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An in Vitro Screening System for Protein Splicing Inhibitors Based on Green Fluorescent Protein as an Indicator

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This paper describes an in vitro fluorometric assay system for protein splicing based on the RecA intein of *Mycobacterium tuberculosis* and a modified green fluorescent protein (GFP). The assay takes advantage of the fact that polypeptides inserted adjacent to residue 129 of GFP cause the protein to form inclusion bodies when expressed in *Escherichia coli* and to be incapable of fluorophore formation. However, when the inserted polypeptide is an intein, the renatured fusion protein can undergo protein splicing and chromophore formation. Comparison of chromophore formation by renatured GFP−intein fusion and renatured GFP showed that under optimal conditions (pH 6.5 and 20 °C) protein splicing is significantly slower than GFP chromophore formation. Taking advantage of the reversible inhibition of protein splicing by zinc ion, a fluorometric protein splicing assay was developed in which the denatured fusion protein of GFP and the RecA intein was purified on a metal ion affinity column and renatured in the presence of 2 mM $ZnCl_2$. When diluted into appropriate buffers, protein splicing could be initiated by the addition of a molar excess of EDTA and followed fluorometrically. This assay should be valuable as a high-throughput screening system for protein splicing inhibitors as potential antimycobacterial agents and as tools for studying the mechanism of protein splicing

Inteins are intervening sequences that interrupt the coding regions of microbial genes, giving rise to nonfunctional protein precursors that can be activated by protein splicing. Protein splicing is a self-catalyzed process that leads to the ligation of the polypeptide segments (exteins) flanking the intein by a native peptide bond, shown diagrammatically in Figure 1A, as described in a recent review.[1] Often, the interrupted genes have important functions in DNA metabolism, and protein splicing is therefore essential for normal growth and survival. Because inteins are found in no bacterial pathogens other than mycobacteria and are absent from multicellular organisms, inhibitors of protein splicing would potentially be highly specific antimycobacterial antibiotics. Consequently, there is considerable interest in developing efficient screening systems for protein splicing inhibitors.

Several in vivo screening systems for identifying protein splicing inhibitors have been developed. The first of these involved disruption of the thymidylate synthase coding region from bacteriophage T4 by the *Mycobacterium tuberculosis* RecA intein.[2] Plasmids encoding such a fusion protein can complement *Escherichia coli thyA* mutants if protein splicing occurs. In the absence of thymine, complementation of *thyA* allows growth and provides a method to select for protein splicing, but in the presence of thymine and trimethoprim, complementation leads to growth inhibition, thus allowing selection against protein splicing.[3] Recently, Perler and colleagues described an assay for the inhibition of protein splicing mediated by the *Mycobacterium xenopi* GyrA intein, based on the conditional dominant lethality of quinolone-sensitive GyrA expressed in a quinolone-*resistant E. coli* host.[4] A screening system developed in our laboratory involved the protein splicing-dependent expression of the CcdB toxin, which allows survival of the host cells only when protein splicing is blocked.[5]

A disadvantage of in vivo systems for screening antibacterial drugs against a specific target is that they involve monitoring the growth of bacteria and are therefore subject to interference by unspecific antibacterial agents. Accordingly, we have focused on developing an in vitro assay system for inhibitors of protein splicing, which examines the inhibition of a specific reaction and is therefore not susceptible to false signals that inhibit other reactions essential for growth. It takes advantage of the observation that green fluorescent protein (GFP) fails to form its chromophore in vivo when interrupted by an intein inserted adjacent to residue 129, but that subsequent protein splicing allows fluorescence to develop.[6] Ozawa and co-workers used this system in a trans-splicing mode as an in vivo assay for protein−protein interactions that bring the intein fragments together and thereby promote protein splicing.[6−8] In this paper, we describe the insertion of *M. tuberculosis* RecA intein at residue 129 of a modified

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(1) Paulus, H. *Annu. Rev. Biochem.* **2000**, *69*, 447−496.

(2) Derbyshire, V.; Wood, D. W.; Wu, W.; Dansereau, J. T.; Dalgaard, L. Z.; Belfort, M. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 11466−11471.

(3) Belfort, M. U.S. Patent 5,798,731, 1998.

(4) Adam, E.; Perler, F. B. *J. Mol. Microbiol. Biotechnol.* **2002**, *4*, 479−487.

(5) Lew, B. M.; Paulus, H. *Gene* **2002**, *282*, 169−177.

(6) Ozawa, T.; Nogami, S.; Sato, M.; Ohya, Y.; Umezawa, Y. *Anal. Chem.* **2000**, *72*, 5151−5157.

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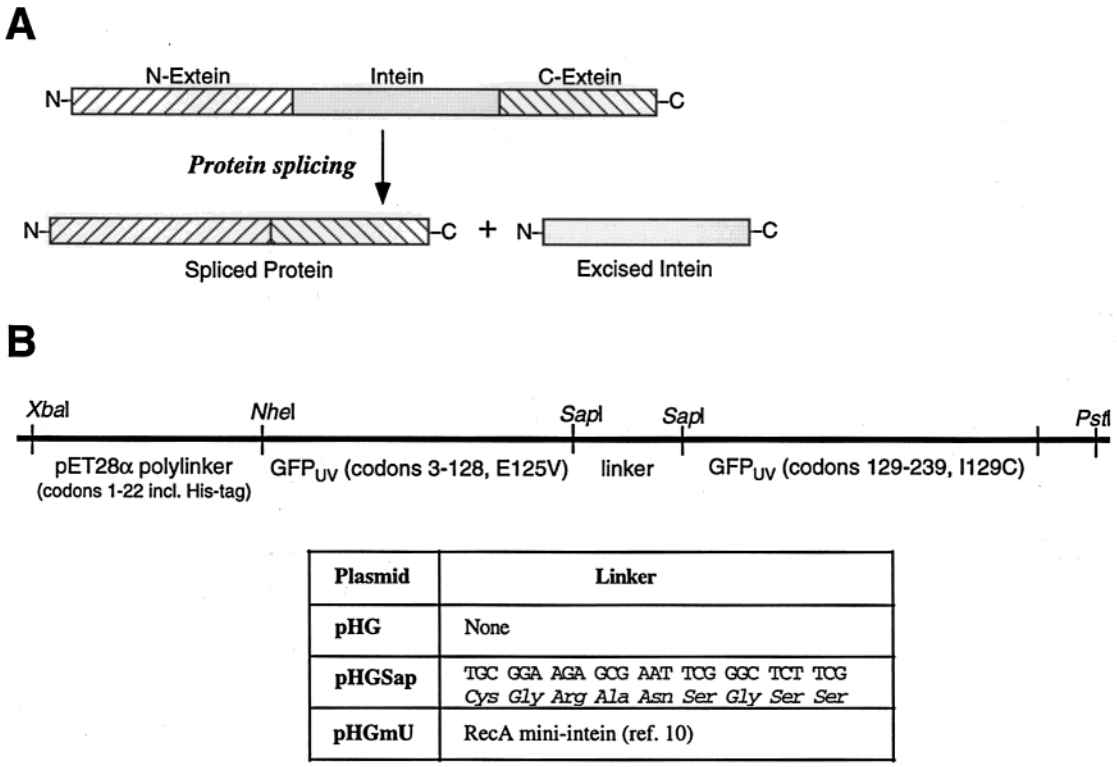

Plasmid	Linker
pHG	None
pHGSap	TGC GGA AGA GCG AAT TCG GGC TCT TCG *Cys Gly Arg Ala Asn Ser Gly Ser Ser*
pHGmU	RecA mini-intein (ref. 10)

Figure 1. (A) Schematic illustration of the protein splicing process. (B) Structure of the plasmids used in this work. Shown is the *Xba*I−*Pst*I segments that replaces the corresponding segments in plasmid pTYB2 (New England Biolabs) as described in the text. The residues in GFP are numbered as in wild-type GFP_{UV}. The DNA segment inserted between the *Sap*I sites in the various plasmids are shown in the box.

GFP, the isolation of the denatured fusion protein, and its use to measure protein splicing in vitro and assay for protein splicing inhibitors.

EXPERIMENTAL PROCEDURES

Plasmid Construction. The coding sequence of GFP_{UV}, a GFP variant with improved properties,[9] was subjected to mutagenesis using the polymerase chain reaction (PCR) with plasmid pGFPUV (Clontech) as the template in a two-stage process. The N-terminal segment was amplified with primers P1 (5′ C GCG GCT AGC AAA GGA GAA GAA CTT TTC AC) and P2 (5′-GA ATT CGC TCT TCC GCA ACC TTT TAA CAC GAT AC) and the C-terminal segment with primers P3 (5′ G AAT TCG GGC TCT TCG TGT GAT TTT AAA GAA GAT G) and P4 (5′- C TGC AGC GGG AAT TCA TTA TTT GTA GAG CTC ATC). Primer P1 contained the *Nhe*I site near the N-terminus of GFP, and primer P2 introduced two amino acid substitutions (E125V and I129C), followed by tandem *Sap*I and *Eco*RI recognition sites at the C-terminal end of the amplified segment.. Primer P3, which partially overlapped primer P2, introduced tandem *Eco*RI and *Sap*I recognition sites and an I129C substitution at the N-terminal end of the amplified segment, which was not otherwise modified, and primer P4 contained the *Pst*I site just beyond the GFP termination codon. These amplification products were ligated through their terminal *Eco*RI sites and inserted into the *Nhe*I and *Pst*I sites of plasmid pHYB2, which was derived from pTYB2 (New England Biolabs) by replacing the *Xba*I−*Nhe*I segment at the N-terminus of the fusion protein controlled by the bacteriophage T7 promoter by the corresponding His-tag-containing segment from plasmid pET28a (Novagen). The resulting plasmid, pHGSap, encoded a variant of GFP_{UV} in which the 24 N-terminal residues were replaced by the 20 N-terminal residues of the plasmid pET28a polylinker containing a His-tag and which carried the substitutions E125V and I129C and a nonapeptide insertion, GRANSGSSC, following residue C129. This insertion was flanked by tandem *Sap*I sites, and cleavage by *Sap*I followed by self-ligation led to plasmid pHG, which encoded for a functional GFP variant lacking the nonapeptide insertion.

The insertion of the RecA intein adjacent to C129 was effected by the *Sap*I strategy described earlier,[10] using the coding region for the RecA mini-intein flanked by *Sap*I sites to replace the nonapeptide insert in plasmid pHGSap with the mini-intein, yielding plasmid pHGmU. A splicing incompetent variant of plasmid pHGmU was prepared by using a mini-intein in which the essential C-terminal Asn codon was replaced by an Ala codon.[11]

The structure of the plasmids used in this work is summarized in Figure 1B.

Protein Expression and Purification. Cultures (50 mL) of *E. coli* JM109(DE3) (Promega) transformed with appropriate plasmids were grown at 37 °C in Luria broth supplemented with 100 μg/mL ampicillin. At a culture density ($A_{600\ nm}$) of 0.5, the

(7) Ozawa, T.; Takeuchi, M.; Kaihara, A.; Sato, M.; Umezawa, Y. *Anal. Chem.* **2001**, *73*, 5866−5874.
(8) Ozawa, T.; Umezawa, Y. *Curr. Opin. Chem. Biol.* **2001**, *5*, 578−583.
(9) Crameri, A.; Whitehorn, E. A.; Tate, E.; Stemmer, W. P. C. *Nat. Biotechnol.* **1996**, *14*, 315−319.
(10) Gangopadhyay, J. P.; Jiang, S.-q.; Berkel, P. v.; Paulus, H. *Biochim. Biophys. Acta* **2003**, *1619*, 193−200.
(11) Shingledecker, K.; Jiang, S.-Q.; Paulus, H. *Arch. Biochem. Biophys.* **2000**, *375*, 138−144.

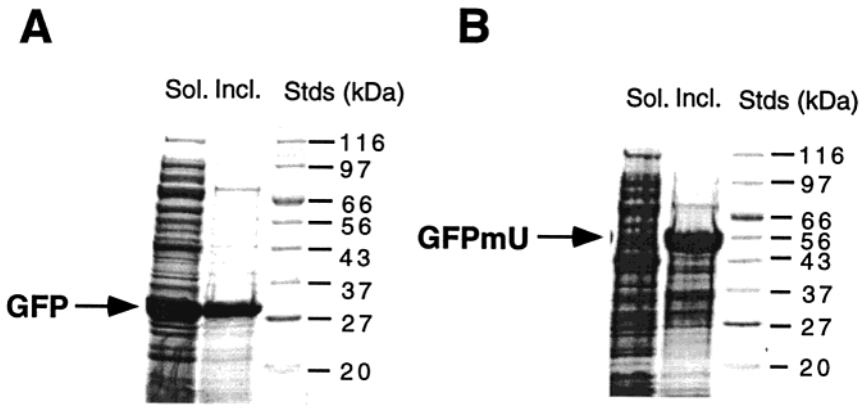

Figure 2. Expression of GFP fusion proteins in the soluble and inclusion body fractions of *E. coli* carrying plasmids (A) pHG and (B) pHGmU. Cell extracts were subjected to centrifugation at 16500*g* for 20 min, and equivalent samples of the soluble (sol.) and insoluble (incl.) fractions were subjected to SDS−PAGE together with protein standards. The gels were stained with Coomassie Blue. The bands corresponding to GFP and the GFPmU fusion protein are indicated by arrows.

cultures were induced with 0.4 mM isopropyl β-D-thiogalactoside (IPTG) and allowed to grow for another 3 h at 37 °C. Cells were harvested by centrifugation at 6500*g* for 15 min, resuspended in 3 mL of buffer A (20 mM sodium phosphate, pH 7.5, 0.5 M NaCl), and disrupted by passage through a French pressure cell. The pellet obtained by centrifugation at 16500*g* for 20 min was resuspended in buffer B (buffer A supplemented with 8 M urea) to extract the inclusion bodies and centrifuged at 16500*g* for 20 min to remove insoluble material. The solubilized inclusion bodies were loaded on a Talon metal affinity column (Clontech) equilibrated with buffer B and washed twice with 1 mL of buffer B and twice with 1 mL of buffer B supplemented with 10 mM imidazole. The His-tagged protein was then eluted with 1 mL of buffer B supplemented with 100 mM imidazole.

Renaturation Procedure. Renaturation of the purified inclusion bodies in 8 M urea was effected by either dialysis or dilution. Dialysis was done at 4 °C using Spectra Pro 8000 MWCO dialysis tubing (Spectrum Laboratories, Inc.) against at least 200 volumes of buffer C [20 mM sodium phosphate buffer, pH 7.0, supplemented with 0.5 M NaCl, 1 mM EDTA, 0.5 M arginine, and 1 mM tris(2-carboxyethylphosphine) (TCEP)] with three buffer changes at 30-min intervals. When the objective was the isolation of the unspliced precursor, the EDTA in the refolding buffer was replaced by 2 mM $ZnCl_2$. Renaturation by dilution involved diluting a small sample (20−30 μL) of the purified precursor protein in 8 M urea into 1 mL of buffer C. The dilution buffer was appropriately modified when the effects of pH or other parameters on protein splicing and chromophore formation were studied. When the effect of pH was studied, 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris-Propane) was substituted for sodium phosphate in buffer C.

Detection of Protein Splicing. Protein splicing was monitored by SDS−PAGE and fluorescence measurements. SDS−PAGE was performed on precast 10−20% gradient Tris-glycine gels (Bio-Rad) and appropriate protein markers (New England Biolabs).[12] Gels were stained with Coomassie Blue and scanned with a Supervista S12 scanner (Umax Data System). For detection of the spliced GFP, its fluorescence was measured with a Perkin-Elmer spectrofluorometer using a path length of 10 mm and excitation at 395 nm by scanning the emission spectra between 450 and 600 nm. The bandwidth for both excitation and emission spectra was 5 nm.

(12) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.

RESULTS

Expression of GFP Fusion Proteins. After induction with IPTG at 37 °C, cultures of *E. coli* transformed with plasmid pHG, which encodes a modified form of GFP_{UV} with an N-terminal His-tag, showed bright green fluorescence. On the other hand, no fluorescence was exhibited by induced cultures transformed with plasmid pHGmU, in which a 220-residue *M. tuberculosis* RecA mini-intein had been introduced at position 129, nor with cultures transformed with plasmid pHGSap, which encodes the same modified form of GFP_{UV} but with nine amino acids inserted adjacent to position 129. Examination of extracts of bacteria transformed with the two types of plasmids showed that plasmid pHG led to the synthesis of GFP (29 kDa), which was found primarily in the soluble fraction, with ∼30% in inclusion bodies, whereas plasmid pHGmU led to the synthesis of unspliced 53-kDa GFP precursor (GFPmU), which was found exclusively as inclusion bodies (Figure 2). When the nonfluorescent inclusion body fractions from *E. coli* transformed with plasmids pHG or pHGmU were extracted with 8 M urea and renatured by dilution or dialysis under appropriate conditions, significant amounts of fluorescent protein were recovered. In contrast, no fluorescence was observed with renatured inclusion bodies obtained from plasmid pHGSap transformants (Figure 3). This indicates that even a small peptide insertion adjacent to residue 129 interferes with chromophore formation and suggests that the fluorescence observed upon renaturing the inclusion bodies encoded by plasmid pHGmU required prior excision of the intein by protein splicing. This was confirmed by the use of a mutant construct in which the C-terminal Asn residue of the intein was replaced by Ala, rendering the intein incapable of splicing. This mutant protein was unable to develop fluorescence upon renaturation (data not shown).

Renaturation and Splicing of Inclusion Bodies. The GFP fusion protein from the insoluble fraction derived from cells transformed with GFPmU was solubilized in 8 M urea and purified by binding to a Talon metal affinity column and elution with 100 mM imidazole in the presence of 8 M urea. Upon dialysis to

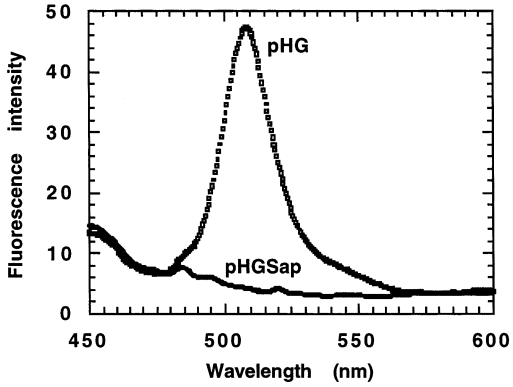

Figure 3. Fluorescence of renatured inclusion bodies derived from *E. coli* transformed with plasmids pHG and pHGSap. The insoluble fractions were redissolved in buffer B and renatured by dialysis against buffer C as described under Experimental Procedures. After 18 h at 25 °C, equivalent samples were analyzed for fluorescence in response to excitation at 395 nm.

remove the denaturant in the presence of 0.5 M arginine at pH 7.0, but in the absence of a thiol reducing agent, a 53-kDa polypeptide was observed as the major component upon SDS−PAGE, corresponding to the unspliced precursor. Dialysis against the same buffer supplemented with 1 mM TCEP yielded additional bands, whose electrophoretic mobility was consistent with that predicted for the products of protein splicing, GFP (29 kDa) and the excised intein (24 kDa) (Figure 4A). In addition, a small amount of a 41-kDa polypeptide was observed, which probably is the N-terminal GFP segment fused to the intein, produced by cleavage at the C-terminal splice junction. The protein splicing efficiency did not exceed 50%, even after 18 h of incubation at 25 °C, perhaps owing to misfolding of the precursor protein during the renaturation procedure. Samples refolded in the absence of reducing agent showed no fluorescence upon excitation of 395 nm, whereas significant fluorescence at 510 nm was observed when refolding was carried out in the presence of 1 mM TCEP (Figure 4B). Refolding could also be effected by diluting the denatured inclusion bodies in 8 M urea at least 30-fold into urea-free buffer, followed by incubating at 25 °C. Indeed, the fluorescence signal, which depended on the presence of TCEP, was about 3−4 times stronger than that observed after renaturation by dialysis (Figure 4C). Owing to the low protein concentration, it was not possible to assess the protein splicing efficiency after renaturation by dilution using SDS−PAGE, but it is clear that the increased fluorescence yield could not have been due solely to more efficient protein splicing but probably also involved a reduced level of misfolding.

Some parameters affecting refolding efficiency were examined using the dilution procedure. A critical element was the concentration of L-arginine in the refolding buffer, which enhanced the yield of fluorescence nearly 3-fold at a concentration of 0.5 M (Table 1). Refolding in 1 M urea reduced the yield of fluorescence by only 20%, but no fluorescence was obtained in 2 M urea (Table 1).

Optimization of Protein Splicing and Chromophore Formation. The development of fluorescence following refolding of the precursor protein from inclusion bodies involves two steps: the excision of the intein from the GFP precursor by protein splicing and the chemical rearrangement of GFP residues 65, 66, and 67 to form the chromophore.[13] To optimize these reactions, we examined separately the effect of reaction conditions on the development of fluorescence by refolded unspliced precursor isolated from cells transformed with pHGmU or by refolded inclusion bodies from cells transformed with pHG. At 25 °C and pH 7.0, the $t_{1/2}$ of fluorescence formation from refolded unspliced GFP precursor and from refolded GFP was 8 and 1.7 h, respectively (data not shown), indicating that protein splicing

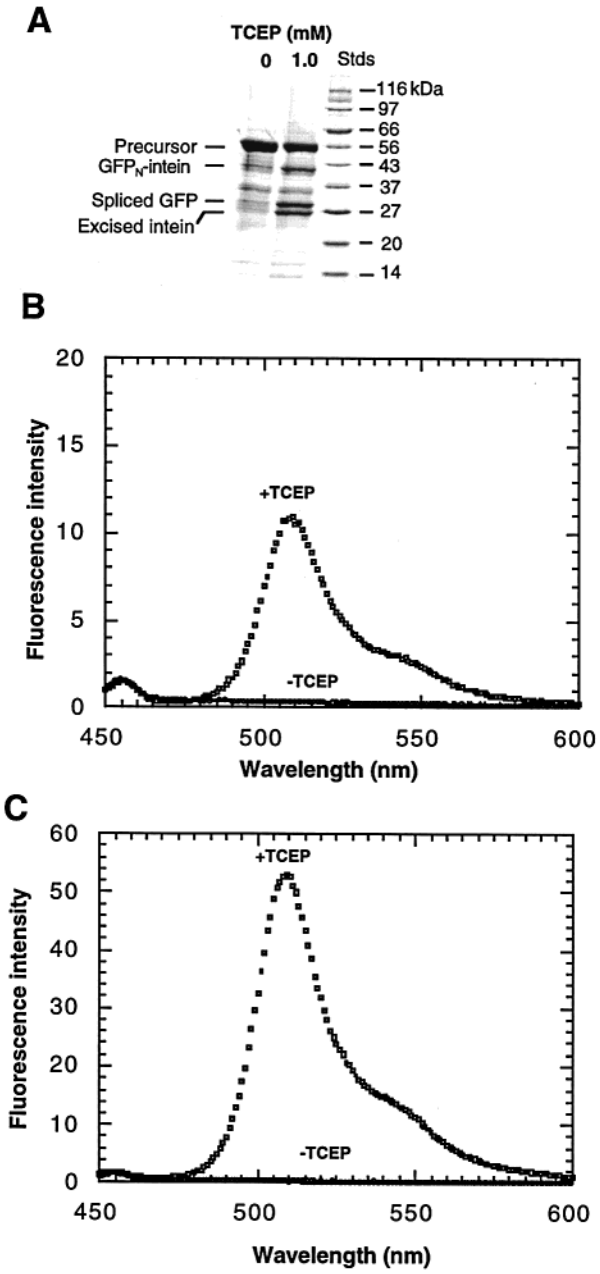

Figure 4. Renaturation of inclusion bodies from *E. coli* transformed with plasmid pHGmU by dialysis and dilution. The insoluble fraction was redissolved in buffer B. One sample was subjected to dialysis against buffer C and after 18 h at 25 °C was analyzed (A) for protein splicing by SDS−PAGE or (B) for fluorescence in response to excitation at 395 nm after dilution to a concentration of 8.5 μg/mL. (C) Another sample was diluted 50-fold into buffer C and analyzed after 18 h at 25 °C for fluorescence at a concentration of 12 μg/mL.

(13) Reid, B. G.; Flynn, G. C. *Biochemistry* **1997**, *36*, 6786−6791.

Table 1. Effect of Solutes on the Renaturation of the Unspliced GFP Precursor (GFPmU)[a]

L-arginine (M)	urea (M)	fluorescence intensity
none	none	11
0.1	none	22
0.2	none	26
0.3	none	28
0.4	none	30
0.5	none	32
0.5	1	25
0.5	2	1

[a] Metal ion affinity-purified precursor from inclusion bodies produced by *E. coli* transformed with plasmid pHGmU in buffer B was renatured by dilution into modified buffer C containing L-arginine or urea at the concentrations indicated. Fluorescence emission was measured at 508 nm after 18 h at 25 °C upon excitation at 395 nm and is expressed in arbitrary units.

rather than chromophore formation is the major rate-determining step in the overall process.

The effect of pH on the efficiency of protein splicing and GFP chromophore formation was examined by diluting the denatured protein samples into buffer C containing 50 mM Bis-Tris-Propane buffer of appropriate pH, incubating at 25 °C for 18 h, and measuring fluorescence. The unspliced GFP precursor showed a distinct optimum at pH 6.5, with little fluorescence development at pH values of 8.0 and higher (Figure 5A), whereas the extent of GFP chromophore formation increased progressively, reaching a plateau between pH 8.0 and 9.0 (Figure 5A). The time period allowed for fluorescence development in these experiments was 10 times longer than the half-time of GFP chromophore formation to ensure that protein splicing rather than chromophore formation was limiting in the studies with the unspliced precursor. However, a similar pH dependence was observed when rates of fluorescence development were measured over much shorter time periods (data not shown).

Protein splicing and GFP chromophore formation had a similar dependence on temperature (Figure 5B). Both processes occurred with maximum efficiency at 20 °C, but protein splicing showed a more pronounced temperature dependence than chromophore formation. To ensure that the temperature dependence observed with the unspliced precursor was not a reflection of the effect of temperature on chromophore formation, the reaction was allowed to proceed at the temperature indicated for 6 h, followed by adjusting the pH to 9.0, where protein splicing is very slow but chromophore formation is optimal, and incubation for an additional 12 h at 20 °C before measuring the fluorescence.

DTT was nearly as effective a thiol-reducing agent as TCEP in the renaturation and protein splicing process (~80% of TCEP at 1 mM). However, the latter provides no opportunities for side reactions such as mixed disulfide formation and TCEP was therefore the reducing agent of choice in our experiments.

Stabilization of the Refolded but Unspliced GFP Precursor. The high sensitivity and precision by which fluorescence can be detected and measurements can be automated make the protein splicing-dependent formation of GFP an attractive high-throughput screening system for protein splicing inhibitors. However, to avoid false hits due to substances that interfere with protein folding, it was desirable to separate the renaturation process from protein splicing. Since protein splicing occurs at significant rates even in 1 M urea, renaturation by dialysis will unavoidably be accompanied by protein splicing. This problem could be avoided by taking advantage of the reversible inhibition of protein splicing by zinc ion.[14,15] When the unspliced intein−GFP fusion protein was renatured in the presence of 2 mM $ZnCl_2$ under otherwise optimal conditions, no protein splicing was observed, but protein splicing leading to the formation of GFP and fluorescence could be induced by removing Zn^{2+} through complexation with a molar excess of EDTA (Figure 6). Similar results were obtained by renaturing the unspliced precursor by dilution into refolding buffer supplemented with 2 mM $ZnCl_2$. The functional stability of the inactive zinc complex was assessed by incubating it at 25 °C for various times before adding EDTA to reverse the Zn^{2+} inhibition. As shown in Figure 7, the ability of

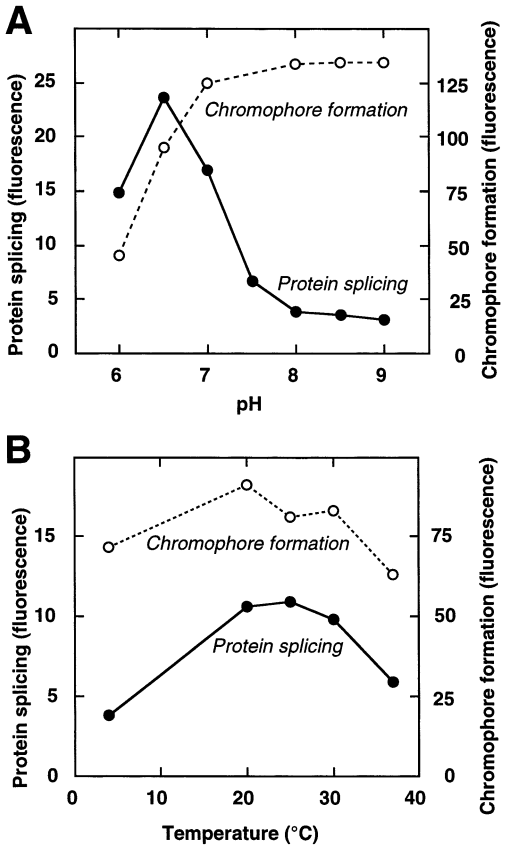

Figure 5. Effect of pH and temperature on protein splicing and chromophore formation. Inclusion bodies produced by *E. coli* transformed with plasmid pHGmU or plasmid pHG were purified by metal ion affinity chromatography in buffer B to yield denatured unspliced precursor or GFP, respectively. The purified proteins were renatured by dilution into (A) modified buffer C containing Bis-Tris-Propane adjusted to the pH values indicated,or into (B) buffer C at pH 6.5 (for the protein splicing reaction) or pH 7.0 (for chromophore formation) at the indicated temperatures. To ensure that chromophore formation was not a limiting factor in protein splicing at the low pH values in (A), the protein splicing samples in this experiment were adjusted to pH 9.0 after 6 h at the pH values indicated and incubated at that pH for 12 h at 20 °C prior to fluorescence measurement. In all other samples, fluorescence was measured after 18 h. Excitation was at 395 nm, and fluorescence emission was measured at 508 nm.

(14) Mills, K. V.; Paulus, H. *J. Biol. Chem.* **2001**, *241*, 10832−10838.
(15) Ghosh, I.; Sun, L.; Xu, M. Q. *J. Biol. Chem.* **2001**, *276*, 24051−24058.

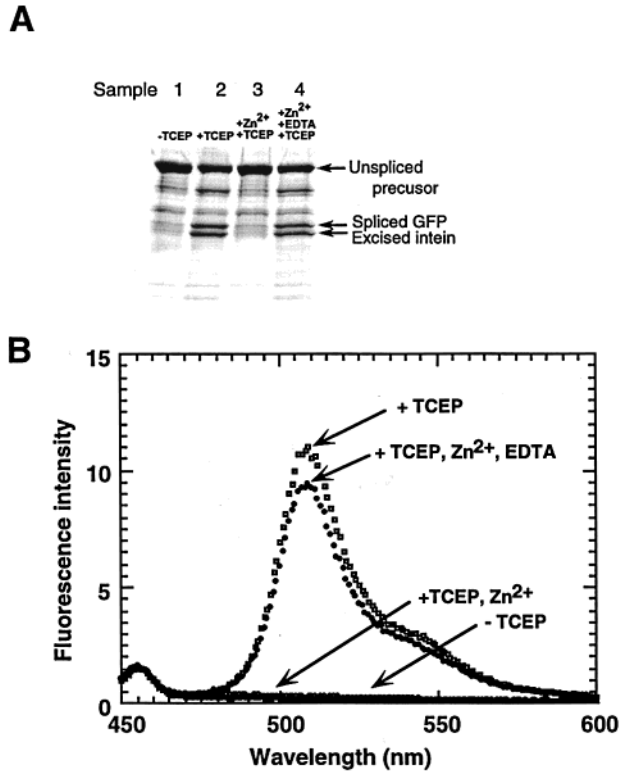

Figure 6. Reversible inhibition of protein splicing by zinc ion. Metal ion affinity-purified precursor from inclusion bodies produced by *E. coli* transformed with plasmid pHGmU in buffer B was renatured by dialysis against modified buffer C as follows. Sample 1 was dialyzed against buffer C without TCEP, sample 2 was dialyzed against buffer C, sample 3 was dialyzed against buffer C supplemented with 2 mM $ZnCl_2$ but without EDTA, and sample 4 was dialyzed with buffer C with 2 mM $ZnCl_2$ and no EDTA for the first dialysis cycle, followed by three cycles of dialysis against buffer C with 4 mM EDTA but no $ZnCl_2$. After dialysis, the samples were incubated for 18 h at 25 °C and analyzed (A) for protein splicing by SDS−PAGE or (B) for fluorescence upon excitation at 395 nm.

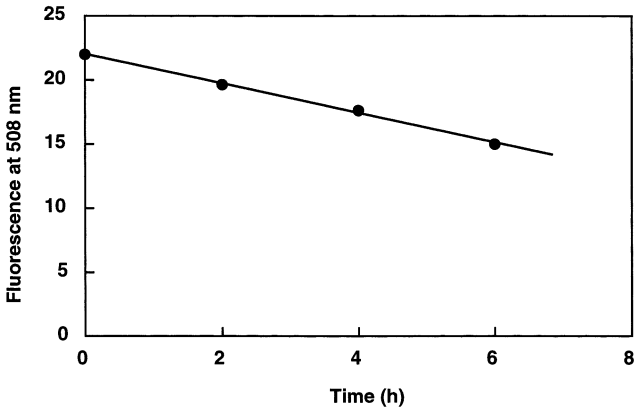

Figure 7. Functional stability of the unspliced GFP precursor renatured in the presence of $ZnCl_2$. Metal ion affinity-purified precursor from inclusion bodies produced by *E. coli* transformed with plasmid pHGmU in buffer B was renatured by dilution with buffer C modified to contain 2 mM $ZnCl_2$ instead of EDTA. At the times indicated, 3 mM EDTA was added to the diluted samples and fluorescence upon excitation at 395 nm for 18 h at 25 °C. A control experiment showed no fluorescence in a sample to which no EDTA was added.

the renatured GFP precursor to undergo splicing and chromophore formation decayed at a rate of only ∼5%/h. The loss of activity during the time needed for setting up a high-throughput screen for protein splicing inhibitors would thus be relatively insignificant.

DISCUSSION

The results described in this paper show that it is possible to develop an in vitro assay system for protein splicing by taking advantage of the fact that insertions adjacent to residue 129 of GFP severely interfere with protein folding and chromophore formation. Whereas GFP_{UV} is expressed in *E. coli* at 37 °C primarily as soluble protein[9] (see Figure 2A), even a nonapeptide inserted adjacent to residue 129 caused GFP to be expressed entirely as inclusion bodies (data not shown) and completely prevented GFP chromophore formation after attempted refolding (Figure 3). Insertion of the RecA intein at this site also caused the resulting fusion protein to be expressed entirely as inclusion bodies (Figure 2B). However, upon solubilization in 8 M urea followed by renaturation, the GFP−intein fusion protein was able to undergo efficient protein splicing to yield GFP capable of undergoing the rearrangements that lead to the formation of the fluorescent chromophore (Figure 4). The efficiency of renaturation was much enhanced by the presence of 0.5 M L-arginine (Table 1), probably by reducing the tendency to form aggregates.[16]

The appearance of fluorescence upon renaturation of the GFP−intein fusion protein involves both protein splicing and the chemical rearrangement of the spliced GFP to form the chromophore. The latter reaction could be studied independently by taking advantage of the fact that, under our expression conditions, ∼30% of GFP_{UV} formed nonfluorescent inclusion bodies (Figure 2A), which were capable of chromophore formation upon renaturation (Figure 3). This allowed us to compare the optimal conditions for protein splicing and chromophore formation. The $t_{1/2}$ of chromophore formation upon renaturation of our GFP_{UV} variant was ∼100 min, close to the value of 84 min described for a slightly different form of GFP.[13] On the other hand, the $t_{1/2}$ for chromophore formation upon renaturation of the GFP−intein fusion protein was ∼8 h, indicating that protein splicing is a limiting step in this process. Examination of the effects of pH and temperature on the two reactions showed that chromophore formation occurred over a much broader range than protein splicing (Figure 5). Although protein splicing proceeded only with very low efficiency under the optimal conditions for chromophore formation (pH 8.5 and 20 °C), under the optimal conditions for protein splicing (pH 6.5 and 20 °C), chromophore formation occurred at 70% of its maximal rate. It is therefore possible to optimize the assay conditions for protein splicing without making chromophore formation a limiting step. Nevertheless, if the formation of GFP fluorescence by the splicing of renatured GFP−intein fusion protein is to be used as an assay for the detection of protein splicing inhibitors, it will be necessary to rescreen inhibitory substances in a secondary assay based on renatured GFP to eliminate compounds that specifically inhibit GFP chromophore formation.

Since protein refolding is relatively sensitive to perturbation, a reliable assay for protein splicing inhibitors should employ fully

(16) Buchner, J.; Pastan, I.; Brinkmann, U. *Anal. Biochem.* **1992**, *205*, 263−270.

renatured GFP−intein fusion protein rather than denatured inclusion bodies. The premature splicing of the refolded fusion protein could be prevented by the reversible inhibition of protein splicing by zinc ion.[14,15] Addition of a molar excess of EDTA to the GFP−intein fusion protein renatured in the presence of $ZnCl_2$ restored 90% of its protein splicing activity (Figure 6); moreover, the renatured Zn^{2+} complex was relatively stable, losing ∼5% of its activity per hour (Figure 7). A robust in vitro screening system for protein splicing inhibitors can thus be constituted by dispensing renatured, Zn^{2+}-inhibited GFP−intein fusion protein into wells containing samples of library to be screened, followed by initiation of the protein splicing reaction by the addition of a molar excess of EDTA and measuring the rate of appearance of fluorescence.

In the experiments described in this paper, we have focused on an intein derived from *M. tuberculosis* in the hope that any inhibitors discovered by this assay could be potential antimycobacterial drugs. However, the procedure described should be applicable to any intein that can be inserted into GFP at position 129 [6] or other suitable insertion sites.[7] Indeed, the intact *Saccharomyces cerevisiae* VMA1 intein has been inserted at that site and shown to be able to undergo protein splicing when expressed in *E. coli* at low temperatures.[6] It should thus be possible to screen for inhibitors of various inteins and to compare their specificity in vitro. The study of the action of inhibitors on enzymes has been a major driving force in the elucidation of enzyme mechanisms. We hope that the availability of a simple and robust in vitro assay system for protein splicing will lead to the discovery of inhibitors that can help us address the many unsolved questions concerning the mechanism of protein splicing.[17]

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(17) Paulus, H. *Bioorg. Chem.* **2001**, *29*, 119−129.