Artificial Modules for Enhancing Rate Constants of a Group I Intron Ribozyme without a P4-P6 Core Element

In this paper we report newly selected artificial modules that enhance the $k_{cat}$ values comparable with or higher than those of the wild-type ribozyme with broad substrate specificity. The elements required for the catalysis of Group I intron ribozymes are concentrated in the P3-P7 domain of their core region, which consists of two conserved helical domains, P4-P6 and P3-P7. Previously, we reported the in vitro selection of artificial modules residing at the peripheral region of a mutant Group I ribozyme lacking P4-P6. We found that derivatives of the ribozyme containing the modules performed the reversal of the first step of the self-splicing reaction efficiently by using their affinity to the substrate RNA, although their $k_{cat}$ values and substrate specificity were uninfluenced and limited, respectively. The results show that it is possible to add a variety of new domains at the peripheral region that play a role comparable with that of the conserved P4-P6 domain.

Group I intron ribozymes catalyze two consecutive trans-esterification reactions to excise themselves from the precursor RNAs and ligate the flanking exons together (1). Their core region consists of two completely conserved helical domains, P3-P7 and P4-P6, which are connected via base-triples, which can be seen in Fig. 1A (2–5). Biochemical studies indicate that P7 and J8/J7 regions in the P3-P7 domain are essential for the catalysis of those reactions mentioned above (6–12). A study employing the T4 td group I intron demonstrated that a mutant ribozyme lacking both the P4-P6 domain and the base triples (M1 mutant, Fig. 1B) can still perform the trans-esterification reactions with moderate activity that was 10^3 times lower than that of the wild-type ribozyme at higher concentrations of magnesium (13).

Previously we performed in vitro selection by employing the M1 mutant ribozyme to obtain artificial modules that enhance the activity of the ribozyme without the conserved P4-P6 that stabilizes the active form of the ribozyme (14). Several modules were selected: L7.1, L8, or L9. The variants with a module efficiently performed the reversal reaction of the first step of self-splicing. However, the modules contained a short sequence complementary to the substrate RNA used in the selection, indicating that the role of the module was likely to assist the substrate recognition by forming extra base pairings. In fact, we confirmed that one variant was unable to perform the reaction with another substrate without the complementary sequence. Thus, it remained unclear whether it is possible to develop a true alternative module substituting the original P4-P6 domain, which does not depend on the affinity to the substrate.

We report here newly selected modules that enhance the activity of the mutant ribozyme lacking P4-P6 domain with broad substrate specificity. After 12 rounds of in vitro selection followed by an additional modification and selection, the selected variants performed the reaction with second order rate constants ($k_{cat}/K_m$ values) one order of magnitude larger than the wild-type ribozyme. Furthermore, their reaction rates ($k_{cat}$ values) were comparable with that of the wild-type ribozyme.

EXPERIMENTAL PROCEDURES

Library Construction—We used the N40 library that was constructed as previously described with minor modifications (14). An oligodeoxynucleotide, td-R (5′-ATTATGGTCCAGATAAGTCGTAATC-3′; all oligodeoxynucleotides were purchased from Hokkaido System Science, Japan), instead of td-Rv, was used as the reverse primer throughout the experiments. In this primer the 5′-G portion was eliminated to inhibit side reactions (circularization). The N70 library was constructed with essentially the same procedure as above using an oligodeoxynucleotide, Ban-N70 (5′-CGTGAGACGCTGAGCAGCCACCCN_7GAGCCCGTGCTCAGGCTGCCTGAC-3′; where N stands for any nucleotide, and restriction sites are shown in italics), as the template for producing the RNA pool. About 1.0 × 10^{14} molecules of each sub-library DNA were transcribed by using T7 RNA polymerase to yield the RNA pools (~20 copies each).

In Vitro Selection—We performed in vitro selection as previously described with minor modifications (14–16). The pool RNA was dissolved in 1.25× reaction buffer (1× reaction buffer contains 50 mM Hepes-KOH, pH 7.5, 2.5 mM spermidine) followed by denaturation by incubating at 65 °C for 10 min. The RNAs were folded by adding 1/5 volume of 100 mM MgCl₂ (final concentration was 10 mM) followed by incubation at 37 °C for 10 min. The reaction was initiated by adding 1/25 volume of the substrate RNA (S-2, 5′-biotin-GUAACACAUUAGCAGAAUGCCAGAG AGAAGUACAGGU-3′, or S-3, 5′-biotin-AUAUCAUCACCAUGCU GAAGAAUCAGGU-3′; Dharmacom Research). The reaction mix was incubated at 37 °C. We arrested the reaction by adding 1/10 volume of 200 mM EDTA. Table I shows that the concentration of the substrate RNA and the reaction time were progressively decreased. Biotin affinity purification, reverse transcription, and cDNA elution were performed as described (14), and selective PCR was carried out by using a selective primer (5′-GTAGCACATCATGAGGCTGCCCCACCAATAACGAG-3′ for the substrate S2-2 and S-5′-ATCATCGCATCAGCCAGAACTTACCTTACG-3′ for the substrate S-3) and td-R. A nested PCR was performed as described.

Assay of Catalytic Efficiency—We assayed the catalytic efficiency of each clone as previously described (14). The folding procedure and the reaction conditions are same as the reaction for the in vitro selection. The values reported in Tables II and III are an average of at least three independent experiments. The sequence of a substrate RNA, S-4, is 5′-GCAUACUGAGAUGUGAGAAUACAGGU-3′ (Dharmacom Research).

Construction and Selection of the Doped Libraries—We constructed the doped clone 10 library as follows. We amplified the 5′ and 3′ fragments of the library separately from clone 10 DNA by PCR using primer (5′-H11011-5′-H11032). We performed an in vitro selection of clones, and the clones that performed with 50% efficiency (in the presence of 100 mM MgCl₂) were selected for doped libraries. We constructed the doped libraries using a selective primer (5′-H11032-3′). The doped clone 10 libraries were constructed with essentially the same procedure as above using an oligodeoxynucleotide, Ban-N70 (5′-CGTGAGACGCTGAGCAGCCACCCN_7GAGCCCGTGCTCAGGCTGCCTGAC-3′; where N stands for any nucleotide, and restriction sites are shown in italics), as the template for producing the RNA pool. About 1.0 × 10^{14} molecules of each sub-library DNA were transcribed by using T7 RNA polymerase to yield the RNA pools (~20 copies each).
FIG. 1. **Secondary structure of the ribozymes.** A, wild-type T4 td ribozyme. B, M1 mutant ribozyme. The insertion sites for random sequences (N40 or N70) are shown by gray boxes. An arrowhead indicates the 5' splice site. Gray lines indicate known tertiary interactions.

FIG. 2. **Scheme for the in vitro selection.** Circled B and the black and white region in the substrate RNA indicate the 5’ biotin moiety and the sequence switched after each cycle (black) and the constant sequence (white), respectively. SA, streptavidin beads.
KOD DNA polymerase (Toyobo, Japan). A set of two primers, td-Fw and DPD10-R (5'-TAACGAACTGACGACGATGTT-3') or another set of primers, td-R and DPD10-P (5'-AGGGAGCCAGTTGAGTGAGCCCTGTCCACGCTCGCTCAC-3') underlined nucleotides were 21% doped (79% of original nucleotide and 3% of the other nucleotides); the 5' end was phosphorylated with T4 polynucleotide kinase (Takara Shuzo) were used for the amplification of 5' or 3' fragments, respectively. The PCR products were assembled by blunt-end ligation (T4 DNA ligase, Takara Shuzo). We employed the ligated DNAs (~1.0 × 10^13 molecules) purified by native PAGE for transcription by using T7 RNA polymerase (~50 copies each).

A doped clone 22 library was constructed as follows. We amplified the fragments for insertion with an oligodeoxynucleotide, DPD22 (5'-CGTGACAGGGTGAGCCCTGTCCACGCTCGCTCAC-3') underlined nucleotides were 21%-doped (79% of original nucleotide and 3% of the other nucleotides), by employing ExTag DNA polymerase (Takara Shuzo). Restriction sites are in italics. The PCR product was digested with BamI and BanII. This fragment was ligated with a 5' and 3' fragment of the BanI/BanII digest of clone 22 DNA by using T4 DNA ligase. The ligated DNAs (~1.2 × 10^13 molecules) purified by native PAGE were used for transcription by using T7 RNA polymerase (~40 copies each). Two rounds of in vitro selection from the doped libraries were carried out as described above under the following conditions; reaction time was 15 s, and the substrates for round 1 and round 2 were 2 μM S-2 and 2 μM S-3, respectively.

Hydroxyl Radical Footprinting — We conducted hydroxyl radical footprinting as described in Celerand (17). The RNAs (1–2 μg) were dissolved in a 2× folding buffer (1× folding buffer contains 50 mM MOPS, pH 7.3, 100 mM NH₄Cl, 5 mM dithiothreitol) followed by denaturation by incubation at 65°C for 10 min. The RNAs were folded in the presence of 10 or 50 mM MgCl₂ followed by incubation at 37°C for 10 min. The cleavage reaction was initiated by adding free radical generator. The resulting mixture contains 5 mM EDTA, 2.5 mM Fe(NH₄)₂(SO₄)₂, 5 mM dithiothreitol, 5 mM ascorbic acid, and 0.05% H₂O₂. The cleavage reaction was carried out at 37°C for 1–3 min and arrested by adding an equal volume of 1 M thiourea followed by ethanol precipitation. The cleavage sites were mapped by reverse transcription with a 5' end-labeled primer, 8-BanI-Rv (14), and ReverTra Ace (Toyobo) followed by 7% denaturing PAGE.

RESULTS

In Vitro Selection — To select artificial modules that enhance the activity of the M1 mutant ribozyme lacking the P4-P6 domain together with the base triples, we constructed its derivatives, which possess random sequences for the selection. The random sequence consisting of 40 or 70 nucleotides was inserted into peripheral loop L7.1, L8, or L9 (boxes in Fig. 1B) of the M1 mutant. Each pool of RNA contained ~1.0 × 10^14 different molecules. Three pools containing either a 40- or 70-nucleotide random sequence were mixed to form the N40 and N70 libraries, respectively. Active ribozymes were selected from the libraries by attempting the reversal reaction of the first step of self-splicing (Fig. 2). The selection conditions were restricted gradually by reducing the reaction time. Two substrates, S-2 and S-3, with a different 5' tag sequence were employed alternately after the completion of each cycle to inhibit the enrichment of the clones specialized for accepting a particular substrate (Table I). The variants in Round 12 pools were cloned because the increased activity was observed for the two pools containing N40 or N70 libraries (Fig. 3) that can utilize both S-2 and S-3 as the substrate. Sequences of the clones are shown in Supplemental Figs. 1 and 2.

Ten selected clones were randomly chosen from the N70 library, and these included the selection at L8 (Supplemental Fig. 1). Five shared the sequence of clone 01. Clone 01 and four

| TABLE I | Conditions for in vitro selection |
|----------|----------------------------------|
| Round    | Substrate | Time       |
| 1        | 30 μM S-2 | 1 hr       |
| 2        | 30 μM S-3 | 5 min      |
| 3        | 2 μM S-2  | 5 min      |
| 4        | 2 μM S-3  | 5 min      |
| 5        | 2 μM S-2  | 2 min      |
| 6        | 2 μM S-3  | 2 min      |
| 7        | 2 μM S-2  | 1 min      |
| 8        | 2 μM S-3  | 1 min      |
| 9        | 2 μM S-2  | 30 s       |
| 10       | 2 μM S-3  | 30 s       |
| 11       | 2 μM S-2  | 15 s       |
| 12       | 2 μM S-3  | 15 s       |

The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; FE, final extent.

![Fig. 3. Increase in the activity due to the in vitro selection. A, the reaction of the wild-type (lanes 1 and 2), M1 mutant (lanes 3 and 4), Round 12 pools of the N70 library (lanes 5 and 6), or Round 12 pools of the N40 library (lanes 7 and 8) were carried out with 10 μM substrate RNA (lanes 1, 2, 3, and 7; S-2, lanes 2, 4, 6, and 8; S-3) for 1 min. B, each pool was reacted with 10 μM substrate RNA (S-2 or S-3) for 2 min.](image-url)
other clones (clones 02, 05, 07, and 12) were found to belong to the same family, whereas the sequence of clone 10 was different from the rest. Ten clones from the N40 library were also analyzed and categorized into four types (Supplemental Fig. 2). Five (clone 22, 23, 27, 29, and 30) with the insertion at L9 belonged to the same type. Clones 21 and 28, with the insertion at L8, possess a similar sequence, whereas clones 25 and 26, also with the insertion at L8, share their own similar sequence. The sequence of clone 24 with the insertion at L8 was different from the rest. There were no clones obtained with the insertion at L7.1 from any of the Round 12 pools. However, digestion with the restriction enzyme showed that 10% of the clones from Round 8 pools did have the insertion at L7.1 (data not shown), suggesting that the ribozyme is able to accept the insertions at different sites as previously noted (14). Without exception, the clones from both the N40 and N70 libraries had mutations at P2, suggesting that they are responsible for conducting the reaction efficiently (see “Evaluation of Mutations in P2” below). We will discuss this later.

To determine whether each clone has broad substrate specificity, we attempted the reaction with a new substrate (S-4) with a different sequence to that of S-2 or S-3 except the P1 region (Fig. 4). The S-4 substrate served as the substrate for clone 10 from the N70 pool to the extent comparable with S-2 or S-3. The clones from the N40 pool reacted efficiently with S-4 without exception, and the results indicate that the ribozymes with broad substrate specificity were selected as anticipated. However, all clones from the N70 pool except clone 10 failed to react with S-4. This may be attributed to a partial sequence of their insertion, GUGUGUG (178–184, Supplemental Fig. 1), which is partially complementary to S-2 (CACN10CAC) and S-3 (CACCAC).

Directed Evolution of Clone 10 and Clone 22—We subjected two highly active clones, clones 10 and 22, from the N70 and
The Mfold program predicted that the clones from the doped 10 library contained two conserved helices, two base pairs extension of P8 (G1:C77 and G2:C76, Fig. 5, A and C) and a stem consisting of seven base pairs (base pairs between A4-C10 and G32-U38, Fig. 5, A and C). It was also observed that the sequence of U56-U67 was highly conserved (Fig. 5, A and C, see also Supplemental Fig. 3). Fig. 5C also shows that clone 107 was predicted to possess an extra stem consisting of seven base pairs (base pairs between U12-A18 and U24-A30) that was not found in clone 10. The corresponding stems were also observed for the majority of the clones (green in Supplemental Fig. 3). The predicted secondary structure of clone 107 suggests that the base pairings may stabilize the conserved structure and/or inhibit the formation of alternative forms (Fig. 5C).

We identified one highly conserved helix with small mismatch(es) (boxed regions in Fig. 5, B and D) and one completely conserved sequence consisting of five nucleotides (G42-U46 in Fig. 5, B and D) in the clones from the doped 22 library. The conserved sequence motif is complementary to the sequence of L1 (Fig. 6A). To see whether they are associated physically, three variants of clone 204 were prepared (Fig. 6B). We observed drastic reductions in activity (102–103-fold) when the base substitutions for disrupting the base pairings were introduced into the motif (204-m1 and 204-m2), whereas ~40% of the original activity was observed when the substitutions designed for maintaining the base pairings were introduced into both the motif and L1 (204-m3). The data strongly support the formation of predicted base pairings.

We determined kinetic parameters of clones 107 and 204 by employing substrates S-2, S-3, and S-4 (Table II). The secondary rate constants ($k_{on}/K_m$ values) of clone 107 are $10^4$–$10^5$-fold and 6.5–22-fold higher than those of the M1 mutant and the wild-type ribozyme, respectively, whereas the $k_{on}/K_m$ value of clone 204 is more than $10^3$-fold and 34–44-fold higher than that of the M1 mutant and the wild-type ribozyme, respectively. The selected modules in clones 107 and 204 contribute not only to the high affinity to the substrate RNA but also to the primary rate constant ($k_{cat}$ value), which is comparable with (or even higher than) that of the wild-type ribozyme.

The Structural Roles of the Inserted Modules—That clone 107 and 204 can efficiently carry out the reaction in the presence of relatively low concentrations of magnesium ions (10 mM) indicates that the inserted modules play a role in stabilizing the active structure. To determine whether the core regions of the clones are highly structured, as is the case for the wild-type ribozyme, we performed hydroxyl radical footprinting experiments (28). The core region, P3, P7, and flanking regions between P3 and P7 of clones 107 and 204 were highly protected (Fig. 7, B and C; see also Supplemental Fig. 5, B and C) at 10 mM magnesium ions, where the corresponding region of the wild-type ribozyme was also highly protected (Fig. 7A and Supplemental Fig. 5A). However, the core region of M1 mutant was not protected or moderately protected or in the presence of 10 or 50 mM magnesium ions, respectively (Fig. 7D and Supplemental Fig. 5D). The results indicate that the inserted modules stabilize the catalytic core directly by interacting with the core region and/or indirectly by stabilizing the overall structure.

Evaluation of Mutations in P2—We observed enrichment of the mutations at P2 after the selection. It is unlikely that these mutations improve efficiency of PCR, reverse transcription, or transcription because they were not observed in the previous selection that employed the same materials (14). To analyze

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Table II  
Kinetic parameters

| Substrate          | S-2          | S-3          | S-4          |
|--------------------|--------------|--------------|--------------|
| Wild type          | $k_{cat}$    | $K_m$        | $k_{cat}$    | $K_m$        | $k_{cat}$    | $K_m$        |
|                    | 0.22 ± 0.03 min$^{-1}$ | 2.07 ± 0.55 μM | 0.25 ± 0.04 min$^{-1}$ | 2.31 ± 0.77 μM | 0.34 ± 0.03 min$^{-1}$ | 2.25 ± 0.63 μM |
| M1 mutant$^a$      | $k_{on}/K_m$ | $K_m$        | $k_{on}/K_m$ | $K_m$        | $k_{on}/K_m$ | $K_m$        |
| Clone 107          | 0.11 ± 0.01 min$^{-1}$ μM$^{-1}$ | 1.8 ± 1.6 × 10$^{-6}$ min$^{-1}$ μM$^{-1}$ | 0.11 ± 0.02 min$^{-1}$ μM$^{-1}$ | 1.8 ± 3.1 × 10$^{-6}$ min$^{-1}$ μM$^{-1}$ | 0.15 ± 0.03 min$^{-1}$ μM$^{-1}$ | 2.8 ± 4.2 × 10$^{-6}$ min$^{-1}$ μM$^{-1}$ |
| Clone 204          | 0.22 ± 0.11 μM | 2.33 ± 2.99 min$^{-1}$ μM$^{-1}$ | 0.23 ± 0.05 μM | 1.23 ± 0.05 min$^{-1}$ μM$^{-1}$ | 0.24 ± 0.05 μM | 1.23 ± 0.05 μM$^{-1}$ |

$^a$ Because of its high values of $K_m$ (more than 50 μM), $k_{cat}$ and $K_m$ values of the M1 mutant were undetermined.
the effect of the P2 mutations, they were eliminated from clone 107 and 204. The elimination reduced the final extent (FE) of the reaction or the fraction of active molecules, although it did not affect the reaction rates ($k_{\text{obs}}$ values) (Table III). Next, we introduced the corresponding P2 mutations into the wild-type ribozyme. The mutation from clone 107 or 204 decreased or increased FE values, respectively, although the resulting derivatives exhibited increased $k_{\text{obs}}$ values (Table III).

According to the recently refined model structure, the T4 td ribozyme has a shorter P1 and longer P2 compared with the that previously proposed (Fig. 8A) (20). The substrate RNAs used in the selection were designed based on the previous

| TABLE III Effect of the P2 mutations |
|--------------------------------------|
| The reaction of each variant was carried out with 0.5 μM S-2. |
|                                        |
|                                        |
| Original P2 | P2 from clone 107a | P2 from clone 204b |
| Wild-type   | $k_{\text{obs}}$: 0.042 min$^{-1}$ | 0.079 min$^{-1}$ | 0.129 min$^{-1}$ |
|            | FE: 0.30 | 0.16 | 0.60 |
| Clone 107   | $k_{\text{obs}}$: 0.308 min$^{-1}$ | 0.298 min$^{-1}$ | NA |
|            | FE: 0.39 | 0.86 | NA |
| Clone 204   | $k_{\text{obs}}$: 0.663 min$^{-1}$ | NA | 0.512 min$^{-1}$ |
|            | FE: 0.42 | NA | 0.73 |

a U34C/U35C/A36G/U40C.

b U35C/A36G/U40C.

NA, not analyzed.

Fig. 7. Hydroxyl radical footprinting of the wild-type ribozyme (A), clone 107 (B), clone 204 (C), and M1 mutant (D). Lanes 1, 2, 3, and 4 are sequence ladders of A, C, G, and U, respectively. Lanes 5 and 6 in A–C and lanes 5–7 in D are the reverse transcription products using template RNAs without the hydroxyl radical cleavage as the control. Quantified data are shown in Supplemental Fig. 5.

Fig. 8. Model of the P1-P2 structure. A, the refined model (wild-type) structure of P1-P2. B, a predicted structure of the modified P1-P2 with the P2 mutations based on the originally predicted model. The nucleotides involved in the rearrangement and the mutated sites are highlighted by blue boxes and gray circles, respectively. The consensus mutations are shown with red letters.
model (Fig. 8B). When a substrate RNA with complete Watson-Crick base pairs in the extended P1 was used for the cleavage reaction, the reaction of the wild-type ribozyme was biphasic (an initial fast and following slow phase) (20).

The fast and slow phase have been considered to reflect the population containing the correct P1-P2 structure and the extended P1 structure, respectively; the population containing the extended P1 structure is supposed to rearrange and correct the P1-P2 structure before catalysis. Thus, the fact that the FE value of the wild-type ribozyme was low (0.30) indicates that the rearrangement did not proceed under these conditions. However, the mutations at P2 including U40C resulted in efficient catalysis that cannot be explained by the hypothesis described above; the reduction of the FE value is anticipated due to unwinding of the P2 extension. One possible explanation for the contradiction is that other mutations cause unpredictable effect on the structure (note that introduction of the P2 mutations into the M1 mutant affected neither $k_{\text{obs,1}}$ value nor FE value, presumably due to its fragile structure (data not shown)).

**DISCUSSION**

The essential elements required for catalysis in Group I introns are concentrated in P3-P7 domain of the conserved core region, which also contains the P4-P6 domain. Previously, we reported the *in vitro* selection of modules residing at L7.1, L8, or L9 of a mutant T4 ts ribozyme (M1 mutant) lacking the P4-P6 domain that is known to assist the functioning of P3-P7. The variants containing the modules performed the reversal reaction of the first step of self-splicing efficiently. No enhancement, however, was observed for the primary rate constant ($k_{\text{cat,1}}$ value). In this study our new method of *in vitro* selection by employing the pools at the peripheral region of the ribozyme effectively contributes to the catalysis by using its L4 loop (21, 22).

The variants were highly active at a concentration of 10 mM magnesium ions due to the structural stabilization of the inserted modules, as shown by hydroxyl radical footprinting. The higher $k_{\text{cat,1}}$ values of the variants suggest that the modules enhance the catalysis, such as the P4-P6 domain, which moderately contributes to the catalysis by using its L4 loop (21, 22).

The variants were selected modules that may not assist or allow the orientation of the 3\' end of the ribozyme to be set properly in the substrate helix, called P10, that is responsible for conducting the second step (2). For example, the base pairings between the inserted module in clone 204 and L1 should prevent P10 formation (Fig. 6). Furthermore, some variants, including clone 107, could not conduct the first step reaction presumably due to the weak affinity to the guanosine cofactor (data not shown). Consequently, the modules obtained by the selection method employing either first- or second-step reactions may not allow a smooth transition between the two steps; thus, a selection procedure based on the splicing activity may be required to select a module whose function is completely equivalent to that of the P4-P6 element.

These results may be clues for understanding the origin of Group I intron ribozymes. The ribozyme can perform the reaction in a similar way to the reversal reaction of the first step by using a dinucleotide, GpN, as a substrate (27). The repetition of the reaction that adds an N at the 3\' end of the substrate on the guide sequence is a template-directed RNA polymerization. Thus, it has been proposed that the ancestral form of Group I intron ribozymes might be an RNA polymerase (27). The role of the P4-P6 element that switches the first and second step in the splicing is not required for conducting this reaction. Our results show that a variety of modules that enhance the activity of the ribozyme without the P4-P6 element can be obtained, suggesting that a primitive RNA containing only P3-P7 element and its fairly simple helper module may be sufficient to compose a primordial RNA polymerase. The P4-P6 element might have evolved from such a helper module for composing the highly sophisticated self-splicing intron RNA.

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