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Emergence of a replicating species from an *in vitro* RNA evolution reaction

(RNA amplification/selfish RNA/RNA polymerase/promoter)

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ABSTRACT **The technique of self-sustained sequence replication allows isothermal amplification of DNA and RNA molecules *in vitro*. This method relies on the activities of a reverse transcriptase and a DNA-dependent RNA polymerase to amplify specific nucleic acid sequences. We have modified this protocol to allow selective amplification of RNAs that catalyze a particular chemical reaction. During an *in vitro* RNA evolution experiment employing this modified system, a unique class of "selfish" RNAs emerged and replicated to the exclusion of the intended RNAs. Members of this class of selfish molecules, termed RNA Z, amplify efficiently despite their inability to catalyze the target chemical reaction. Their amplification requires the action of both reverse transcriptase and RNA polymerase and involves the synthesis of both DNA and RNA replication intermediates. The proposed amplification mechanism for RNA Z involves the formation of a DNA hairpin that functions as a template for transcription by RNA polymerase. This arrangement links the two strands of the DNA, resulting in the production of RNA transcripts that contain an embedded RNA polymerase promoter sequence.**

In vitro evolution techniques make it possible to generate and isolate nucleic acids with novel structures and functions (for reviews see refs. 1 and 2). An important aspect of these techniques is the integration of a selection process with a procedure for amplification of nucleic acids, such as the polymerase chain reaction (PCR) (3, 4) or self-sustained sequence replication (3SR) (5, 6). We have been interested in the directed evolution of RNAs with novel catalytic function (7, 8), and in this work have made extensive use of *in vitro* RNA amplification. We employ a simplified version of 3SR that relies on the combined activities of a reverse transcriptase (RT) and a DNA-dependent RNA polymerase (RNAP) (9). This method makes use of two DNA primers to confer specificity for RNA amplification. Primer 1 selectively hybridizes to the 3′ end of the target RNA template and initiates cDNA synthesis, catalyzed by RT. Primer 2, in turn, selectively hybridizes to the 3′ end of the cDNA and introduces an RNAP promoter sequence. RT converts this complex into a double-stranded DNA template that is suitable for transcription by RNAP. Amplification occurs because multiple copies of RNA are produced per copy of DNA template and because each RNA copy is able to enter another round of amplification.

In the present study, we sought to carry out continuous RNA amplification in concert with RNA catalysis, so that RNAs that perform a particular chemical reaction would be immediately eligible for amplification, and RNAs produced by amplification would be immediately eligible to perform the chemical reaction. A variation of the 3SR method was devised for this purpose (Fig. 1). This coupled catalysis and amplification system was designed to selectively amplify RNAs that covalently attach a substrate oligonucleotide to their 5′ terminus with concomitant cleavage of a phosphoanhydride bond. The same catalytic activity is intrinsic to certain constructs of the bI1 group II self-splicing intron of yeast mitochondria (10). Moreover, a precedent exists for the *in vitro* evolution of ribozymes that catalyze this reaction (11).

The substrate oligomer used in the coupled system contains a phage T7 RNAP promoter sequence. Consequently, only functional ribozymes that covalently attach a substrate molecule will give rise to DNA templates that can be transcribed by T7 RNAP. RNAs produced by transcription contain a 5′-terminal triphosphate moiety and thus are able to initiate the next round of the coupled system. In this way, multiple "generations" of ribozymes would be produced during a single incubation.

A major concern when amplifying nucleic acids in a continuous manner is the emergence of rogue templates that evolve to replicate more rapidly than the desired templates (12). The first examples of such "selfish" RNAs were observed by Spiegelman and coworkers (13), who used the RNA replicase from Qβ bacteriophage to amplify Qβ genomic RNA and obtained small, fast-replicating RNAs that retained structural features necessary for recognition by the replicase. A second example of *in vitro* generated selfish RNAs are the small (64-nt) X and Y RNAs, which replicate with the assistance of T7 RNAP (14, 15). Similarly, the DNA-dependent RNAP polymerase of *Escherichia coli* can generate and replicate small selfish RNAs (16).

While implementing the coupled catalysis and amplification system described above, a family of RNA molecules, which we collectively term RNA Z, arose and replicated to the exclusion of the desired molecules. These variant RNAs do not catalyze the intended chemical reaction. Instead they have evolved a mechanism that eliminates the need for covalent attachment of a substrate molecule during the amplification cycle, thereby avoiding the selection constraint.

MATERIALS AND METHODS

Enzymes. Avian myeloblastosis virus RT was obtained from Life Sciences and Moloney murine leukemia virus RT was obtained from United States Biochemical. T7 RNAP was prepared as described (17) and was purified according to a modification of a procedure originally developed for SP6 RNAP (18).

DNA Constructs. Plasmids peI and peT7I were derived from plasmid BS/bI1Δ-1, which contains the gene for the bI1 self-splicing group II intron of yeast mitochondria (10).

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Abbreviations: 3SR, self-sustained sequence replication; RT, reverse transcriptase; RNAP, RNA polymerase; EBS, exon-binding site.

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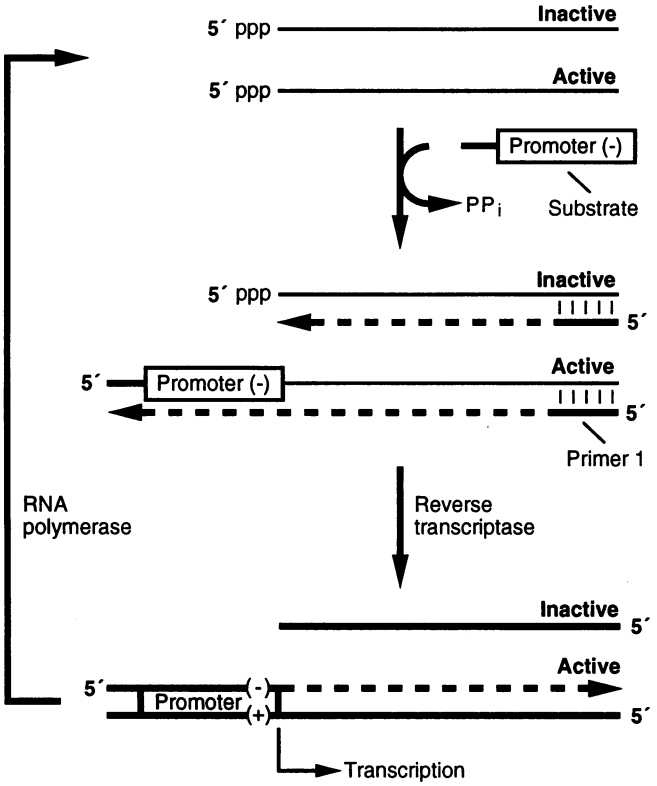

FIG. 1. Coupled catalysis and amplification. RNA and DNA molecules are represented by thin and thick lines, respectively. Newly synthesized DNA is depicted by dashed lines. RNA molecules with the desired activity catalyze covalent attachment of a substrate molecule to their 5′ end. Active RNAs acquire an RNAP promoter (−) sequence, corresponding to the portion of the RNAP promoter element encoded by the nontemplate strand of the double-stranded DNA. All RNAs are eligible to direct the synthesis of cDNA, but only those cDNAs that are derived from active ribozymes will include a promoter (+) sequence, corresponding to the portion of the RNAP promoter element encoded by the template strand of the double-stranded DNA. The resulting partial duplex DNA, which includes a functional double-stranded promoter, can be converted to a complete duplex by RT and transcribed by RNAP to produce copies of the active ribozymes.

BS/bI1Δ-1 was used as the input for PCR amplification (3, 4) employing oligodeoxynucleotide primers 1 (5′-GTGCCAAGCTTGTGATAGGTAGATCTTTACAAATTTTCCC-3′) and 2 (5′-CTGCAGAATTCTAATACGACTCACTATAGGGAACAAAAGCTGAGACAAGTATAAG-3′). Primer 1 is complementary to the 3′ terminus of the ribozyme. Primer 2 contains a region that corresponds to the 5′ terminus of the RNA and in addition carries the promoter consensus sequence for T7 RNAP (underlined). The PCR products were digested with *Eco*RI and *Hin*dIII and cloned into a pUC18-derived plasmid vector to generate recombinant plasmid peI. Plasmid peT7I is identical to peI, except that the exon-binding sites (EBS1 and EBS2) have been changed to 5′-UAUAGU-3′ and 5′-GAGUCGU-3′, respectively. These changes were made by site-directed template mutagenesis (19) of peI, using the mutagenic oligodeoxynucleotides 5′-GTATATATATGTATTCTAACTATAATTGAATATCTTACTTCT-3′ and 5′-ATAATAGAAAAAAATATTACGACTCTTACGATATTTTATAATA-3′ and were verified by DNA sequence analysis.

***In Vitro* Transcription.** Templates for the synthesis of partially randomized peT7I-derived RNA were generated by mutagenic PCR (20) using primers 1 and 2 and peT7I input DNA. The PCR products were transcribed as described (7). The RNA product was purified by denaturing 5% PAGE followed by affinity chromatography on DuPont NENsorb. The RNA substrate 2 (5′-pppGGGACGAAUUCUAAUACGACUCACUAUA-3′) was prepared by *in vitro* transcription of synthetic DNA (21) and purified as described above.

3SR and Coupled Reactions. 3SR reaction mixtures (20 μl) contained 200 fmol of mutagenized peT7I RNA, 0.5 μM each primers 1 and 2, 50 mM Hepes (pH 7.5, 23°C), 50 mM NaCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM spermidine, 2 mM each NTP, 0.2 mM each dNTP, 5 μCi (185 kBq) of [α-^{32}P]GTP, 100 units of T7 RNAP, and 100 units of Moloney murine leukemia virus RT and were incubated at 37°C. Conditions for the initial round of the coupled catalysis and amplification reaction were similar to those employed for 3SR, except that the reaction volume was increased to 200 μl, 2 pmol of mutagenized peT7I-derived RNA was used as input, and primer 2 was replaced by substrate 1 [5′-d(GGGACGAATTCTAATACGACTCACTAT)rA-3′] and substrate 2. Subsequent rounds of RNA amplification were carried out in 20 μl under the same conditions as above, except that various combinations of enzymes and primers were utilized (see *Results*). All reaction products were separated by denaturing 5% PAGE and visualized by autoradiography.

RESULTS

Origin of RNA Z. RNA amplifications were conducted with input RNA derived from the bI1 group II ribozyme. Certain modified forms of this RNA can undergo a reaction in which the 3′-hydroxyl of a ribozyme-bound substrate attacks the 5′-terminal triphosphate of the ribozyme itself, resulting in the liberation of inorganic pyrophosphate and the formation of a covalent linkage between the substrate and the ribozyme (10).

For this study, the substrate specificity of the ribozyme was altered by site-directed mutagenesis changing the substrate-binding domains EBS1 and EBS2 (22) to be complementary to the last 13 nt of the T7 RNAP promoter consensus sequence (23). Both the wild-type (peI-derived) and altered (peT7I-derived) ribozymes catalyze the triphosphate-dependent cleavage–ligation reaction with their corresponding (matched) RNA substrate, but not with each other's (mismatched) substrates (data not shown). The peT7I-derived ribozyme is unable to catalyze this reaction with a comparable DNA substrate (0.1 μM ribozyme, 0.05 μM substrate). Consequently, an all-RNA substrate (substrate 2) and a largely DNA substrate (substrate 1), both of which contained the T7 promoter sequence, were included in the coupled reaction mixture. The rationale for supplying both substrates was to present the ribozyme with an acceptable substrate for catalysis (substrate 2), as well as a more challenging substrate (substrate 1) that would serve to support amplification by allowing formation of a functional double-stranded promoter.

Amplification of mutagenized peT7I-derived RNA under standard 3SR conditions produced templates of the expected size (≈800 nt) (Fig. 2*A*, lane 1). The corresponding coupled catalysis and amplification reaction failed to produce RNA products of similar length. Instead, after incubation for 4 hr, several smaller RNA species (≈100 nt) came to dominate the population of amplifying molecules (Fig. 2*A*, lane 2). These RNAs can be propagated by a serial transfer procedure whereby a portion of the original reaction mixture is transferred to a fresh reaction mixture. Upon transfer of 1 μl of the initial reaction mixture to a new reaction mixture, two distinct replicating RNAs arose, RNA Z_1 and RNA Z_2 (Fig. 2*B*, lane 1). If the amplification reactions were deficient in either Moloney virus RT or T7 RNAP, then neither RNA Z_1 nor RNA Z_2 could be propagated (Fig. 2*B*, lanes 2 and 3). Withholding various primer and substrate oligonucleotides

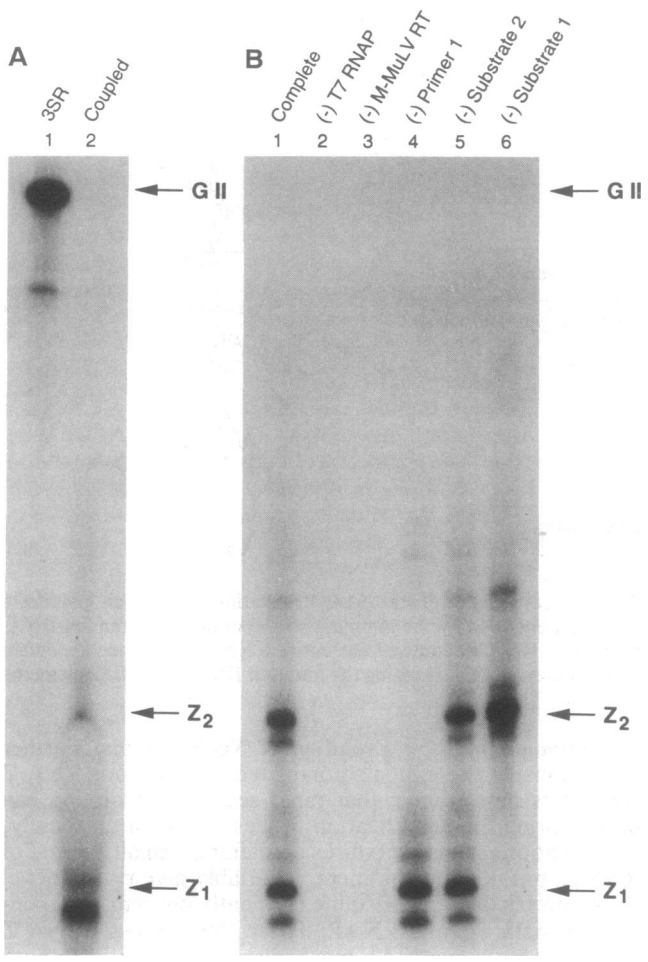

FIG. 2. Emergence and propagation of RNA Z. (*A*) Autoradiograph of a denaturing 5% polyacrylamide gel showing the RNA products of the standard 3SR reaction (lane 1) and the coupled catalysis and amplification reaction (lane 2), initiated with mutagenized peT7I-derived RNA. (*B*) Autoradiograph of a denaturing 5% polyacrylamide gel showing the second round of a serial-transfer reaction, initiated by the products of the coupled reaction mixture. Reaction mixtures were incubated for 2 hr under standard conditions (lane 1) or in the absence of various constituents (lanes 2–6). M-MuLV, Moloney murine leukemia virus; GII, peT7I-derived RNA; Z_1, RNA Z_1; Z_2, RNA Z_2.

from the reaction mixture revealed that RNA Z_1 was dependent upon substrate 1 whereas RNA Z_2 was dependent upon primer 1, but neither RNA was dependent on substrate 2 for its amplification (Fig. 2*B*, lanes 4–6).

Sequence Analysis of RNA Z_1 and RNA Z_2. The sequence of RNA Z_1 was determined by the dideoxy chain-termination method (24) using RT and 5′-^{32}P-labeled substrate 1 (Fig. 3). A pause was found to occur in the extension reaction after 15 min of incubation. However, incubation for 120 min revealed that primer extension could continue for an additional 28 nt beyond the pause site. Examination of the nucleotide sequence that was completed during this secondary extension revealed its complementarity to substrate 1, including the portion that corresponds to the T7 promoter element (Fig. 4*A*). Sequencing of RNA Z_2 was conducted in a similar fashion using 5′-^{32}P-labeled primer 1 (data not shown). The template characteristics of RNA Z_2 are similar to those of RNA Z_1 (Fig. 4*A*).

The sequence of the 73-nt RNA Z_1 is intriguing because it contains an embedded T7 promoter sequence. In addition, it contains a portion of the peT7I-derived group II ribozyme (nt 229–267) corresponding to a small hairpin structural domain

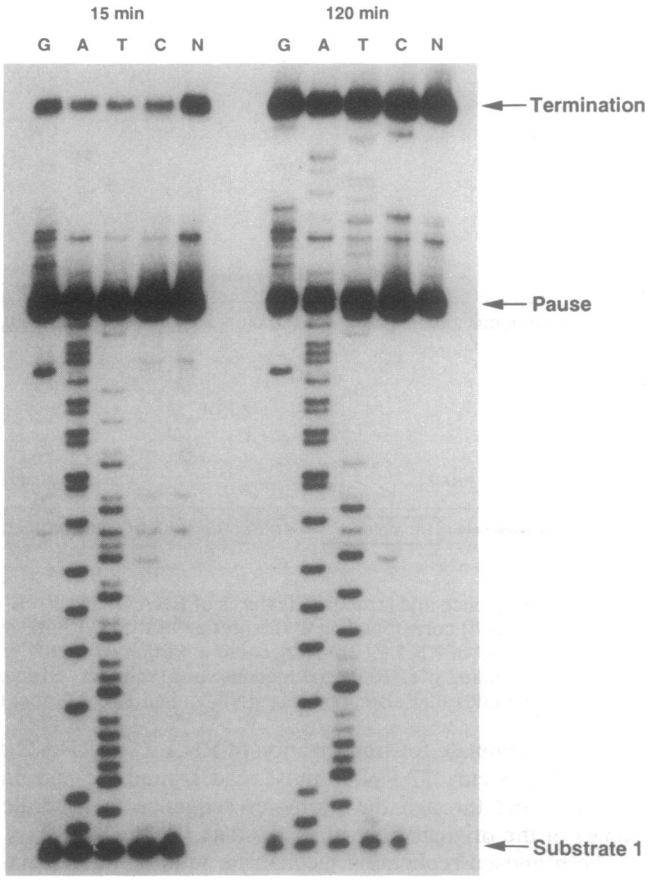

FIG. 3. Sequence analysis of RNA Z_1. Autoradiograph of products of dideoxy sequencing reactions of RNA Z_1, primed by 5′-^{32}P-labeled substrate 1, after 15 and 120 min of incubation.

(Fig. 4*B*). The RNA Z_1 sequence is a subset of the RNA Z_2 sequence. RNA Z_2 consists of 113 nt comprising the 40-nt primer 1 hybridization site appended to the 3′ end of RNA Z_1 (Fig. 4*A*). This arrangement is supported by the observation that addition of both primer 1 and substrate 1 to an amplification reaction mixture containing only RNA Z_2 gave rise to equivalent amounts of RNA Z_1 and RNA Z_2 (data not shown).

Amplification Mechanism of RNA Z_1 and RNA Z_2. An amplification mechanism for the two selfish RNAs can be inferred from the sequence data and from the characteristics of the primer-extension sequencing reactions. Amplification relies on three critical RNA structural features inherent to both RNA Z_1 and RNA Z_2: the embedded T7 RNAP promoter sequence, the primer binding site, and the hairpin domain (Fig. 4*A*). A model for the mechanism of replication of RNAs Z_1 and Z_2 is shown in Fig. 5.

According to this model, synthesis of Z_1 cDNA is initiated by hybridization of substrate 1 to the 3′ end of RNA Z_1 (Fig. 5, stage I). Similarly, synthesis of Z_2 cDNA is dependent on hybridization of primer 1 to the 3′ end of RNA Z_2. Synthesis of the cDNA strand proceeds to the end of the RNA template, giving rise to the major pause site that was seen during the primer-extension reaction (Fig. 3). After cDNA synthesis is complete, the DNA strand undergoes a rearrangement to form a 3′-terminal hairpin structure (Fig. 5, stage II). The hairpin structure self-primes additional DNA synthesis, with RT operating as a DNA-dependent DNA polymerase to produce the second strand of the T7 RNAP promoter element (Fig. 5, stage III). The product of this "fill-in" reaction is identical to the final termination product identified by primer-extension sequence analysis (Fig. 3). Completion of the double-stranded T7 RNAP promoter allows the DNA to

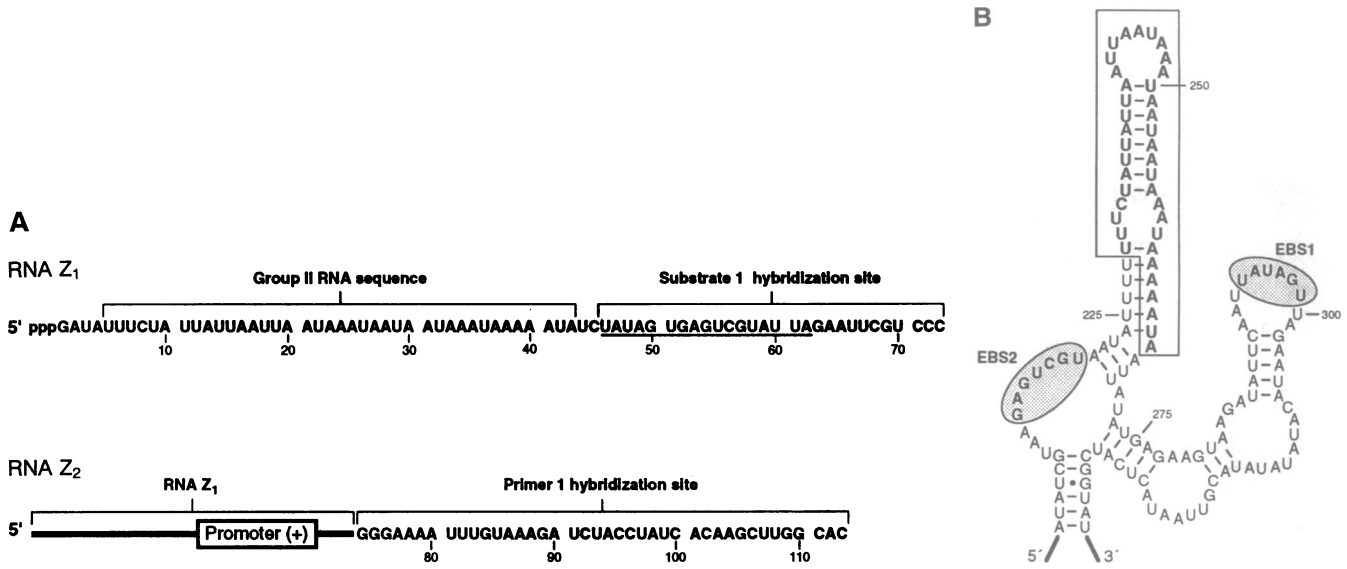

FIG. 4. Sequence and structural features of RNA Z_1 and RNA Z_2. (*A*) RNA Z_1 contains a substrate 1 hybridization site, which includes a region (underlined) corresponding to the portion of the T7 RNAP promoter element encoded by the template strand of double-stranded DNA. Nucleotides 5–43 of RNA Z_1 are identical to a portion of the bI1 group II ribozyme. RNA Z_2 contains the entire RNA Z_1 sequence appended to a primer 1 binding site. (*B*) Partial sequence and secondary structure of the bI1 ribozyme (22), showing the mutated EBS1 and EBS2 elements and the nucleotides that also appear in RNA Z_1 and RNA Z_2 (boxed).

serve as a template for transcription of RNA Z_1 or RNA Z_2. For this to occur, T7 RNAP must read around the hairpin structure and through the promoter sequence to generate copies of the original RNA (Fig. 5, stage IV).

The proposed replication mechanism requires the formation of a double-stranded T7 RNAP promoter, made possible by the self-primed fill-in reaction that produces a double-stranded DNA. Using a trace amount of [α-^{32}P]dATP in the amplification reaction, we confirmed that cDNA synthesis occurs during the synthesis of RNA Z_2 (data not shown). Most of the cDNA that is present after a 15-min incubation is converted to a higher molecular weight DNA upon longer incubation. Primer 1, which initiates the synthesis of Z_2 cDNA, contains a *Hin*dIII recognition sequence near its 5′ end. If hairpin formation and self-priming occur, a functional *Hin*dIII site should be generated. Indeed, *Hin*dIII digestion of DNA produced from RNA Z_2 gave rise to the expected cleavage products (data not shown).

Continuous *in Vitro* Evolution of Nucleic Acids. Further serial-transfer experiments conducted with various RNA Z constructs have shown that rapid sequence evolution can occur during the amplification process. Most dramatically, amplification reaction mixtures containing a small amount of RNA Z, but no added primers, invariably give rise to novel RNA species that amplify quite efficiently but remain dependent on both RT and RNAP. These "primerless" RNA Z molecules have evolved a mechanism for self-priming both cDNA synthesis and second-strand DNA synthesis (M. C. Wright, R.R.B., and G.F.J., unpublished results). More commonly, RNA Z templates acquire mutations in the hairpin domain, most likely due to occasional errors by RT or RNAP. These mutations were never observed to result in the complete disruption of the hairpin structure.

DISCUSSION

Origin and Amplification Mechanism of RNA Z. Linking of RNA catalysis and RNA amplification in controlled *in vitro*

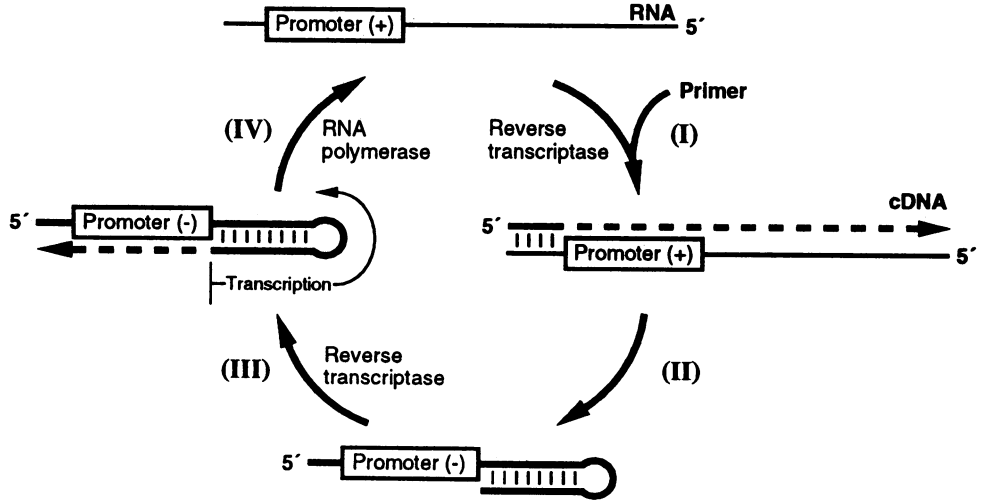

FIG. 5. Amplification mechanism of RNA Z. (Stage I) A primer binds to the 3′ end of RNA Z (thin line) and initiates cDNA synthesis (dashed line), catalyzed by RT. (Stage II) The completed cDNA (thick line), which contains a promoter (−) sequence, rearranges to form a hairpin structure. (Stage III) RT carries out a "fill-in" reaction to generate a functional double-stranded promoter element. (Stage IV) The DNA hairpin serves as a template for transcription by T7 RNAP; the enzyme passes around the hairpin to produce complete copies of the starting RNA.

evolution experiments promises to facilitate the rapid and continuous generation of nucleic acids with novel functions. However, the occurrence of mutations during amplification can lead to the formation of RNAs that amplify selfishly and compete effectively for the resources of the reaction mixture. In 3SR reactions, such selfish RNAs typically arise as a result of mis-priming events, which produce truncated molecules that amplify faster than their full-length competitors. The coupled catalysis and amplification reaction was designed to avoid the emergence of selfish RNAs because mis-hybridization of the primer that contains the promoter sequence would give rise to transcription products that lack the promoter sequence and thus are incapable of hybridizing the same primer in the next round of amplification.

RNAs Z_1 and Z_2 have overcome the selection criterion by internalizing the T7 RNAP promoter sequence (Fig. 4A). As a result, they have eliminated the requirement for covalent attachment of a substrate molecule during successive rounds of amplification. In addition, the cDNAs formed from both RNA Z_1 and RNA Z_2 include a 3′-terminal hairpin structure that allows self-priming to produce a functional double-stranded promoter element (Fig. 5, stages II and III). The hairpin also covalently joins the template and nontemplate strands of the DNA so that the promoter sequence and primer-binding domain are included in the newly synthesized RNAs (Fig. 5, stage IV).

We can only speculate concerning the molecular events that led to the origin of RNAs Z_1 and Z_2. The initial coupled catalysis and amplification reaction actually produced several new species that are visible by autoradiography (Fig. 2). The main product of this reaction was an RNA molecule a few nucleotides shorter than RNA Z_1. Subsequent reactions, initiated by serial transfer, resulted in the synthesis of RNAs Z_1 and Z_2. The major structural domain found in RNAs Z_1 and Z_2 is a hairpin sequence derived from the original group II ribozyme. This hairpin is flanked by EBS1 and EBS2, which were mutated to recognize either substrate 1 or substrate 2. A primer mis-hybridization event involving substrate 1, followed by RT-mediated primer extension, may have resulted in the formation of a covalent linkage between the hairpin domain of the ribozyme and the substrate that contains the T7 promoter sequence. This construct, if converted to a double-stranded hairpin (analogous to the structure in Fig. 5, stage III), would produce an RNA product that is 4 nt shorter than RNA Z_1. A simple 2-nt insertion would have given rise to the final structure of RNA Z_1. RNA Z_2 appears to be the result of fusion of a primer 1 binding site to the 3′ end of RNA Z_1, although the exact mechanism of this event is obscure. We find that other oligodeoxynucleotides, when added to an RNA Z_1 amplification mixture, can give rise to fusion products analogous to RNA Z_2 (data not shown).

Applications of RNA Z-Based Amplification Systems. Synthetic DNAs can be used to initiate the amplification of RNA Z molecules of defined sequence. This makes it possible to study the effects of individual mutations on the functional properties of RNA Z. For example, we used the RNA Z system to quickly obtain functional promoters for various RNA polymerases, beginning with either random or partially randomized promoter domains (R.R.B., A. Banerji, and G.F.J., unpublished results). In addition, it is possible to alter the hairpin and primer-binding domains of RNA Z, allowing rapid amplification of heterologous sequences in the context of an RNA Z-based evolution vector.

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