues—The construction of pMYB129 containing the wild-type Gly -1 residue was described previously (9). pMYB129 contains an XhoI site and a KpnI site flanking the N-terminal splice junction including the -1 position (9). These unique sites allowed convenient substitution of the Gly -1 residue with the remaining 19 naturally occurring amino acids through linker insertion. pMYB129 was digested with XhoI and KpnI and then ligated with complementary oligomers 5′-TC CAC NNN TGG TTT GGC AAG GGT AC-3′ and 5′-CT TGG GCC AAG GGA CNA CNG TTT GGA-3′, which were synthesized to contain each of the 19 amino acids (NNN) at the -1 position. The resulting constructs were named pMYB130(X-1), e.g. pMYB130(Ala -1) containing Ala as the -1 residue, pMYB130(Asn -1) containing Asn as the -1 residue, etc. The substitutions of the -1 residues in pMYB130(X-1) were further confirmed by DNA sequencing (New England Biolabs). Both pMYB129 and pMYB130(X-1) contained the N454A mutation, which blocked splicing and C-terminal cleavage and therefore were fusion constructs for the study of the N-S acyl rearrangement and N-terminal cleavage. Unless otherwise stated, all enzymes and plasmids used were the products of New England Biolabs, Inc.

Construction of pMYKX -1 and pMYK Containing His 653 Substitutions—The T4 polymerase kind gene (16) was synthesized by the polymerase chain reaction using the primers 5′-GCC GGT ACC GGT AAA AAG ATT ATT TGT ACT ATT GGC-3′ and 5′-GGT GGT CTG CAG TCA AAA TCC TTC GCC AGC ACC GAT CTT CCA-3′. Polymerase chain reaction mixtures (100 μl) contained Vent DNA polymerase buffer (New England Biolabs), 3 mM MgSO4, 300 μM each of the 4 dNTPs, 10 μM each primer, 1 μl of T4 phage particle suspension, and 1 unit of Vent DNA polymerase. Amplification was carried out for 25 cycles using a Perkin-Elmer thermal cycler at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. The product was digested with AgeI and PstI and ligated with pMYK (15) digested with AgeI and PstI, to yield pMYK. pMYK contained the wild-type splice junction residues including Gly as the -1 residue. To construct pMYKX -1 containing different -1 substitutions, pMYB130(X-1) was digested with XhoI and BamHI, and the resulting linear fragment was used to replace the corresponding fragment from pMYK to yield pMYKX -1. pMYKX -1 contained each of the 20 amino acid residues at the -1 position and the wild-type splice junction residues and was used to study the effect of the -1 substitutions on splicing.

Unique BamHI and AgeI sites flanking the C-terminal splice junction of pMYK allowed convenient substitution of the pentadecapeptide His653 through linker insertion. pMYK was digested with BamHI and AgeI and then ligated with the complementary oligomers, 5′-GA TCC GGT GAT GTA GTA CTT GAC ATT CAC GAA AAT GCA-3′ and 5′-GGT GGT CTG CAG TCA TAG TAC AAC GGT CAG GGG AT 3′, corresponding to the reaction conditions were essentially as described above. The product was digested with AgeI and PstI and ligated with pMYB (15) that had been digested with AgeI and PstI, yielding pMYT4, which contains the wild-type splice junction residues including Gly -1. pMYT4 was digested with BamHI and AgeI and ligated with the complementary oligomers, 5′-GA TCC GGT GAT GTA GTA CTT GAC ATT CAC GAA AAT GCA-3′ and 5′-GGT GGT CTG CAG TCA TAG TAC AAC GGT CAG GGG AT 3′, corresponding to the reaction conditions were essentially as described above. The product was digested with AgeI and PstI, yielding pMYT4, which contains the wild-type splice junction residues including Gly -1. pMYT4 was digested with BamHI and AgeI and ligated with the complementary oligomers, 5′-GA TCC GGT GAT GTA GTA CTT GAC ATT CAC GAA AAT GCA-3′ and 5′-GGT GGT CTG CAG TCA TAG TAC AAC GGT CAG GGG AT 3′, corresponding to the reaction conditions were essentially as described above. The product was digested with AgeI and PstI, yielding pMYT4, which contains the wild-type splice junction residues including Gly -1. pMYT4 was digested with BamHI and AgeI and ligated with the complementary oligomers, 5′-GA TCC GGT GAT GTA GTA CTT GAC ATT CAC GAA AAT GCA-3′ and 5′-GGT GGT CTG CAG TCA TAG TAC AAC GGT CAG GGG AT 3′, corresponding to the reaction conditions were essentially as described above. The product was digested with AgeI and PstI, yielding pMYT4, which contains the wild-type splice junction residues including Gly -1. pMYT4 was digested with BamHI and AgeI and ligated with the complementary oligomers, 5′-GA TCC GGT GAT GTA GTA CTT GAC ATT CAC GAA AAT GCA-3′ and 5′-GGT GGT CTG CAG TCA TAG TAC AAC GGT CAG GGG AT 3′, corresponding to the reaction conditions were essentially as described above. The product was digested with AgeI and PstI, yielding pMYT4, which contains the wild-type splice junction residues including Gly -1.
FIG. 1. A proposed mechanism for protein splicing and thiol-induced cleavage at splice junctions of the Sce VMA intein. The Sce VMA intein (white box) is inserted between the N-extein (black box) and the C-extein (striped box). The protein splicing pathway of the Sce VMA intein (5) is indicated by the bold arrows. I, precursor; II, ester intermediate; III, branched intermediate; IV, excised intein; V, spliced exteins. The proposed mechanism for thiol-induced cleavage reactions is shown on the upper right. The cleavage reactions were examined in the MYB fusion system, in which the intein contained an N454A substitution (not shown) or in the MYT4 fusion system in which the intein contained a double substitution, H453Q/C455A. See text for more details. The residues at the splice junctions are shown as follows: X, the last N-extein residue (−1 residue); Cys1, the first intein residue; His453, the intein penultimate residue; Asn454, the last intein residue; Cyss455, the first C-extein residue. B, schematic diagram of the Sce VMA intein fusion systems. The same shading scheme as A is used to represent the intein, N-extein and C-extein. The arrows and numbers below indicate the amino acid positions. The splice junction residues are shown essentially the same as A. The decapeptide motifs are indicated as shaded boxes. The features of the fusion constructs are described in Table I.

RESULTS

Fusion Constructs of the Sce VMA Intein for Modulation of Protein Splicing—Protein splicing of the Sce VMA intein was examined by fusing the intein between two independent extein domains (Fig. 1B). E. coli maltose-binding protein (21) (MBP) was used as the N-extein to facilitate purification of splicing products that contained the MBP moiety. C-exteins of varying molecular masses were used for easy resolution of splicing products on SDS-PAGE. The MBY, MYB, or MYT4 fusion systems contained Bacillus circulans chitin-binding domain (CBD or B), T4 polynucleotide kinase (K), or T4 DNA ligase (T4) as the C-extein, respectively (Table I). In the case of the wild-type Sce VMA intein with Gly−1 and Cys as the first C-extein residue (residue 455), complete in vivo splicing occurred in all three fusion systems (data not shown). The MYB fusion system, including pMYB129, pMYB130, pMYB131, pMYB132, and pMYB133, was constructed to examine the effect of the −1 substitutions on thiol-induced N-terminal cleavage and N-S acyl rearrangement and the effect of deletion and insertion mutations (Table I). The MYK fusion system, including pMYK(A)−1, pMYK(B), pMYK(C), pMYK(D), and pMYK(E), was constructed to examine the effect of substituting the −1 residue and the intein penultimate residue on protein splicing (Table I). The MYT4 fusion system, including pMYT4, pMYT4(1), pMYT4(2), and pMYT4(3), was constructed to examine the effect of substituting the −1 residue and the intein penultimate residue on protein splicing (Table I).

Effect of the −1 Substitutions on Induction of N-terminal Cleavage—An N-S acyl rearrangement at the N terminus of the Sce VMA intein forms a thioester bond between Cys2 and the −1 residue (Fig. 1A). Exogenous thiols, e.g. DTT and cysteine, can attack the thioester bond to induce cleavage at the intein N terminus (Fig. 1A) (5, 9). This thiol-induced cleavage has been previously examined in pMYB129 (9). In this study, we determined the effect of pH and temperature on the half-time and efficiency of the cleavage reaction (Table II). The rate of the DTT-induced cleavage of the fusion proteins from pMYB129 increased 4-fold at pH 8.0 versus pH 6.0 at 4–16 °C and almost 50-fold at 23 °C. At pH 8.0, the rate of cleavage increased 30-fold at 23 versus 4 °C (Table II).

To investigate the effect of substitutions at the −1 position on thiol-induced N-terminal cleavage and N-S acyl rearrange-
The presence of DTT or incubation at basic pH (pH 9.5) had no effect on native MYB precursors. In the absence or presence of DTT, only trace amounts of the N-terminal cleavage products (M and YB) were observed for the denatured MYB precursors containing Gly-1 (Fig. 2, left panel). The presence of DTT or incubation at basic pH (pH 9.5) had no effect on N-terminal cleavage suggesting that the N-S equilibrium was disrupted after the denaturation. Similar results were obtained with Asn-1 (Fig. 2, middle panel), Ala-1, Ile-1, Ser-1, His-1, and Pro-1 substitutions (data not shown). However, significant amount of DTT-induced N-terminal cleavage

**Table I**

| Fusion constructs for modulation of protein splicing of the Sce VMA intein |
|-----------------------------|-----------------------------|
| pMYB129             | MBP (42 kDa)  |
| pMYB130(X-1)       | MBP            |
| pMYB1(N)(N454A)    | MBP            |
| pAMYB1(Bam)        | MBP            |
| pMYKX-1            | MBP            |

**Table II**

DTT-induced N-terminal cleavage of the MYB fusion precursor from pMYB129

| Temperature | pH 6.0 | pH 8.0 |
|-------------|--------|--------|
| °C          | Half-time (% cleavage after 16 h) | Half-time (% cleavage after 16 h) |
| 4           | 12%    | 92%    |
| 16          | 7.4%   | 95%    |
| 23          | 4.8%   | 96%    |

**Table III**

Effect of the –1 substitutions on N-terminal cleavage and protein splicing

| Minus 1 residue | In vivo | In vitro in the presence of DTT (%) |
|-----------------|---------|-----------------------------------|
| Gly             | <10     | <5                                |
| Ala             | <10     | <5                                |
| Val             | <10     | <5                                |
| Leu             | <10     | <5                                |
| Ile             | <10     | <5                                |
| Ser             | <10     | <5                                |
| Thr             | 25%     | 32%                               |
| Cys             | <10     | 10%                               |
| Asp             | >95     | ND                                |
| Glu             | 50%     | 58%                               |
| Arg             | 75%     | ND                                |
| His             | 50%     | <5                                |
| Phe             | >10     | 50%                               |
| Tyr             | >10     | 66%                               |
| Trp             | >10     | 52%                               |
| Pro             | <10     | <5                                |
| Met             | >10     | 72%                               |

**Table IV**

Protein splicing of MYK fusion protein

| Protein splicing of MYK fusion protein | 4 °C | 16 °C |
|--------------------------------------|------|-------|
| Native MYB | 95 (1.7) | 95 (1.7) |
| Urea-denatured MYB | 95 (1.5) | 95 (1.5) |

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*a* The approximate percentage of N-terminal cleavage in vivo or in the course of purification was estimated from SDS-PAGE as the ratio of MBP versus the MYB precursor in the amylose-purified proteins.

*b* Cleavage reactions were in 30 mM Hepes, pH 8.0, 0.5 mM NaCl, and 40 mM DTT. The percentage of in vivo N-terminal cleavage was determined by comparing the MYB precursors from the DTT-treated samples with those from the samples without the DTT treatment in scanned images of Coomassie Blue-stained SDS-polyacrylamide gels. Each percentage determination is typical of 2 or 3 determinations with a variation of <10%. ND, not determined.

The numbers in parentheses are half-times (hour) of the cleavage process, i.e., the incubation times required for DTT to cleave 50% of the native MYB precursors.
products (M and YB) was observed for the denatured MYB precursors containing the Lys<sup>−1</sup> residue (Fig. 2, right panel). Incubation of the precursors at pH 7.4 and 9.5 in the absence of DTT yielded the same cleavage products, M and YB (in only trace amount) (Fig. 2, right panel). The missing YB and smeared bands on the SDS-polyacrylamide gel could be due to protein aggregation or precipitation during incubation in the absence of DTT (Fig. 2). These results suggest that Lys<sup>−1</sup> position shifted the N-S equilibrium resulting in accumulation of thioster intermediates prior to induction of cleavage by DTT. Similar results were obtained with the Val<sup>−1</sup>, Leu<sup>−1</sup>, Thr<sup>−1</sup>, Glu<sup>−1</sup>, Phe<sup>−1</sup>, Tyr<sup>−1</sup>, Trp<sup>−1</sup>, and Met<sup>−1</sup> substitutions (Table III, SDS-PAGE data not shown).

**Effect of the −1 Substitutions on Protein Splicing**—Since N-S acyl rearrangement initiates protein splicing (Fig. 1A) (5), it is probable that the −1 substitutions that shift the N-S equilibrium also affect protein splicing. This was examined in the MYK fusion system using pMYK(X<sup>−1</sup>), in which the intein contained all wild-type catalytic residues for splicing. After expression of pMYK(X<sup>−1</sup>) in E. coli, the splicing products were purified on amylose resin. The predicted splicing products were identified by their apparent molecular masses on SDS-PAGE.

As shown in Fig. 3A, the majority of −1 substitutions in MYK resulted in the production of a predominant 75-kDa protein whose size corresponded to the ligated exteins, MK, indicating that efficient splicing occurred in vitro. The spliced intein (Y) was identified in the crude extract and the flow-through (data not shown). However, some −1 substitutions produced significant amounts of the linear precursors, MYK (e.g. Val (lane 3), Leu (lane 4), Ile (lane 5), Cys (lane 8), Asn (lane 11), and Pro (lane 19)), or C-terminal cleavage products, MY and K (e.g. Leu (lane 4), Ile (lane 5)). In the case of the Cys<sup>−1</sup> substitution, significant splicing also occurred (lane 8). A minor component, corresponding to the size of the intein (Y), was detected in samples of many −1 substitutions (e.g. the Cys<sup>−1</sup> and Asn<sup>−1</sup>) possibly due to splicing of the MYK precursors during purification.

Accumulation of the linear precursors in some −1 substitutions suggests that protein splicing was attenuated in vivo despite the fact that the intein contains all wild-type catalytic residues. To examine whether these −1 substitutions allowed splicing to continue in vitro, purified proteins from the Asn<sup>−1</sup>, Cys<sup>−1</sup>, Val<sup>−1</sup>, Ile<sup>−1</sup>, and Pro<sup>−1</sup> substitutions were incubated in Hepes buffer for up to 40 h. In vitro splicing of the linear
precursors from the Cys-1 and Asn-1 substitutions (Fig. 3A, lane 8 and 11) was also examined at different pH values. As shown in Fig. 3B, incubation of the precursors containing the Asn-1 substitution resulted in almost complete splicing at pH 6.0, yielding MK and Y, whereas significant amount of the precursors remained at pH 8.5. Similarly, incubation of the purified proteins from the Cys-1 substitution at pH 6.0 allowed the remaining linear precursors to splice, but splicing was significantly inhibited at pH 8.5 (data not shown). It is apparent that both Asn and Cys as the -1 residue allowed completion of protein splicing without side reactions. More importantly, these results suggest that overall splicing reaction of the wild-type Sce VMA intein was favored at pH 6.0 versus pH 8.5. In vitro splicing (at pH 6.0) of the purified proteins from the Ile-1 substitution, on the other hand, resulted predominantly in C-terminal cleavage, whereas the Val-1 substitution produced both splicing and C-terminal cleavage products (Table III). No significant splicing or cleavage in vivo was observed for the Leu-1 and Pro-1 substitutions (data not shown).

Effect of Substituting the Intein Penultimate Histidine Residue on Protein Splicing—Succinimide formation by the last intein residue, Asn<sup>1</sup>, is coupled to peptide bond cleavage at the intein C terminus, resulting in branch resolution (Fig. 1A) (5). It is conceivable that the intein penultimate residue, His<sup>453</sup>, may assist the function of Asn<sup>1</sup>. The effects of single and double substitutions of His<sup>453</sup> were therefore examined in the MYK fusion system (Table IV). Analysis of the purified proteins from the single substitution, H453L, indicated partial in vivo splicing and in vivo N-terminal cleavage (Table IV). Incubation of the purified precursors at pH 8.0 resulted in no in vitro splicing; however, significant cleavage at both splice junctions occurred in the presence of DTT (Table IV). Efficient splicing was observed when the H453L substitution was combined with the C455S substitution (Fig. 4). Expression of pMYK(H453L/C455S) resulted in the production of a major 125-kDa protein corresponding to the linear precursor MYK (Fig. 4, lane 1). A slowly migrating component corresponding to the branched intermediate (MYK*) was also observed along with splicing products MK and Y (Fig. 4, lane 1). Incubation of the purified proteins at pH 8.5 resulted in significant in vivo splicing of the linear precursor MYK, as indicated by an increase of MK and Y (Fig. 4, lanes 7–10). The rate of splicing was much lower at pH 6.0 as only a slight increase of MK and Y was observed (Fig. 4, lanes 1–6). The amount of branched intermediate (MYK*) remained unchanged after incubation at pH 6.0 but decreased rapidly at pH 8.5 (Fig. 4), suggesting that the branch resolution reaction was favored at pH 8.5 but inhibited at pH 6.0. Although more in vivo splicing was observed resulting in less linear precursors, the double substitutions, H453Q/C455S, produced similar results as described above (data not shown). In comparison, the double substitutions, H453A/C455S and H453F/C455S, blocked splicing in vivo and allowed partial splicing and some accumulation of the branched intermediate in vivo (Table IV, SDS-PAGE data not shown). These results suggest that substitution of His<sup>453</sup> attenuated succinimide formation by the adjacent Asn<sup>1</sup>, which, in conjunction with the C455S substitution, resulted in accumulation of the branched intermediate. Consistent with this explanation, the H453Q substitution blocked both splicing and cleavage in vivo when a second substitution, C455A, rendered the intein incapable of undergoing trans-esterification, i.e., branch formation (Fig. 1A) (Table IV). However, efficient cleavage at both splice junctions could occur in vitro in the presence of DTT (Table IV). This DTT-induced cleavage at both splice junctions was further examined in the MYT4 fusion system as described below.

Induction of C-terminal Cleavage in Vitro—Expression of the fusion construct pMYT4 (H453Q/C455A) resulted in the production of a single 150-kDa protein corresponding to the linear precursor MYT4 (Fig. 5A, lane 1). The MYT4 precursors were very stable in vitro as no significant cleavage at both splice junctions was observed after incubation at 4 °C for 72 h or 23 °C for 16 h (Fig. 5, A and B, lane 2). However, treatment of MYT4 with DTT at 23 °C immediately induced cleavage at the intein N terminus to yield YT4 and M (Fig. 5A, lanes 3–5). In addition, cleavage at the intein C terminus was observed, as indicated by the appearance of T4 and Y (Fig. 5A, lanes 4–10). The amount of YT4 declined after an initial increase (Fig. 5A, lanes 3–10) suggesting that T4 and Y were produced from the C-terminal cleavage of YT4 and that the C-terminal cleavage occurred after the initiation of the N-terminal cleavage. Consistent with this explanation, no significant amount of MY, the product of an exclusive C-terminal cleavage, was observed (Fig. 5A).

To examine the effect of pH on C-terminal cleavage, MYT4 was first incubated with DTT at pH 6.0. As shown in Fig. 5B, the incubation resulted in efficient cleavage at the intein N terminus, yielding YT4 and M, but no significant cleavage at the C terminus (lanes 3 and 4). However, efficient C-terminal cleavage occurred after incubation of the DTT-treated samples for additional 16 h at pH 8.0 (yielding T4 and Y) (lane 5). The C-terminal cleavage appeared to occur in YT4 but not in MYT4 as indicated by a decrease of YT4 and no apparent change of acidity.

### Table IV

| Substitutions          | Splicing | N-terminal cleavage | C-terminal cleavage |
|------------------------|----------|---------------------|---------------------|
|                        | In vivo<sup>a</sup> | In vitro<sup>b</sup> | In vitro<sup>c</sup> + DTT<sup>d</sup> | In vivo<sup>a</sup> | In vitro<sup>c</sup> + DTT<sup>d</sup> |
| H453L                  | +        | –                   | +                   | +                   | +                   |
| H453A/C455S            | +        | –                   | +                   | –                   | +                   |
| H453F/C455S            | +        | –                   | +                   | –                   | +                   |
| H453L/C455S            | +        | +                   | +                   | –                   | +                   |
| H453Q/C455S            | +        | +                   | +                   | –                   | +                   |
| H453Q/C455A            | –        | –                   | –                   | –                   | –                   |

<sup>a</sup>Protein splicing or cleavage in vivo or in the course of purification was determined by inspection of the splicing or cleavage products in crude extract, flow-through, and amylase-purified proteins resolved by SDS-PAGE.

<sup>b</sup>In vitro splicing or cleavage was determined by comparing the splicing or cleavage products after incubation of the amylase-purified proteins at 23 °C for 16 h without or with 40 mM DTT (+DTT).

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**Fig. 4. Examination of branch resolution in vitro.** Amylase-purified proteins from pMYK(H453L/C455S) (lane 1) were incubated at 16 °C in 30 mM Hepes, 0.5 mM NaCl for up to 20 h at pH 6.0 (lanes 2–6) or at pH 8.5 (lanes 7–10). The samples taken at different times were resolved by SDS-PAGE followed by Coomassie Blue staining. Based on their molecular masses, the predicted splicing products are indicated on the right: MYK*, branched intermediate; MYK, unspliced precursor; MK, the ligated exteins; Y, the spliced intein. The molecular mass standards (in kDa) are indicated on the left.
and incubated at 4 °C at pH 8.0 for an additional 8 h.

Effect of Deletion and Insertion on Protein Splicing—We have demonstrated efficient splicing in pΔMYB1(NG) in which the endonuclease domain of the intein was removed by deletion (15). To determine if the intein deletion mutant could be modified to undergo cleavage reactions similar to the full-length intein in the MYB fusion system, the N454A substitution was introduced in pΔMYB1(NG) to yield pΔMYB1(NG)(N454A). Expression of pΔMYB1(NG)(N454A) resulted in the production of a predominant linear precursor (Fig. 6A, lane 1). Induction of N-terminal cleavage by DTT was conducted at different temperatures. As shown in Fig. 6A, incubation of the purified precursors with DTT at 4 and 16 °C resulted in significant N-terminal cleavage, yielding M and ΔΔYB(NG)(N454A) (lanes 2–7), whereas incubation at 23 °C blocked DTT-induced cleavage (lanes 8–10). As a control, the linear precursors were incubated without DTT at 4–23 °C for 16 h, resulting in no significant N-terminal cleavage (data not shown). The double substitution, H453Q/C455A, which led to the DTT-induced splice junction cleavage in MYT4 (Fig. 5), was also introduced in the pΔMYB1(NG) construct. Incubation of the purified precursors with DTT resulted in no significant cleavage at either splice junction (data not shown), suggesting that the H453Q/C455A substitution may disrupt the structure of the intein deletion mutant for efficient cleavage.

To determine whether we could replace the endonuclease domain of the Sce VMA intein with other protein domains without significantly affecting splicing, the gene for BamHI was inserted into the deletion site of pΔMYB1(NG) to yield

**Fig. 5.** Induction of C-terminal cleavage of the MYT4 fusion proteins. A, purified fusion proteins from pMYT4(H453Q/C455A) (lane 1) were incubated at 23 °C with 40 mM DTT in 30 mM Hapes, 0.5 mM NaCl, pH 8.0, for up to 8 h (lanes 3–10), or without DTT for 16 h (lane 2). Schematic representations of the structures of the fusion precursor and cleavage products, using the same shading scheme as Fig. 1, are shown on the right. MYT4, unspliced precursor; YT4, N-terminal cleavage intermediate; T4, T4 DNA ligase (striped box); Y, the intein (white box); M, MBP (black box). B, purified fusion proteins from pMYT4(H453Q/C455A) (lane 1) were dialyzed against 30 mM Hapes, 0.5 mM NaCl, pH 6.0, and incubated at 4 °C in the same buffer from pMYT4(H453Q/C455A) (lane 10) were incubated at 4 °C without DTT (lane 11) or with DTT at pH 6.0 (lane 12) or pH 8.0 (lane 13) for 16 h. All samples were resolved by SDS-PAGE followed by Coomassie Blue staining.
pMYB1(Bam). Following the expression of the fusion construct in E. coli, the proteins were purified by amylase affinity chromatography and analyzed by SDS-PAGE and Western blot analysis. As shown in Fig. 6B, the major component was a 60-kDa protein corresponding to the ligated exteins, MB (lane 4) and by Western blot analysis using antibodies against the maltose-binding protein (data not shown). The other splicing product, a chimeric fusion of the intein splicing domain and BamHI (1α), was detected in the crude extract as a 60-kDa protein (Fig. 6B, lane 2) and by Western blot analysis (Fig. 6B, lane 1). ΔY(Bam) reacted specifically with antibodies raised against BamHI (Fig. 6B, lane 1) and the Sce VMA intein (data not shown). Some minor components with high molecular masses were also detected in the amylose-purified proteins (Fig. 6B, lane 4), the 2% SDS eluates (Fig. 6B, lane 5), and by Western blot analysis (Fig. 6B, lane 1), suggesting that they were probably unspliced precursors and splicing intermediates.

In conclusion, efficient splicing occurred after the endonuclease domain of the Sce VMA intein was replaced with the restriction endonuclease BamHI. Thus, insertion of a heterologous protein domain in the Sce VMA intein is possible without significantly affecting the splicing activity.

**DISCUSSION**

In this study, we present a comprehensive analysis of the first three reactions in the protein splicing pathway of the Sce VMA intein: N-S acyl rearrangement, trans-esterification, and succinimide formation coupled to C-terminal cleavage (Fig. 1A). By substituting the −1 residue and the intein penultimate residue, we were able to attenuate the splicing process and allow investigation of each splicing reaction in *in vitro* systems. Appropriate substitution of the −1 residue enabled us to examine *in vitro* splicing of the Sce VMA intein containing all wild-type catalytic residues. Induction of branch resolution and C-terminal cleavage *in vitro* was made possible by substitution of the intein penultimate residue in conjunction with substitution of the first C-extein residue. In addition, the study of the effect of insertion and deletion provides the first evidence suggesting that intein can tolerate the insertion of a foreign protein domain and that the structure of the intein for protein splicing may be destabilized by the deletion or insertion mutation. Our data yield further insights into the mechanism of protein splicing and strategies for modulating protein splicing reactions.

**Roles of the Last N-extein Residue and Modulation of Protein Splicing with −1 Substitutions—**Efficient splicing occurs when inteins are transferred into heterologous proteins, suggesting that the inteins plus the first C-extein residue contain sufficient information for catalyzing splicing reactions (3, 5, 7, 23, 24). On the other hand, it is conceivable that splicing in a foreign context can be affected by the proximal extein sequences since the catalytic residues of protein splicing are located at the intein termini (4, 5). It has been recently proposed that splicing of the Sce VMA intein involves interactions between the intein residues upstream of the C-terminal splice junction and the proximal extein residues upstream of the N-terminal splice junction (25). The N-S acyl rearrangement initiates protein splicing, forming a thioester bond between Cys1 and the −1 residue (5). Glycine is the −1 residue in the 69-kDa vacuolar ATPase subunit of *S. cerevisiae* in which the Sce VMA intein is embedded prior to protein splicing (26). The question of how substitution of the −1 residue may affect protein splicing has not been examined by previous studies. In this study, we addressed this question by substituting the −1 residue with each of the 20 naturally occurring amino acids.

Based on their effects on N-terminal cleavage and/or the N-S acyl rearrangement, the 20 −1 substitutions in the MYB fusion system can be divided into four groups. The first group of substitutions, including the Gly−1, Ala−1, Ile−1, Ser−1, Cys1, and His−1 substitutions, resulted in efficient (30–95%) DTT-induced N-terminal cleavage of the native precursors but almost no (<5%) cleavage of the uba-denatured precursors (Table III). These substitutions allowed the N-S acyl rearrangement to favor peptide bond formation prior to DTT treatment. Incubation with DTT shifted the N-S equilibrium by cleaving the thioester intermediate and inducing the formation of a thioester bond. The second group of substitutions, including the Cys−1, Asn−1, and Pro−1 substitutions, blocked DTT-induced N-terminal cleavage of both native and urea-denatured precursors (Table III). One possible explanation is that these substitutions blocked the formation of thioester bond in the N-S equilibrium. Alternatively, these substitutions may cause a structural change in the active site that prevents the access of DTT to the thioester bond. The third group of substitutions includes the Val−1, Leu−1, Thr−1, Glu−1, Lys−1, Phe−1, Tyr−1, Trp−1, and Met−1 substitutions (Table III). Significant amounts of the DTT-induced N-terminal cleavage occurred in both native and urea-denatured precursors. N-terminal cleavage was also observed when the precursors were denatured in urea at high pH without DTT treatment (Fig. 2) or denatured in 6 M guanidinium HCl followed by DTT treatment (data not shown). The data suggest that these substitutions caused a significant shift in the N-S equilibrium, resulting in accumulation of thioester intermediates prior to DTT treatment. These thioester intermediates could include both linear precursors and branched intermediates, as cleavage of both results in the identical products (i.e. M and YB) (Fig. 1A). The MYB fusion proteins contained the wild-type first C-extein residue Cys455 and, therefore, were capable of forming the branched intermediate (Fig. 1A). As shown in Fig. 2, there was a component in the untreated samples migrating slower than the expected linear precursor. Whether this slow-migrating component was the branched intermediate or the result of protein aggregation requires further investigation. Assuming that urea denaturation immediately disrupted the N-S equilibrium and no further thioester or peptide bond formation occurred during denaturation, the percentage of N-terminal cleavage of the urea-denatured precursors (Table III) may represent the percentage of thioester intermediates in the native fusion precursors. Nevertheless, without detailed kinetic studies, we cannot rule out the possibility that these substitutions might cause accumulation of thioester intermediates during denaturation. The fourth group of the −1 substitutions, including the Asp−1 and Arg−1 substitutions, caused substantial N-terminal cleavage *in vivo* or in the course of purification (Table III). It is possible that these substitutions may disrupt the structure of the splice junction, resulting in proteolytic cleavage or hydrolysis.

The majority of the −1 substitutions in the MYB fusion system had no significant effect on protein splicing even though many substitutions had been shown to shift the N-S equilibrium in the MYB fusion (Fig. 3A and Table III). Nevertheless, certain −1 substitutions clearly disrupted the course of normal protein splicing, and their effects on proteins splicing paralleled their effects on the N-S equilibrium (Table III). For instance, the Pro−1 substitution, which blocked DTT-induced N-terminal cleavage in the MYB fusion, also inhibited protein splicing in the MYB fusion. The Val−1, Leu−1, and Ile−1 substitutions, which retarded the thiol-induced N-terminal cleavage in the MYB fusion, resulted in mostly unspliced precursors and/or C-terminal cleavage products in the MYK fusion (Table III). The effects of the Asn−1 and Cys−1 substitutions were
somewhat different. Although DTT-induced N-terminal cleavage was almost completely blocked by the Asn$^{-1}$ and Cys$^{-1}$ substitutions in the MYB fusion, efficient splicing in the MYF fusion occurred in vitro (Table III and Fig. 3B). A combination of the N454A mutation and the Asn$^{-1}$ or Cys$^{-1}$ substitution in the MYB fusion resulted in inhibition of the N-terminal cleavage, whereas restoration of the intein residue 454 to the wild-type residue Asn in the MYK fusion in conjunction with the Asn$^{-1}$ or Cys$^{-1}$ substitution allowed completion of protein splicing reactions. In conclusion, our results indicate that glycine at the −1 position of the Sce VMA intein is not essential for efficient protein splicing and can be substituted by other amino acid residues. A subset of amino acids at the −1 position shifts the equilibrium position of the N-S acyl rearrangement thereby affecting protein splicing. Appropriate substitutions of the −1 residue enabled us to modulate the rate of protein splicing and convert splicing into C-terminal cleavage.

Effect of Substituting the Intein Penultimate Histidine—Substitution of the penultimate histidine of the Psp pol-1 intein (His$^{539}$) has been shown to block both splicing and C-terminal cleavage and lead to accumulation of branched intermediate (4). Substitution of the penultimate histidine of the Sce VMA intein (His$^{453}$) on the other hand, produced different results. Neither splicing nor cleavage was completely inhibited by the H453L substitution (Table IV). Double substitutions, such as H453L/C455S, are required in order for efficient splicing to occur without in vivo cleavage. The observations of the accumulation of a branched intermediate and in vitro splicing of the precursors in the double substitutions, H453L/C455S (Fig. 4) and H453Q/C455S (data not shown), suggest that the His$^{453}$ substitution attenuates but does not block succinimide formation or C-terminal cleavage, whereas the C455S substitution may help to allow normal splicing to proceed without side reactions. In vitro protein splicing was less efficient when His$^{453}$ was substituted with Ala or Phe (i.e. in H453A/C455S and H453P/C455S) (Table IV). Splicing was completely blocked, and efficient DTT-induced cleavage was observed when Cys$^{455}$ was substituted with Ala to block the trans-esterification in the double substitution, H453Q/C455A (discussed below). It is apparent that substituting the intein penultimate histidine residues in conjunction with the first C-extein residue provides an alternative for modulating protein splicing and converting splicing to cleavage. These results illustrate the intricate coordination of the active site residues of the Sce VMA intein in catalyzing protein splicing and the possible role of His$^{453}$ in assisting succinimide formation and branch resolution.

Induction of C-terminal Cleavage and Simulation of Protein Splicing Reactions in Vitro—The double substitution, H453Q/C455S, resulted in accumulation of a branched intermediate and in vitro splicing (Table IV) suggesting that, albeit at an attenuated rate, the H453Q substitution could still allow C-terminal succinimide formation and cleavage. However, C-terminal cleavage in the H453Q/C455A double mutant was completely blocked resulting in isolation of linear unspliced precursors (Fig. 5). As the C455A substitution rendered the mutant incapable of undergoing trans-esterification, the results suggested that by inducing N-terminal cleavage to mimic trans-esterification, we were able to induce C-terminal cleavage. This was indeed the case when the linear precursors carrying the H453Q/C455A double substitution were treated with DTT or cysteine to generate both N-terminal and C-terminal cleavage products (Fig. 5). Unlike thiol-induced N-terminal cleavage (9), C-terminal cleavage was independent of thiols as it could occur after the removal of thiols and was inhibited at pH 6.0 but not at pH 8.0 (Fig. 5B). In addition, no C-terminal cleavage was observed before thiol treatment (Fig. 5A, lanes 1 and 2), suggesting that C-terminal cleavage occurred after the induction of N-terminal cleavage. We speculate that thiol-induced N-terminal cleavage in the H453Q/C455A double mutant triggers a conformational change in the intein structure thereby allowing succinimide formation and C-terminal cleavage to proceed at basic pH. Whether or not this reflects the actual protein splicing of the wild-type Sce VMA intein remains unanswered. Nevertheless, the induction of cleavage at both splice junctions by exogenous cysteine simulated each step of the splicing reactions as follows: the exogenous cysteine functioned as Cys$^{455}$ to attack the thioester bond formed by the N-S acyl rearrangement; C-terminal cleavage occurred after the N-terminal cleavage; and the exogenous cysteine formed a covalent bond with the N-extein through an S-N acyl rearrangement (Fig. 1A) (5).

Further Examination of the Mechanism of Protein Splicing of the Sce VMA Intein—Studies of protein splicing of a thermophilic archael intein, i.e. Psp pol-1 intein, have been facilitated by the fact that in vivo splicing of the wild-type intein in a foreign protein context could be attenuated by low growth temperatures (12–15 °C) (3, 27). Consequently, sufficient precursors could be isolated to demonstrate in vitro splicing (3). The Sce VMA intein, on the other hand, underwent rapid splicing in vivo even at low growth temperatures (5, 7). We utilized amino acid substitutions of catalytic residues of the Sce VMA intein to block or attenuate the splicing process to isolate sufficient precursors and intermediates (5). Since the mechanism of protein splicing was deduced from analyses of mutants containing altered catalytic residues (5, 7), little is known about the close coupling and intricate balancing of the wild-type splicing reactions of the Sce VMA intein. In this study, we modulated protein splicing through appropriate substitutions of the −1 residue and the intein penultimate residue. As a result, we were able to examine protein splicing of the Sce VMA intein without altering its essential catalytic residues. In particular, we focused on the effect of pH on splicing reactions. Thiol-induced cleavage at the N terminus proceeded efficiently at pH values ranging from 5.5 to 9.0, but the rate of cleavage was significantly higher at pH 8.0 than pH 6.0 (Table II) (9). Similarly, our results suggest that branch resolution (Fig. 4) and C-terminal cleavage (Fig. 5B) were inhibited at pH 6.0 but proceeded efficiently at pH 8.0–8.5. It is possible that the nucleophilic displacements at the intein upstream and downstream splice junctions are assisted by similar groups of residues that act as acid or base catalysts. Completely different pH effects were observed when in vitro splicing of the fusion precursors from pMYK(Asn$^{-1}$) was examined. The rate of splicing was higher at pH 6.0 than pH 8.0 (Fig. 3B). As no catalytic residues of the intein in pMYK(Asn$^{-1}$) were altered, it is possible that a similar pH profile may also apply to protein splicing of the wild-type intein in vivo. Since it has been shown that the N-S (or N-O) rearrangement favors ester bond formation at low pH and amide bond formation at high pH (28, 29), the results suggest that the rate of overall splicing is primarily determined by the N-S acyl rearrangement. Consistent with this explanation, we have shown that thiol-induced N-terminal cleavage (Table II), branch resolution (Fig. 4), and C-terminal cleavage (Fig. 5B) proceed more efficiently at high pH than low pH. Therefore, both trans-esterification and branch resolution are unlikely to be the rate-limiting step in the protein splicing pathway. In addition, we were able to modulate the rate of protein splicing by substituting the −1 residue that directly affects the N-S acyl rearrangement (Table II). It has been shown that the rate of splicing for the thermophilic Psp pol-1 intein is also favored at pH 6 and inhibited at pH 9 or above (3).
Our data are consistent with the proposal that both the ther-
mophilic Psp pol-1 intein and the mesophilic Sce VMA intein follow the same protein splicing pathway (5).

Effect of Deletion and Insertion in the Sce VMA Intein—
Previously, we deleted the central region of the Sce VMA intein and showed that the remaining intein structure (splicing do-
main) is sufficient to catalyze protein splicing (15). In this study, we examined how the deletion might affect the structure and catalysis of the splicing domain. Our results indicated several differences between the deletion mutant (ΔY(NG)) and the full-length intein. First, splicing efficiency of the intein deletion mutant was affected by heterologous extein domains. For instance, when the intein deletion mutant was fused between MBP and CBD in ΔMYB(NM), more than 80% of the fusion precursors spliced in vivo (15). However, changing the C-extein into the E. coli thioredoxin (T) in ΔMYT(NM) resulted in only 50% of the precursors splicing in vivo (data not shown). In comparison, the full-length intein underwent complete splicing in vivo in all fusion systems that we constructed (i.e. MYB, MYT, MYK, and MYT4) (5). Second, the induction temperature for protein expression affected protein splicing of the deletion mutant but not the full-length intein. In vivo splicing of the intein deletion mutant was efficient when induction of protein expression of pΔMYB(NM) in E. coli was conducted at 15–20 °C but was completely blocked when the induction was at 30 °C or above (data not shown). The full-length intein, on the other hand, catalyzed efficient splicing in vivo at induction temperatures ranging from 15 to 37 °C (5, 9). The sensitivity of the intein deletion mutant to induction temperature could be due to the effects of the deletion on protein folding in vivo. Consistent with this explanation, expression of pΔMYB(NM) at induction temperatures of 30 °C or above resulted in only unspliced linear precursors incapable of undergoing in vitro splicing or cleavage (data not shown). Third, the amino acid substitutions that resulted in thiol-induced splice junction cleavage of the full-length intein had different effects on the intein deletion mutant. The rate of DTT-induced cleavage of the full-length intein with the N454A substitution increased significantly upon increase of the incubation temperature (between 4 and 23 °C) (Table II). In contrast, DTT-induced cleavage of the deletion mutant was efficient only at 4–16 °C and was completely inhibited at 23 °C (Fig. 6A). It is possible that the deletion mutation destabilized the structure of the deletion mutant, and consequently an increase in temperature perturbed the alignment of catalytic residues. The active site structure of the full-length intein, on the other hand, was more stable, and higher temperatures simply increased the rate of trans-esterification reaction by DTT.

Based on the crystal structure, Duan et al. (6) hypothesized that the Sce VMA intein may have evolved from a composite gene that resulted from the invasion of an endonuclease open reading frame into a pre-existing gene encoding a protein splicing element. In this study, we recreated this gene invasion scenario through genetic engineering. The gene encoding the endonuclease BamHI was inserted into the Sce VMA intein replacing the native endonuclease domain. Subsequently, the chimeric fusion was allowed to splice in ΔMYB(Bam) resulting in >80% splicing in vivo (Fig. 6B). Although no endonuclease activity was detected in the spliced ΔY(Bam) (data not shown), possibly due to misfolding of BamHI or the intein moiety interfering with dimerization and/or DNA binding of BamHI, the result clearly demonstrated the feasibility of an endonuclease gene invasion of a protein splicing element. Other heterologous proteins, e.g. chitin-binding domain (CBD) from B. circulans (22) and green fluorescent protein (GFP) from Aequorea victo-
rria (30), have also been inserted into the Sce VMA intein, and between 50 and 95% splicing efficiencies were obtained.2 In addition, both CBD and GFP in the spliced chimeric fusions appeared to fold correctly as the CBD fusion was able to bind chitin and the GFP fusion was fluorescent.2 Efficient splicing of the Sce VMA intein with insertion of different heterologous protein domains suggested a tightly packed structure in the intein splicing domain. This is consistent with the crystal structure of the Sce VMA intein which indicates that domain I, essentially the same as the splicing domain in our deletion mutant, consists of predominately closely packed β-sheets (6).

Perspectives—The work presented here has advanced our understanding of the mechanism of protein splicing. As analogous reactions have been found in the autoprocessing of some biologically important proteins, e.g. hedgehog proteins (31–33), glycosylasparaginases (34), etc., our results should enhance the understanding of the biological functions of these processes. Although the crystal structure of the excised Sce VMA intein has been solved, the active site structure at the extein-intein junctions prior to splicing has yet to be determined. It is apparent that complete characterization of the residues involved in four nucleophilic displacements of the splicing pathway requires further mutagenesis and crystallographic studies.

Protein splicing illustrates a highly specific and efficient way to cleave and religate peptide bonds. It is possible that all inteins may share a similar splicing pathway. As more inteins are being identified in various organisms (2), our study of the Sce VMA intein may reveal common strategies for modulating the rate of protein splicing, converting splicing into efficient and controllable peptide cleavage, and re-engineering inteins through deletion and insertion. The results presented in this study suggest many potential applications. The endonuclease activity of an intein is responsible for the intein-mediated gene mobility that allows site-specific insertion of an intein into an intein-less allele (12). By replacing the endonuclease domain of an intein with an endonuclease of different specificity, we can potentially redirect intein lateral transmission to a designated site. Intein insertion into a heterologous protein could be used to regulate its biological function through protein splicing. Our studies of the modulation of protein splicing and the effect of the −1 substitutions may provide valuable information regarding the choice of insertion sites and the strategy for conditional gene knock-out. Our results also suggest that inteins may tolerate insertion of heterologous protein domains. By inserting a functional protein domain (e.g. GFP, transcriptional factors, protein kinases, etc.) into an intein without diminishing its splicing activity, we may be able to monitor in situ protein splicing processes or link protein splicing to other biological events. The thiol-induced N-terminal cleavage of the Sce VMA intein has been used for protein purification in which the target protein is fused to the N terminus of the intein (9). Alternatively, we can use the C-terminal cleavage activity to purify a target protein fused to the C terminus of the intein. One advantage of the C-terminal fusion is that the N terminus of the fusion protein can be optimized for high level protein expression. With similar modifications, inteins from other organisms can also be used for protein purification or other intein-based applications. As nature reveals its unique ways to break and join peptide bonds, we will undoubtedly discover more usage of this novel class of proteins.

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2 S. Chong, unpublished results.
Note Added in Proof—The crystal structure of GyrA intein has recently been solved that showed the active site structure of the intein plus Ala as the –1 residue (35).

REFERENCES
1. Perler, F. B., Davis, E. O., Dean, G. E., Gimble, F. S., Jack, W. E., Neff, N., Noren, C. J., Thorner, J., and Belfort, M. (1994) *Nucleic Acids Res.* 22, 1125–1127
2. Perler, F. B., Olsen, G. J., and Adam, E. (1997) *Nucleic Acids Res.* 25, 1087–1094
3. Xu, M.-Q., Southworth, M. W., Mersha, F. B., Hornstra, L. J., and Perler, F. B. (1995) *Cell* 75, 1371–1377
4. Xu, M.-Q., and Perler, F. B. (1996) *EMBO J.* 15, 5146–5153
5. Chong, S., Yang, S., Paulus, H., Benner, J., Perler, F. B., and Xu, M.-Q. (1996) *J. Biol. Chem.* 271, 22159–22168
6. Duan, X., Gimble, F. S., and Quiocho, F. A. (1997) *Cell* 89, 555–564
7. Cooper, A. A., Chen, Y., Lindorfer, M. A., and Stevens, T. H. (1993) *EMBO J.* 12, 2575–2583
8. Hirata, R., and Anraku, Y. (1992) Biochem. Biophys. Res. Commun. 188, 40–47
9. Chong, S., Mersha, F. B., Comb, D. G., Scott, M. E., Landry, D., Vence, L. M., Perler, F. B., Benner, J., Kucera, R. B., Hirvonen, C. A., Pelletier, J. J., Paulus, H., and Xu, M.-Q. (1997) *Gene* (Amst.) 192, 271–281
10. Lambowitz, A. M., and Belfort, M. (1993) *Annu. Rev. Biochem.* 62, 587–622
11. Shah, D. A., and Goodrich-Blair, H. (1992) *Cell* 71, 183–186
12. Gimble, F. S., and Thorner, J. (1992) *Nature* 357, 301–306
13. Gimble, F. S., and Stephens, B. W. (1995) *J. Biol. Chem.* 270, 5849–5856
14. Gimble, F. S., and Wang, J. (1996) *J. Mol. Biol.* 263, 163–180
15. Chong, S., and Xu, M.-Q. (1997) *J. Biol. Chem.* 272, 15587–15590
16. Midgley, C. A., and Murray, N. E. (1985) *EMBO J.* 4, 2695–2703
17. Armstrong, A., Brown, R. S., and Tsugita, A. (1983) *Nucleic Acids Res.* 11, 7145–7156
18. Brooks, J. E., Benner, J. S., Heiter, D. F., Silber, K. R., Szynter, L. A., Jager-Quinton, T., Moran, L. S., Slatko, B. E., Wilson, G. G., and Nwankwo, D. O. (1989) *Nucleic Acids Res.* 13, 979–997
19. Dorner, L. F., and Schildkraut, L. (1994) *Nucleic Acids Res.* 22, 1068–1074
20. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
21. Guan, C. D., Li, P., Riggs, P. D., and Inouye, H. (1988) *Gene* (Amst.) 67, 21–30
22. Watanabe, T., Ito, Y., Yamada, T., Hashimoto, M., Sekine, S., and Tanaka, H. (1994) *J. Bacteriol.* 176, 4465–4472
23. Davis, E. O., Thangaraj, H. S., Brooks, P. S., and Colston, M. J. (1994) *EMBO J.* 13, 699–703
24. Davis, E. O., Jenner, P. J., Brooks, P. C., Colston, M. J., and Sedgwick, S. G. (1992) *Cell* 71, 201–210
25. Nogami, S., Satow, Y., Ohyu, Y., and Anraku, Y. (1997) *Genetics* 147, 73–85
26. Kane, P. M., Yamashiro, C. T., Wolczyk, D. F., Neff, N., Goebl, M., and Stevens, T. H. (1990) *Science* 250, 651–657
27. Xu, M.-Q., Comb, D. G., Paulus, H., Noren, C. J., Shao, Y., and Perler, F. B. (1994) *EMBO J.* 13, 5517–5522
28. Bruce, T. C., and Benkovic, S. J. (1966) in *Bioorganic Mechanisms* (Breslow, R., and Karplus, M., eds) Vol. 1, pp. 266–297, W. A. Benjamin, Inc., New York
29. Iwai, K., and Ando, T. (1967) *Methods Enzymol.* 11, 263–283
30. Shimomura, O., Johnson, F. H., and Saiga, Y. (1962) *J. Cell. Comp. Physiol.* 50, 223–227
31. Porter, J. A., von Kessler, D. P., Ekker, S. C., Young, K. E., Lee, J. J., Moses, K., and Beachy, P. A. (1995) *Nature* 374, 363–366
32. Porter, J. A., Ekker, S. C., Park, W.-J., von Kessler, D. P., Young, K. E., Chen, C.-H., Ma, Y., Woods, A. S., Cotter, R. J., Koain, E. V., and Beachy, P. A. (1996) *Cell* 86, 21–34
33. Porter, J. A., Young, K. E., and Beachy, P. A. (1996) *Science* 274, 255–259
34. Guan, C., Cui, T., Rao, V., Liao, W., Benner, J., Lin, C.-L., and Comb, D. (1996) *J. Biol. Chem.* 271, 1732–1737
35. Klabunde, T., Sharma, S., Telenti, A., Jacobs, W. R., Jr., and Saccettini, J. C. (1998) *Nat. Struct. Biol.* 5, 31–36