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# Protein splicing removes intervening sequences in an archaea DNA polymerase

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## ABSTRACT

**The Vent DNA polymerase gene from *Thermococcus litoralis* contains two in-frame insertions that must be spliced out to form the mature polymerase. Primer extension and cDNA PCR revealed no evidence of spliced RNA to account for this editing. In contrast, pulse-chase analysis indicated that expression constructs lacking the first insertion produced a protein precursor in *Escherichia coli* that was processed post-translationally to form polymerase and I-*Tli*I, the endonuclease protein that is the product of the second insertion. At least one intermediate, which migrated more slowly than the precursor and may be branched, was also detected. Amino acid substitutions at the splice junction slowed or blocked the protein splicing reaction. Processing occurs in several heterologous systems, indicating either self-splicing or ubiquitous splicing factors. Processing occurs in a mutant lacking I-*Tli*I endonuclease activity, establishing the independence of splicing and endonuclease activities.**

## INTRODUCTION

Production of mature proteins involves the flow of information from DNA to RNA to protein. Precise excision of DNA and RNA elements which interrupt that information have previously been described (1–3). More recently, evidence for the precise excision of intervening protein sequences has also been described in the *TFP1* allele from *Saccharomyces cerevisiae* (4,5) and *recA* gene from *Mycobacterium tuberculosis* (6,7). Each contains internal in-frame peptide segments which must be removed to produce the mature protein. Expression of Tfp1 and RecA each results in two polypeptides: one representing the intervening protein sequence (IVPS), and the other the ligated product of the external protein sequences (EPS).

Similarly, the Vent DNA polymerase gene from the hyperthermophilic archaea *Thermococcus litoralis* contains two in-frame IVPSs (8; Fig. 1) that can be deleted at the DNA level without affecting the kinetic and biochemical properties of the expressed polymerase (9). Correct processing of the Vent DNA polymerase gene containing both IVPSs occurs in the native archaea, *T.litoralis*. In addition, correct processing of expression constructs lacking IVPS1 has been observed in eubacterial *E.coli* (8) and in eukaryotic baculovirus-infected insect cells (10). Furthermore, rabbit reticulocyte and *E.coli in vitro* transcription/translation systems correctly remove IVPS2 sequences to produce the mature polymerase (11). The diversity of hosts capable of processing the polymerase, and reports of similar processing in *S.cerevisiae* and *M.tuberculosis*, suggest that either the Vent DNA polymerase precursor carries all required splicing factors, or that host-derived splicing factors have been conserved across all three kingdoms. We describe here experiments which define the nature of the splicing event for the Vent DNA polymerase.

## MATERIALS AND METHODS

### Strains and plasmids

Polymerase expression constructs used the pET11 derivative pAII17 (9) in which transcription initiates from the T7 promoter (8, Fig. 1). pNEB687 expresses a Vent DNA polymerase gene containing IVPS2 but lacking IVPS1. pAKK4 expresses a Vent DNA polymerase gene lacking both IVPSs. The host for expression from the T7 promoter was BL21(DE3)pLysS (12). Expression was also monitored from the tac promoter in ER2315, an *E.coli recF*143 strain. Ptac expression constructs included: pAJQ10, containing the wild type gene lacking IVPS1, pAKO7, a frameshift mutant derived from pAKC1 (8), and pAID1, a deletion mutant lacking coding sequences for amino acid residues L1205-N1209, replacing those residues with a single phenylalanine codon. This deletion was created by Bal31 exonuclease degradation starting from the *NdeI* site within IVPS2. pAID1 produces an unspliced precursor, but does not produce the mature polymerase. Trans-complementation expression of I-*Tli*I was from pAKN5, an IVPS2-containing *Hinc*II to *Eco*RI fragment from the Vent DNA polymerase gene under control of the lac promoter in a plasmid (pIH919) with the P15A replication origin. The vector lacking I-*Tli*I is pIH919, and was provided by Iris Hall (New England Biolabs).

### Enzymes

All enzymes, except where noted, were from New England Biolabs and used under recommended conditions.

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### Poison primer detection of spliced RNA

*RNA production and isolation.* Total RNA was isolated from actively expressing BL21(DE3)pLysS/pNEB687 using the guanidinium isothiocyanate, cesium chloride cushion method (13). Total RNA was isolated from RR1/pAJQ10 by a variation of the phenol/guanidinium isothiocyanate extraction method (14). Cells from 100 ml of an induced mid-log-phase culture were suspended in 1.6 ml phenol/guanidinium isothiocyanate (RNAzol B, Biotecx). After addition of 180 μl chloroform:isoamyl alcohol (24:1), the suspension was vigorously shaken, incubated on ice 5 min, and spun 15 min in a microcentrifuge. RNA was precipitated from the upper layer with an equal volume of isopropanol, washed with 70% ethanol, dried and treated with 10 units of DNase I (Promega) in 400 μl 40 mM Tris-HCl (pH 8.1), 6 mM $MgCl_2$, 10 mM NaCl for 20 min at 37°C. RNA was recovered by phenol extraction and ethanol precipitation (total yield 660 μg). In vitro transcripts were synthesized from indicated plasmids as previously described (15).

*Poison primer reactions.* Reactions used the antisense primer NOR3 (5′-GTGGCATAAAAGCCGTCA-3′) which annealed downstream of IVPS2. Annealing reactions (7 μl), containing RNA and 200 fmol (0.6 μCi) 5′-$^{32}$P-labeled NOR3 (13) in 70 mM Tris-HCl (pH 8.0), 85 mM NaCl, 14 mM DTT, were incubated at 65°C for 3 min, then transferred to a dry ice bath for 1 min. To each reaction was then added 2 μl Extension Mix (625 μM each dGTP, dCTP, dTTP, 2.5 mM ddATP in 50 mM Tris-HCl (pH 8.0), 60 mM NaCl, 6 mM $Mg(OAc)_2$, 10 mM DTT) and 4 units AMV reverse transcriptase (Amersham). After incubation at 48°C for 30 min, reactions were terminated by addition of 10 μl formamide. Samples were analyzed by 12% denaturing PAGE followed by autoradiography.

### Pulse-chase analysis of protein splicing

BL21(DE3)pLysS cells containing the indicated T7 expression constructs were grown with vigorous shaking at 30°C to early log phase in M9 salts containing 0.2% glucose, 0.1 mg/ml ampicillin and 30 μg/ml chloramphenicol (13). Expression from the T7 promoter was induced by the addition of IPTG to 0.3 mM. After incubating 30 min at 37°C, *E.coli* host expression was inhibited by addition of rifampicin to 0.2 mg/ml, and incubation at 37°C was continued for 1 h. Cells from 3 ml of culture were concentrated 7.5-fold in the culture media and placed in a 37°C bath. After addition of 80 μCi of [$^{35}$S]methionine (1200 Ci/mmol; DuPont NEN) cultures were incubated 2 min at 37°C, at which point 27 μl of 20 mg/ml unlabeled methionine chase was added. Immediately, and at indicated times after cold methionine addition, 35 μl aliquots were removed from the cultures, mixed with 70 μl of 1.5×SDS gel loading buffer (13) and incubated in a boiling water bath for 5 minutes. Samples were separated by SDS-PAGE (10%; Daiichi, Tokyo, Japan). The gel was fixed by washing in three changes of 25:65:10 isopropanol:water:acetic acid (30 min per change), dried and autoradiographed.

### Assays of I-*Tli*I endonuclease cleavage

I-*Tli*I endonuclease cleavage was assayed at 70°C for 20 min in reactions containing 25 μg/ml linearized DNA substrate (containing the Vent DNA polymerase gene EPS2/EPS3 junction region), 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT. The extent of cleavage was monitored by agarose gel electrophoresis.

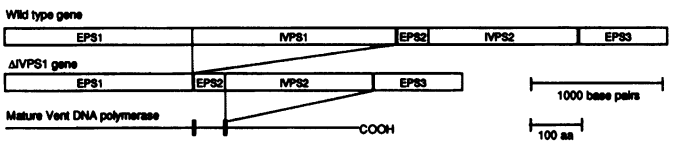


**Figure 1.** Vent DNA polymerase gene structure. The wild type Vent DNA polymerase gene contains two in-frame intervening protein sequences (IVPS). The polymerase coding regions interrupted by these insertions have been labeled as external protein sequences (EPS). Constructs lacking IVPS1 (ΔIVPS1) include pNEB687 (8) and pAJQ10.

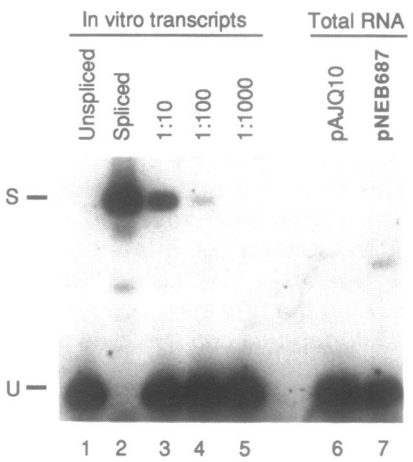


**Figure 2.** Detection of spliced mRNA by 'poison' primer extension. Primer NOR3 was designed to anneal 2 nucleotides downstream from the IVPS2/EPS3 splice junction. Extension of NOR3 annealed to spliced or unspliced Vent DNA polymerase mRNA in the presence of dGTP, dCTP, dTTP and ddATP should result in products of 27 (S) or 21 (U) nucleotides in length, respectively, which correspond to the position of the first U encountered in the respective templates. Primer extension reactions were carried out as described in MATERIALS AND METHODS. Lane 1. 120 ng *in vitro* transcript of *Bam*HI-linearized pNEB687 (containing IVPS2). Lane 2. 120 ng *in vitro* transcript of BamHI-linearized pAKK4 (IVPS2 deleted). Lanes 3–5. Mixture of 120 ng pNEB687 transcript and 12 ng, 1.2 ng and 0.12 ng pAKK4 transcript (10:1, 100:1 and 1000:1 ratios unspliced:spliced mRNA, respectively). Lane 6. 33 μg total RNA from induced *E.coli* RR1/pAJQ10 (Vent polymerase with IVPS2, tac promoter) cells. Lane 7. 7.5 μg total RNA from induced *E.coli* BL21(DE3)pLysS/pNEB687 (Vent polymerase with IVPS2, T7 promoter). Extension products were sized by comparison with RNA sequencing reactions of pNEB687 and pAKK4 transcripts (not shown).

## RESULTS AND DISCUSSION

### Possible involvement of RNA splicing in Vent DNA polymerase production

The well-established precedents of RNA splicing guided our early studies of Vent DNA polymerase processing. The conserved sequences and structures found in previously described RNA splicing systems were not evident in the Vent DNA polymerase gene (1,2,8). Additionally, multiple silent mutations at the splice junction had no effect on polymerase production, discounting the involvement of RNA secondary structure at that site (8). The absence of detectable spliced RNA was confirmed using cDNA PCR (8,16) and 'poison-primer' extension (17). cDNA PCR analysis of total RNA from *T.litoralis* yielded products corresponding to unspliced, but not spliced message across either IVPS junction (data not shown). Primer extension analysis of

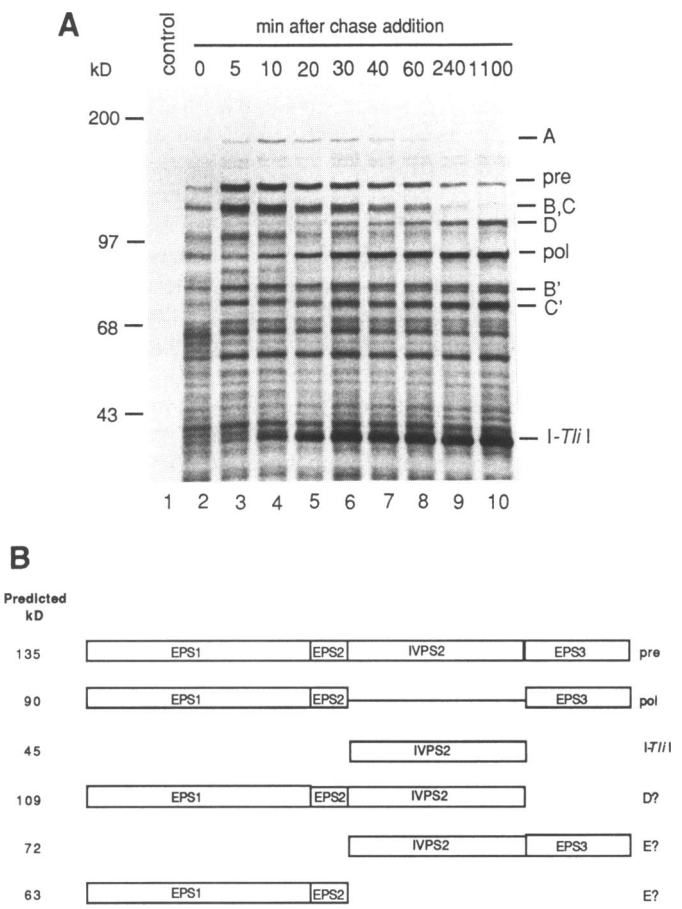


**Figure 3. A.** Pulse-chase analysis of Vent DNA polymerase. *E.coli* . Pulse-chase analysis of indicated expression constructs was performed as described in MATERIALS AND METHODS. Lane 1 pAII17, the T7 expression vector without a gene insert. Lanes 2 – 10, Vent DNA polymerase construct pNEB687. Numbers at the top of the gel indicate the length of time between cold methionine addition and sample collection. The mobility of $^{14}C$ protein molecular weight standards is indicated at the left of the autoradiograph. Bands corresponding to the primary translation product of the Vent DNA polymerase gene (pre), mature Vent DNA polymerase (pol), and the I-*Tli*I endonuclease are indicated, as well as potential splicing intermediates described in the text. Apparent molecular weights determined by SDS-PAGE have previously been reported (8) for the polymerase (93,000) and I-*Tli*I (42,000). **B.** Predicted sizes of Vent DNA polymerase gene products based on the deduced amino acid sequence.

total RNA isolated from *E.coli* expressing the polymerase gave similar results (Fig. 2). Primer extension across the downstream IVPS2 splice junction in the presence of ddATP yielded only products diagnostic of unspliced message. Comparison with mixtures of 'spliced' and 'unspliced' RNA standards indicated that no more than 0.1% of the Vent DNA polymerase mRNA was spliced at that junction, the limit of detection in these experiments (Fig. 2).

### Pulse chase analysis of protein processing

In light of the evidence against RNA splicing, we focused on [$^{35}S$]methionine pulse-chase analysis to detect protein splicing. To simplify the analysis, we examined processing in *E.coli* of constructs lacking IVPS1 (Fig. 1). This analysis was facilitated by use of the T7 promoter in the presence of rifampicin, allowing selective expression of Vent DNA polymerase in the absence of synthesis of *E.coli* proteins (12, Fig. 3 control lane). Within 5 min of cold methionine addition, translation of labeled proteins appeared complete, yielding multiple labeled proteins that correlated with species observed by Western blot analysis (see Fig. 5A below). Since the coding sequence contains numerous codons rarely used in *E.coli*, one possible source of these peptides is premature translation termination. However, the pattern of proteins was not altered by rare codon replacement, coexpression of the rare arginine tRNA argU (18) or linkage to the tac promoter (data not shown). Thus, these smaller products are not the result of premature translation termination. Since most of these products showed no further decay between 5 min and 18 h, they appear to be proteolyzed during or immediately following translation, after which they are not further degraded. Several proteins did show changes in intensity over time, presumably reflecting splicing precursors, intermediates and products. Some of the most prominent processed polypeptides are the sizes predicted from the primary protein sequence for the IVPS2-containing precursor (135 kDa), the mature polymerase (90 kDa) and I-*Tli*I, the 'intron' endonuclease encoded by IVPS2 (45 kDa; Fig. 3B). These time-dependent shifts in the labeled protein pattern persisted in control experiments when new synthesis was blocked by antibiotic protein synthesis inhibitors, demonstrating that these changes resulted from post-translational processing. The quantity of precursor reached a maximum by 5 min, and then decreased over the 18 h time course. Concomitant with this decrease, the polymerase and I-*Tli*I increased in prominence, with a $t_{1/2}$ of about 20 min. Since appearance of polymerase follows that of the precursor, splicing is not obligatorily coupled to translation. The absence of significant amounts of the precursor in the native *T.litoralis*, as well as the absence of corresponding precursors in the native Tfp1 and *M.tuberculosis* RecA sources, makes it seems likely that the slow processing observed in *E.coli* reflects expression at 37°C of a protein normally expressed at much higher temperatures (>80°C). This reduced rate of processing may reflect either slower folding or a decrease in the rate of peptide bond cleavage and rejoining.

While the decrease in precursor paralleled the increase in polymerase, greater amounts of I-*Tli*I were produced during the reaction. This accumulation could arise from processing of truncated precursors that contain all of I-*Tli*I. In fact, the intensity of protein species labeled B and C mimics that of the full-length precursor, and proteins which differ in size by the mass of I-*Tli*I (B′ and C′) appear as these two species disappear (Fig. 3A). Thus, at least some of the external polymerase sequences may be dispensable for the splicing reaction.

### Comparison with known examples of protein splicing and proteolysis

The aligned splice junctions of the known examples of protein splicing reveal several similarities (Fig. 4). In particular, -OH and -SH side chains are found on the N-terminal side of both splice junctions, preceded by the dipeptide His-Asn at the downstream splice junction. The His-Asn-Ser/Thr/Cys motif is reminiscent of the 'catalytic triad' found in serine and cysteine proteases. These classes of proteases proceed via a mechanism where the scissile peptide bond of the substrate is attacked by a nucleophilic residue (Ser or Cys), forming an acyl-enzyme intermediate with the N-terminal peptide that is then hydrolyzed by water to release free enzyme and cleaved substrate (19). The nucleophile is activated for both acylation and deacylation steps by deprotonation/protonation of the side chain by an adjacent histidine, which in turn has its pKa modulated by interactions with an adjacent Asp (in serine proteases) or Asn (in cysteine

proteases) residue. Although these residues are not adjacent in the primary sequence of any known proteases, they are in close proximity in the folded active site, and one can imagine a similar role for the His-Asn-Ser/Thr/Cys motif described here in a protein splicing mechanism. Such a mechanism could potentially involve formation of intramolecular 'acyl-enzyme'-like intermediates at one or both splice junctions, followed by aminolysis of the upstream junction by the free amino group of the downstream EPS to generate the new peptide bond. How such a free amine would be generated is not clear. Experiments involving active site nucleophiles of proteases (20,21) and β-lactamase (22,23) led us to replace the serine at the junction of EPS2 and IVPS2 with threonine (S1082T), cysteine (S1082C), and alanine (S1082A). Mutant protein expression was analyzed by Western blot, DNA polymerase activity and pulse-chase assays. The S1082T mutant displayed 10% of the polymerase activity of the wild-type enzyme, while the S1082C and S1082A mutants gave no detectable activity. Similar results were seen in Western blot (Fig. 5A) and pulse-chase analyses (Fig. 5B), where the Vent DNA polymerase (pol) and I-*Tli*I bands present in the wild type were discernable only at the latest time point in S1082T, only faintly seen in S1082C and not seen in S1082A. The effect on polymerase production parallels the change in character of the side chain from primary to secondary hydroxyl, to sulfhydryl, and finally to aliphatic, which is consistent with involvement of S1082 both as a nucleophile and a leaving group in the splicing reaction. Of course, other structural or catalytic roles are also possible. It is interesting to note that serine residues also occur at positions S1084 and S1087, and are not able to substitute for the loss of S1082.

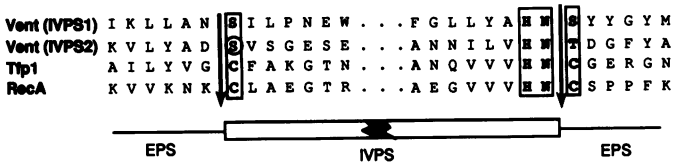


**Fig. 4.** Amino acid sequences at proposed protein splice junctions. Amino-terminal (top) and carboxy-terminal (bottom) splice junctions are shown with splice sites indicated by arrows and conserved or similar amino acids boxed. Tfp1 is the catalytic subunit of the *Saccharomyces cerevisiae* vacuolar proton-translocating adenosine triphosphatase (4,5). RecA refers to *Mycobacterium tuberculosis recA* gene product (6). The mutagenesis target (S1082) from Vent DNA polymerase IVPS2 is circled.

## Appearance of splicing intermediates

The mutants also affect other SDS-PAGE products which appear with kinetics consistent with being splicing intermediates. For example, species A migrates more slowly than the precursor on SDS-PAGE and is reminiscent of branched splicing intermediates observed in Group I RNA introns (1,2; Fig 5B). Alternatively, this could represent a multimeric splicing intermediate. This polypeptide persists in S1082T, and is absent in S1082C and S1082A, as might be expected from the reduced levels of splicing noted in these variants. Other prominent bands correspond to the sizes expected for aborted splicing products (Fig. 3B). These include E (~66 kD; Fig. 5), possibly representing either EPS1/EPS2 or IVPS2/EPS3, and D (~107 kD; Fig. 3A), potentially representing EPS1/EPS2/IVPS2. E is particularly prominent in S1082C, suggesting that cysteine at this position may form a blocked acyl-enzyme-like structure which hydrolyzes either spontaneously or under the conditions of SDS-PAGE.

## Independence of I-*Tli*I endonucleolytic cleavage and protein splicing

The IVPS ORFs found in Tfp1, RecA and Vent DNA polymerase have protein sequence similarity to homing endonucleases, a class of intron-encoded proteins capable of cleaving alleles which lack the intron (4–6,8). In fact, both I-*Tli*I (8) and VDE, the protein product of the *TFP1* IVPS (24), have been shown to cleave DNA lacking the corresponding IVPS at the deletion junction. To test if endonuclease and splicing activities are independent, endonuclease activity was abolished by a single amino acid substitution within one of the conserved dodecapeptide motifs (1,8) of I-*Tli*I (D1236A). Despite the loss of detectable I-*Tli*I activity, protein splicing was unaffected by this mutation as evidenced by polymerase activity assays and Western blot analysis

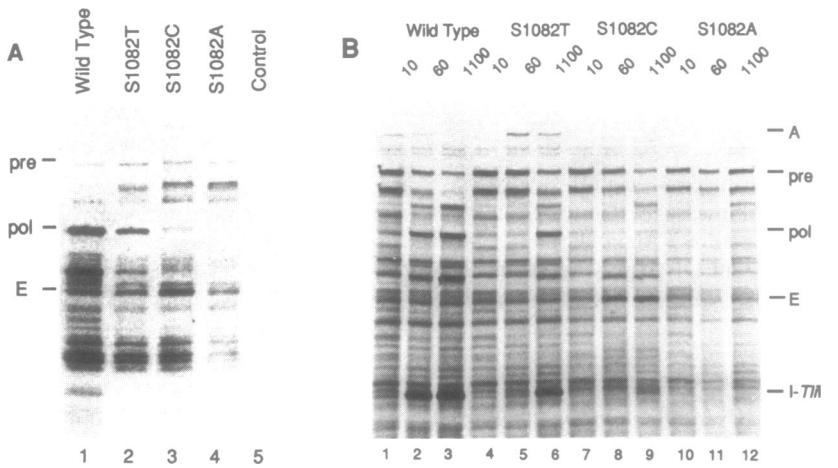


**Figure 5.** Expression of Vent DNA polymerases mutants. The precursor (pre), Vent DNA polymerase (pol), and I-*Tli*I endonuclease are indicated. The proposed splicing intermediate (A) and aborted splicing product (E) are also indicated. (Panel **A**) Western blot of cell extracts of *E.coli* BL21(DE3)pLysS containing indicated expression constructs probed with anti-Vent DNA polymerase mouse sera as described by Perler *et al.* (8). Control lane contains extracts of cells containing the expression vector pAII17. (Panel **B**) Pulse-chase analysis of mutant Vent DNA polymerase gene products (see Fig. 3). Numbers above the lanes indicate the time between cold methionine addition and sample collection.

(data not shown). On the other hand, splicing does not appear to be required for endonuclease function since extracts of S1082A retained reduced levels of cleavage. These results indicate that there is significant separation between the splicing and endonuclease functions of the I-*Tli*I coding region. This is also consistent with the notion that homing endonucleases facilitate transfer of the DNA encoding the IVPS to genes without insertions, as opposed to participating in the splicing reaction itself (1,2).

### I-*Tli*I does not act in *trans* to affect splicing

Frameshift mutations in IVPS2 demonstrated a requirement for a continuous open reading frame to obtain active Vent DNA polymerase (8). A further structural role for IVPS2 sequences is seen in internal IVPS2 deletions that are inactive in splicing (see below). Another possible role for the IVPS2-encoded protein is as a *trans*-acting maturase, similar to RNA maturases encoded within Group I introns (1,2). To test this possibility, we attempted to complement non-functional IVPS2 insertion frameshift and in-frame deletion mutants with I-*Tli*I (Table I). Since polymerase coding regions were not altered, both mutants could potentially be rescued by I-*Tli*I acting as an RNA maturase. Similarly, the in-frame deletion mutant, but not the truncated frameshift mutant, could potentially be rescued by I-*Tli*I acting as a protein maturase. *In vivo* complementation in *E.coli* of both mutants yielded neither polymerase activity (Table I) nor protein corresponding to the mature polymerase on Western blots. Thus, I-*Tli*I either does not act as a maturase, or cannot overcome the distortion in RNA or protein structure caused by these mutations.

Given the diverse origins of the three known examples of protein splicing, it seems likely that other examples will be found. Furthermore, this diversity also suggests that information for IVPS removal will reside largely within the IVPS, although amino acid residue(s) adjacent to the splice junctions may participate directly or influence the reaction. Given the fact that appearance of the full length precursor precedes that of the polymerase, we also consider it likely that protein conformation in the vicinity of the splice will be an important modulator of splicing. We note that removal of the intervening protein sequences can be viewed in the same light as introns removed by RNA splicing (1,2) and mobile DNA elements removed by DNA splicing (3). In all three cases, the efficient removal of intervening information allows normal cellular processes to proceed unabated, and thus these insertions can be maintained without compromising cell viability. Lateral transmission of the IVPS through the mediation of the encoded homing endonuclease is also free from constraints of inserting into non-coding or non-essential regions, perhaps conferring a selective advantage over insertion elements which disrupt gene expression. Alternatively, insertion of the IVPS within a coding sequence may itself be a scheme to ensure translation of the homing endonuclease, facilitating transfer of the insertion to alternate sites.

**Table I.** I-*Tli*I does not rescue frameshift and deletion variants in IVPS2

| | wild type | | frameshift | | deletion | |
|---|---|---|---|---|---|---|
| vector | + | − | + | − | + | − |
| I-*Tli*I | − | + | − | + | − | + |
| I. Endonuclease | 100 | 200 | <2(n.d) | 20 | | |
| Polymerase | 100 | 93 | 0 | 2 | | |
| II. Endonuclease | 100 | 100 | | | <2(n.d) | 10 |
| Polymerase | 100 | 84 | | | 0 | 2 |

Polymerase and endonuclease activities are expressed as a percentage of the activity seen in ER2315 containing the two plasmids pIH919 and pAJQ10. n.d. = none detected.

Expression was monitored in the *E.coli* strain ER2315. Cells contained two plasmids, one expressing the polymerase from the tac promoter (wild type, frame shift, deletion) and one either containing or lacking I-*Tli*I (vector or I-*Tli*I, respectively). pAJQ10, pAKO7 and pAID1 were the wild type, frame shift and deletion constructs, respectively

Expression was induced by incubating in the presence of 0.4 mM IPTG for 5 h at 30°C. Polymerase activity assays of crude extracts were performed as previously described (8). Endonuclease activity was monitored as described in MATERIALS AND METHODS.

## ACKNOWLEDGEMENTS

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