

## Restriction-Enzyme-Nondependent Recombination and Rearrangement of DNA (RRR)

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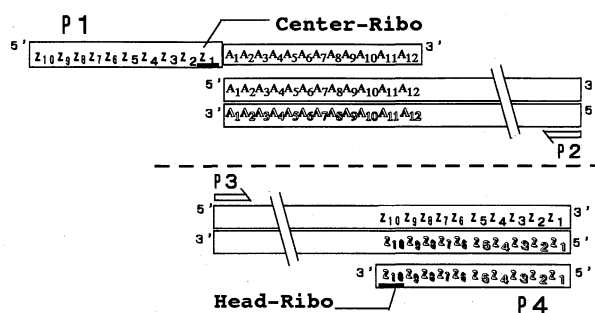
A new technology for recombining DNAs was developed. This method utilizes ribonucleotide-containing DNAs (RCDs) as primers to obtain PCR products and subsequently generate sticky ends by cleaving at the ribonucleotide site. This method also enables us to generate combinatorial diversities of primers, which are useful for *exon-shuffling*.

Recombination of DNA plays important roles in both biological phenomena and DNA technologies.<sup>1,2</sup> Current methods to obtain recombinant DNAs depend on restriction enzymes to generate sticky ends, which limits candidates for ligation of DNA. The size of protruding sticky ends is also confined to those which restriction enzymes offer: at most 4 nucleotides in length,<sup>3</sup> which are often insufficient in specificity or/and stability for ligation.

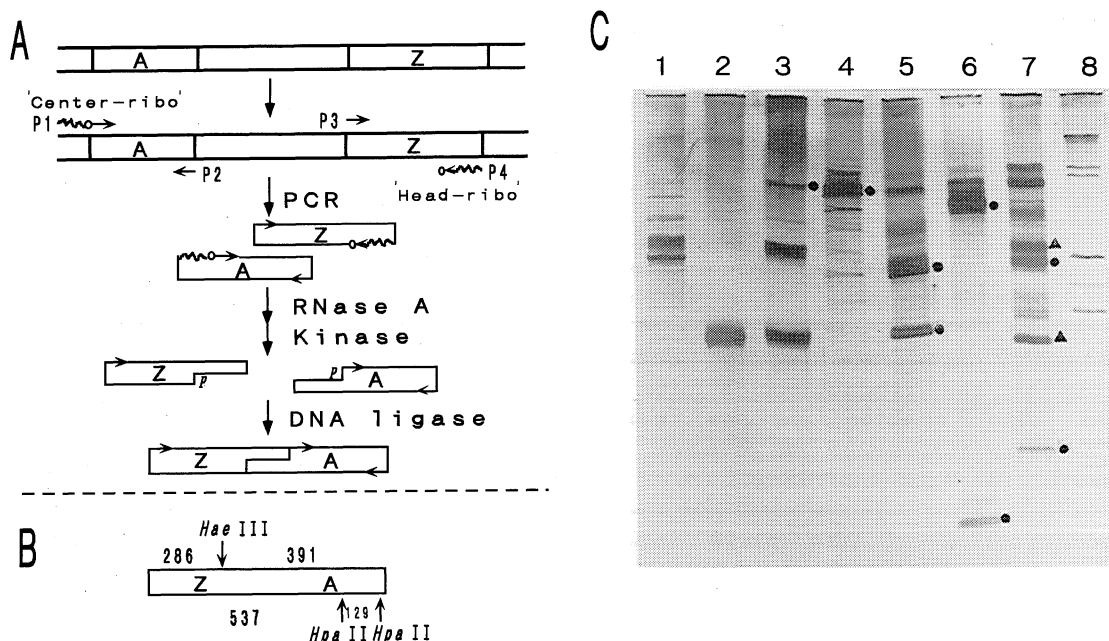
Thereon, we devised a new method to recombine DNAs at arbitrary sites, with an arbitrary size of sticky ends, and independently of restriction enzymes: *i.e.*, restriction-enzyme-nondependent recombination and rearrangement of DNA (RRR). It was achieved by specially designed, two kinds of RCD primers such as shown in Figure 1. They were designated as 'head-ribo' and 'center-ribo' primers. A *head-ribo* is complementary to the 5'-side from the center ribonucleotide of the corresponding *center-ribo* so that they can generate mutually complementary sticky ends (Figures 1 & 2A). A *head-ribo*, which has a ribonucleotide tip at the 3'-end to a normal PCR primer, can be made by one step reaction with terminal nucleotidyl transferase (TdT) whereas *center-ribo* can be synthesized by a single additional reaction using T4 RNA ligase, which combines the 3'-end (ribonucleotide) of an acceptor *head-ribo* with the 5'-phosphate of a donor oligonucleotide.<sup>4</sup> These enzymatic reactions can be evidently replaced by organic syntheses using DNA synthesizer. However, the enzymatic approach developed here is incomparably beneficial in obtaining combinatorial diversities of oligonucleotides as explained later. The

DNA products obtained by PCR with these RCD primers can be nicked at the ribonucleotide site either by alkaline treatment (0.1M NaOH, 95°C, 5 min) or by RNase A (in case of pyrimidine), generating sticky ends (Figure 2A). Phosphorylation of 5'-end by polynucleotide kinase and ligation of these DNAs with DNA ligase gave an observable amount of the aimed DNA (Figure 2C). Then, it could be amplified by a second PCR to a major band of the genuine product (Figure 2C). Through this experiment, we could confirm the followings: (i) The *pfu* (Stratagene) as well as *Taq* DNA polymerase was proved to make ribonucleotide-containing DNAs as templates; (ii) *pfu* DNA polymerase, which has 3' to 5' exonuclease activity, can evade the 3'-overhang effect which is unavoidable with *Taq* DNA polymerase; and (iii) RNase A could nick at cytidine (strongly) and uridine (weakly). (Therefore, designing RCD primers needs to conform to this pyrimidine requirement at this moment. Alkaline treatment also inclines to cause a trouble of insufficient renaturation.)

Rearrangement of three DNA fragments was also successful (data not shown). The most advantageous of this technology is



**Figure 1.** The ribonucleotide-containing DNA (RCD) primers used here. Two kinds of DNA fragments to be ligated are obtained by PCR using specifically designed primers (P<sub>1</sub>/P<sub>2</sub> and P<sub>3</sub>/P<sub>4</sub>) as shown here. The 5' half of a *center-ribo* primer is made complementary to the sequence of the *head-ribo* primer. Each nucleotide is expressed by an alphabetical letter with a numerical suffix. A nucleotide in outlined letters is complementary to the nucleotide in filled letters of the same kind. The ribonucleotides, at the centre of *center-ribo* and the head of *head-ribo* are underlined.



**Figure 2.** (A) A basic scheme of recombination of two DNA fragments. The primers,  $P_1$  to  $P_4$ , are constructed as shown in Figure 1. A small circle and a letter  $p$  stand for a ribonucleotide and a phosphate, respectively. (B) The restriction map of recombinant DNA. The sizes of restriction fragments are shown aside. (C) Gel electrophoretic analysis of experimental results. Urea-containing 4% polyacrylamide gel was used. Lanes 1 and 2; the dotted are PCR-prepared "Z" (actually, fd gene VII-IX-VIII (408 bp)) and "A" (actually, fd gene V (269 bp) + cohesive sequence (10 bp)), respectively. The used primers were  $P_1$ , 5'GAAAGCAAGCTCAAAAATGATTA3';  $P_2$ , 5'TTACTTAGCCGG3';  $P_3$ , 5'AGCAGTTCGCGG3'; and  $P_4$ , 5'GCTTGCTTTC3', where the underlined are ribonucleotides and the others deoxyribonucleotides. The A and Z DNAs (10 nmole, respectively) were then treated with RNase A (0.3 mg/ml) in 1xSSC at 50°C for 30 min, followed by 1 min-incubation at 70°C to chase the split oligonucleotides. Lane 3; a product after ligating A with Z at 45°C for 1 h using Taq ligase (NEB, Beverly), which were phosphorylated at the newly generated 5'-termini by T4 polynucleotide kinase<sup>3</sup>. Lane 4; Re-PCR of the lane 3 products using the primers,  $P_2$  and  $P_3$  (these two primers do not work unless a template is the aimed ligation product (see Figure 2A)). In both lanes 3 and 4, the dot indicates the aimed product (677 bp). Lanes 5-7; the Re-PCR products treated with restriction enzymes, *Hae III*, *Hpa II* and *Alu I*, respectively, indicating the expected bands by a dot (see Figure 2A). The partially degraded are marked by a triangle. Lane 8 is fd ss DNA cleaved by *Hae III* as a reference.

readiness to generate diversities: only  $(n+m)$  species of oligonucleotide are necessary to prepare  $(n \times m)$  species of recombinant DNAs because  $(n \times m)$  species of *center-ribo* required can be generated combinatorially from  $(n+m)$  species of oligomers as shown in Figures 1 & 2. This is unattainable for the current methods such as the dUTP/uracyl-DNA-glycosylase method<sup>5</sup> which do not contain combinatorial steps to make oligonucleotides.

Naturally, this method, RRR, will lead to developing *exon shuffling* technology, which must be the next step of *evolutionary molecular engineering*.<sup>6</sup>

#### References and Notes

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