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A method to increase the cumulative cleavage efficiency of ribozymes: thermal cycling

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Catalytic RNAs (ribozymes) cleave target RNAs in a sequence specific manner. The rate of cleavage is influenced by temperature, substrate and ribozyme concentration, and free magnesium (1, 2). Under optimal conditions, some ribozymes will inherently cleave less efficiently than others (3). Some investigators have elevated incubation temperatures in order to observe cleavage (4, 5). Others have increased the incubation time (6, 7). We have devised a method that increases the cumulative cleavage efficiency of any given ribozyme *in vitro*. We reasoned that although ribozyme molecules are not terminally consumed during the cleavage reaction, in order for the same molecule to be used a second time it must be strand separated from the cleaved substrate RNA. This could be accomplished by a high incubation temperature followed by reannealing of the separated catalytic RNA to a new substrate RNA at a lower incubation temperature. We have verified that if a ribozyme/substrate reaction is cycled between 80°C and 37°C, then cumulative cleavage can be increased over that optimally obtained by incubating at constant temperatures of 37°C, 55°C, 68°C or 80°C.

A ribozyme to the U5 region of the human immunodeficiency virus was used in this assay (8) Conditions used to generate RNA transcripts *in vitro* are described elsewhere (8). The substrate transcript is 495 bases in length. When it is cleaved by the U5 ribozyme, two fragments, a 3′ product P1 (380 bases) and a 5′ product P2 (115 bases) are produced. We constrained the system to use ribozyme and substrate RNAs at a molar ratio of 1:1. We cycled the samples for 5, 10, 15 or 20 rounds, and measured cumulative cleavage. Samples were incubated either at a constant

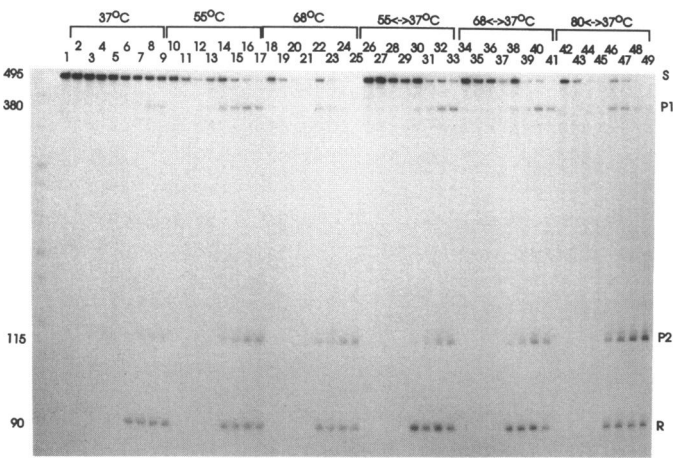

Figure 1. *In vitro* cleavage of the HIV-1 U5 substrate by a ribozyme. Cleavage of substrate (S) by the ribozyme (R) produces products P1 and P2. Lane 1 contains the total amount of substrate at the beginning of each reaction. Each quadruplicate set represents cycled equivalent times of 5, 10, 15 or 20 cycles. Samples in lanes 2 to 9 were incubated at a constant temperature of 37°C without (lanes 2 to 5) or with ribozyme (lanes 6 to 9). Samples in lanes 10 to 17 were incubated at a constant temperature of 55°C without (lanes 10 to 13) or with ribozyme (lanes 14 to 17). Samples in lanes 18 to 25 were incubated at a constant temperature of 68°C without (lanes 18 to 21) or with ribozyme (lanes 22 to 25). Samples in lanes 26 to 33 were thermal cycled between 55°C and 37°C without (lanes 26 to 29) or with ribozyme (30 to 33). Samples in lanes 34 to 41 were thermal cycled between 68°C and 37°C without (lanes 34 to 37) or with ribozyme (38 to 41). Samples in lanes 42 to 49 were thermal cycled between 80°C and 37°C without (lanes 42 to 45) or with ribozyme (46 to 49). Molecular size markers are shown in the left most lane.

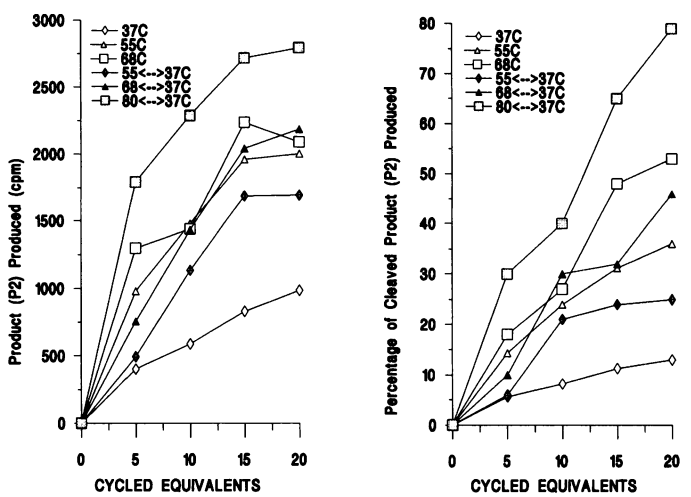

Figure 2. Quantification of HIV-1 U5 RNA cleavage. Panels show cleavage reactions incubated either at a constant temperature of 37°C (open diamond); constant temperature of 55°C (open triangle); constant temperature of 68°C (open square); cycled between 55°C and 37°C (closed diamond); cycled between 68°C and 37°C (closed triangle); and cycled between 80°C and 37°C (closed square). (**A**) Plot of absolute counts contained in the P2 product. (**B**) Plot of the percentage of P2 product normalized to total starting substrate RNA. The x-axis is expressed as 'cycled equivalents'. This represents the particular cycle (for cycled reactions) or the equivalent time period (for constant incubations) at which the data points were measured.

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temperature or the reaction was cycled between two temperatures for exactly the same total duration (Figure 1). Each cycle consisted of heating to the designed maximum temperature for 1 minute, followed by cooling to 37°C for 1 minute. Accounting for time to equilibrate between the two temperatures, each round was 2.5–3 minutes. Samples maintained at a constant temperature were incubated in parallel for the same total time as the cycled reactions. (The total time for the constant incubations is not the sum of reaction times for thermocycled incubations, but actually included the time needed to ramp between the two temperatures of the cycled reaction.) Cleavage was terminated by the addition of formamide followed by boiling and resolution in a 8% polyacrylamide gel containing 7 M urea. Sets of control samples were subjected to thermal cycling in the absence of added ribozyme to gauge the effects of high temperature on the degradation of RNA substrate. For comparison purposes, we quantified either the absolute amount of cleaved P2 product RNA (Figure 2A) or the percentage of P2 normalized to the starting amount of substrate (Figure 2B). At a constant incubation temperature of 37°C, overall cleavage was inefficient; only 13% of the substrate was converted to P2 (Figure 2B). As we increased the incubation temperature, cleavage increased until optimal efficiency was found at a temperature between 55°C and 68°C (Figure 2). Here, the cumulative percentage of P2 produced was about 50–60%. In comparison to the constant temperature incubations, thermal cycling between 55°C and 37°C, or between 68°C and 37°C, did not appreciably enhance the rate of cleavage above that observed for constant incubation at 68°C. However, when we thermal cycled between 80°C and 37°C, the amount of P2 product did increase significantly. The actual amount of increase is hard to assess since the efficiency of cleavage in the last few cycles is underestimated due to progressively smaller amounts of remaining substrate RNA (Figure 1).

These results demonstrate that thermal cycling increases the cumulative efficiency of cleavage by a ribozyme. An explanation for this is that thermal cycling maximizes the number of substrate/ribozyme encounters by successively separating enzyme RNA from cleaved and uncleaved substrate RNA thus allowing for hybridization to new substrate molecules. It may also act by enhancing the number of correctly folded ribozyme molecules. Therefore, thermal cycling could be used to examine cleavage reaction kinetics (Kcat) by removing the constraints of the rate limiting step; hybridization/denaturation (Km). This approach could further be applied to increase the sensitivity of detecting cleavage of highly secondary structured RNAs. Additionally it could also be useful for *in vitro* evolution selection of phenotypically new ribozymes with extremely low efficiencies (9). Finally, industrial applications of ribozymes would benefit from thermal cycling.

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