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An efficient strategy for the synthesis of circular RNA molecules

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We have developed a fast and simple one test-tube procedure for synthesizing large amounts of pure, homogeneous circular RNA molecules of any sequence. This new strategy is based on the exploitation of RNA internal secondary structure to position the 5′ and 3′ termini such that they will be single stranded, but held in close proximity to each other for subsequent ligation by T4 RNA ligase. This method consists of five simple steps (Fig. 1A): (i) use of the circularly permuted RNA strategy (1) to introduce any desired mutations and to position the ligation site; (ii) run-off transcription [using a highly improved commercial kit, RiboMax System, Promega (2)]; (iii) replacement of the ensuing transcript's 5′ triphosphate by a monophosphate (e.g. dephosphorylation–phosphorylation); (iv) ligation of the RNA molecules by T4 RNA ligase (3) (e.g. circularization) and (v) gel purification. The critical step in this procedure is the use of the circularly permuted RNA strategy (1) to produce the desired transcriptional template. This strategy involves a PCR amplification of a unit-length gene from a tandemly duplicated gene template, and is used to position the ligation site and to introduce any desired mutations into the subsequent RNA molecule. The amplification is performed using a forward primer with T7 RNA polymerase promoter sequences at its 5′ end, followed by two or three guanosine residues for more efficient transcriptional initiation, and ending with the 5′ terminal sequence desired for the subsequent RNA molecule. The 3′ terminal of this RNA is similarly determined by the reverse primer utilized. By tandemly moving both primers along the template, effectively any position can be engineered to become the ligation site. The use of a highly processive 5′-3′ DNA polymerase that possesses a 3′-5′ exonuclease activity to effect the amplification ensures the elimination of polymerase errors and, therefore, sequence homogeneity

Conventional recombinant RNA techniques may be used for synthesis of circular molecules; however, they require polyacrylamide gel purification of the products at almost every step resulting in a laborious and inefficient synthesis. Here, we described a procedure that may be performed in <3 days, and requires only a single, terminal gel purification. Moreover, this method complements the T4 DNA ligase strategy for joining RNAs (4), a strategy which we have shown to be unsuccessful for the preparation of large amounts of model viroid using either a complementary oligodeoxyribonucleotide, when the ligation site was located in a loop due to inefficient RNA–DNA heteroduplex formation attributable to the stable secondary structure, or when the ligation site was located in a native stable helix because the 5′ end dephosphorylation–phosphorylation reactions of the previous step were sterically inefficient. Using the strategy described here, the introduction of a mutation in a double-stranded region of the molecule is not limiting because the ligation site is selected to be in a single-stranded region. The limiting factor to the application of this method is some knowledge of the secondary structure in the region of the ligation site. However, this restriction may be easily circumvented by a fast study of the secondary structure near the ligation site, for example by RNase T1 partial hydrolysis or by computer prediction. Use of this simple strategy permits either the circularization of an RNA, or the ligation of two RNAs of any sequence by using primers leading to production of RNA whose extremities fulfil these requirements. A synthesis of mutated circular transcripts of sequences derived from peach latent mosaic viroid (PLMVd) is described here as an example. Since viroids are composed of a series of single- and double-stranded regions, they offer several potential T4 RNA ligase ligation sites. The ligation site chosen in the current example is located in the loop at the end of the left arm region so as to ensure efficient ligation by T4 RNA ligase (Fig. 1A, enlarged circle). This loop has been selected since several positions are mutated as compared with the sequence variants published (5) and, consequently, the replacement of three nucleotides (UCA) by three guanosine residues should allow for an efficient initiation of transcription without interfering in the ensuing studies.

All enzymatic reactions within the method have been optimized to a preparative scale. The enzymatic steps (PCR-amplification, run-off transcription, dephosphorylation, phosphorylation and ligation) are linked sequentially by simple manipulations. Both phenol (1 vol) and phenol–chloroform (0.5 vol:0.5 vol) extractions are performed after each step prior to proceeding. After both the PCR-amplification and transcription reactions, the large nucleic acid products are separated from any remaining nucleotides and primers by isopropanol precipitation; whereas the products resulting from the dephosphorylation, phosphorylation and ligation reactions are ethanol precipitated. All precipitations are followed by 70% ethanol washes and pellet lyophilyzation. Hence, it is therefore possible to interrupt the synthesis at any step.

Step 1, PCR-amplification. PPD1 insert (0.2 ng, a tandemly duplicated sequence derived from PLMVd; ref. 6) was amplified with 2.5 U of Pwo DNA polymerase (Boehringer Mannheim) in a mixture of 10 mM Tris–HCl pH 8.85, 25 mM KCl, 5 mM

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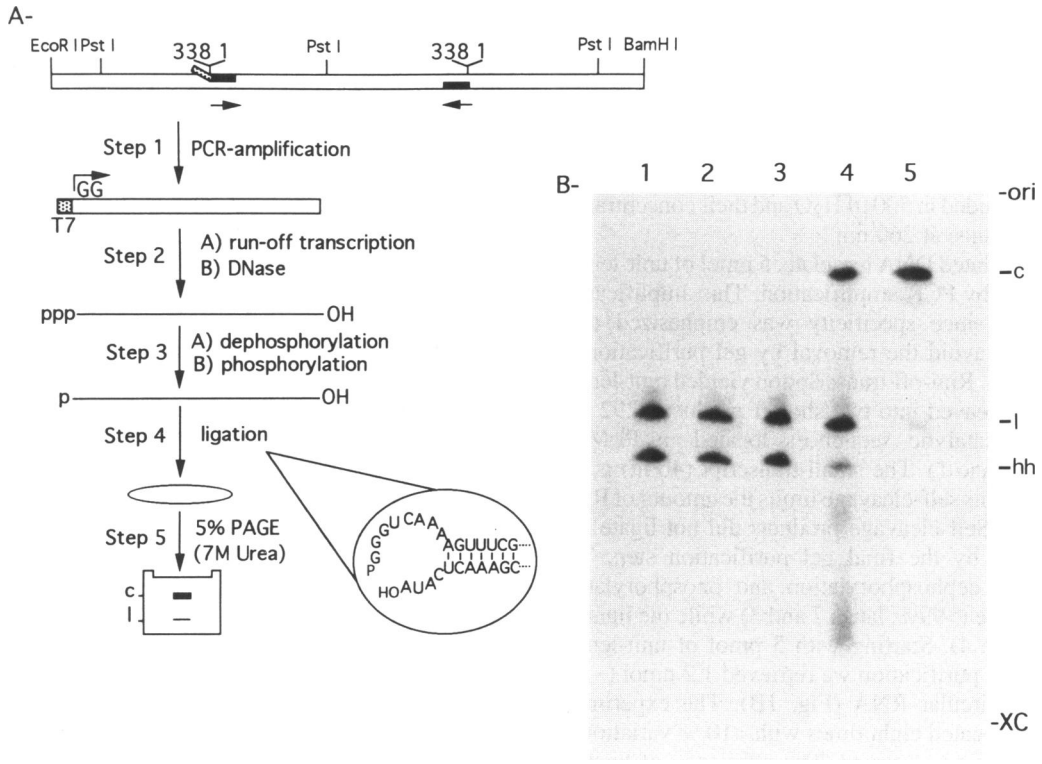

Figure 1. Protocol for the synthesis of large amounts of circular RNA. (**A**) Schematic representation of the procedure. *Eco*RI and *Bam*HI are the restriction sites used to isolate the tandemly duplicated sequence of PLMVd from pPD1 (6). The plasmid was co-digested, electrophoresed on a 1% agarose gel, and the insert band excised and electroeluted. *Pst*I indicates the restriction sites used for pPD1 construction. Black boxes show PCR primers, while the dotted box shows the T7 RNA polymerase promoter sequence. In the enlarged circle, the sequence and secondary structure of the ligation site of PLMVd used for the example reported here are illustrated. (**B**) Analysis of transcripts after each step on a 5% polyacrylamide gel. Radioactive transcripts were obtained by adding [α-^{32}P]UTP to the transcription reaction (see text). Lane 1, after transcription; lane 2, after dephosphorylation; lane 3, after phosphorylation; lane 4, after ligation and lane 5, after purification. Linear and circular 338 nucleotide transcripts are indicated by **l** and **c**, respectively, while hammerhead self-cleavage products are noted by **hh**. **ori** is for origin of migration and **XC** for xylene cyanol.

$(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.2 mM dNTPs and 1 µM of each primer (forward 5′-TAATACGACTCACTATAGGGTCAAAA-GTTTCGCCGC-3′; reverse 5′-TATGAGTTTCGTCTCATT-TC-3′) in a final volume of 100 µl, and the PCR products analyzed by agarose gel electrophoresis. The thermocycle consisted of: pre-incubation for 1 min at 94°C, 35 cycles for 1 min at 94°C/1 min at 44°C/2 min at 72°C and a final 10 min extension at 72°C.

Step 2, run-off transcription. The total PCR product was transcribed using the RiboMax System (Promega) as recommended by the manufacturer for a final reaction volume of 50 µl (2). For random internal labelling, 50 µCi of [α-^{32}P]UTP (3000 mCi/mmol, Amersham) was added to the reaction. After transcription, 5 U RQ1 RNase-free DNase (Promega) was added and the reaction incubated at 37°C for 15 min in order to remove the template DNA.

Step 3, dephosphorylation–phosphorylation. Transcripts were incubated for 60 min at 37°C in a final volume of 200 µl containing 250 mM Tris–HCl pH 8.3, 25 U RNAguard (Pharmacia), and 3 U calf intestinal alkaline phosphatase (Pharmacia). It is important to avoid the presence of magnesium and ensure that the temperature does not exceed 37°C in the dephosphorylation reaction in order to limit RNA hydrolysis. After extraction and precipitation, the pellet was resuspended in 10 µl H_2O and incubated for 60 min at 37°C in a final volume of 50 µl containing 1× One-Phor-All buffer (10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate and 50 mM potassium acetate, Pharmacia), 1 mM ATP, 50 U RNAguard (Pharmacia) and 20 U T4 polynucleotide kinase (Pharmacia). If a single 5′-terminal labelling is desired, [γ-^{32}P]ATP may be introduced during this incubation.

Step 4, ligation. Transcripts were resuspended in 20 µl H_2O and incubated in a final volume of 200 µl containing 1× One-Phor-All buffer, 10% dimethylsulfoxide, 0.2 mM ATP, 50 U RNAguard and 30 U T4 RNA ligase (Pharmacia) at 16°C for 4 h. The phosphorylation and ligation steps may be coupled without organic extraction and precipitation (3). However, we observed substantial RNA hydrolysis under such experimental conditions.

Step 5, gel-purification. The circular transcripts were purified by denaturing polyacrylamide gel electrophoresis where they have a slower mobility as compared to their linear counterparts (Fig. 1A). Transcripts were resuspended in 10 µl H_2O and 10 µl stop-buffer (0.3% each of bromophenol blue and xylene cyanol,

10 mM EDTA pH 7.5 and 97% deionized formamide), denatured for 2 min at 65°C and separated on a 5% polyacrylamide gel in 100 mM Tris-borate pH 8.3, 1 mM EDTA containing 7 M urea at constant temperature (50°C). Circular transcripts were detected by UV shadowing, excised, eluted (with 0.5 M ammonium acetate and 0.1% sodium dodecylsulfate), passed through a microporous membrane (Cronex), ethanol precipitated and washed, dried, resuspended in 500 µl H_2O and their concentration determined by absorbance at 260 nm.

From 0.2 fmol of isolated DNA template, 5 pmol of unit-length product was obtained by PCR amplification. This amplification was not logarithmic since specificity was emphasized over efficiency in order to avoid the removal by gel purification of aberrant PCR products. Run-off transcription yielded unit-length products which self-cleaved into two shorter products (292 and 46 nt) due to autocatalytic sequences located in PLMVd transcripts (Fig. 1B, lane 1). The small transcript (46 nt) is not shown in Figure 1B. This self-cleavage limits the amount of RNA that can be obtained. Self-cleavage products did not ligate and were easily removed by the final gel purification step. The subsequent transcript dephosphorylation and phosphorylation were virtually complete (>99%, lanes 2 and 3) while the ligation reached 48–53% (lane 4). Starting with 5 pmol of unit-length PCR product, after gel purification we retrieved 1.2 nmol (~100 µg) of pure 338 nt circular RNA (Fig. 1B). The experiment described here was repeated eight times with <10 % variation in the yield of product being observed. The efficiency of ligation may be influenced by the identity of both the donor and receptor residues (3). In the present case, the donor guanosine residue may reduce the efficiency of the ligation because it is a poor donor group. However, the preference of T7 RNA polymerase for two or three guanosine residues at the site of transcription initiation can be circumvented by the use of di- or trinucleotide primers to initiate transcription (7).

For the specific aim of viroid study, the use of circular RNAs *in vivo* as well as *in vitro*, instead of cDNA and linear RNA structures, respectively, may be of considerable advantage because this would reflect more accurately the biological behavior of these molecules. More generally, this method has the advantage of allowing the introduction of any desired mutation during the amplification step, and provides the possibility of the incorporation of ribonucleotides analogs, such as photoaffinity agents for cross-linking (1), during transcription. Furthermore, it is not limited by the length of the desired RNAs. Moreover, when combined with RT–PCR amplification, circular RNAs may be newly mutated and their derived sequence transcribed and circularized in order to study a second generation of molecules. The application perspectives for this method are many for studying the involvement of RNA in several significant biological reactions; alternatively, circularization may be used to stabilize RNA molecules and thereby increase their half-lives by several orders of magnitude (e.g. ribozymes). The procedure is a simple, efficient and rapid method of synthesizing from hundred micrograms to milligrams of pure, homogeneous, circular and potentially modified RNA molecules.

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