Extremely High Mutation Rate of a Hammerhead Viroid

Selma Gago, Santiago F. Elena, Ricardo Flores, Rafael Sanjuán

Mutation rates vary by orders of magnitude across species (1, 2), with the highest rates measured so far corresponding to RNA viruses (3), but little is known about other RNA replicons. Viroids are plant pathogens with minimal non-protein-coding RNA genomes replicated by host RNA polymerases (4). We estimated the mutation rate of *Chrysanthemum chlorotic mottle viroid* (CChMVd), a 399-nucleotide chloroplastic viroid with hammerhead ribozymes. Hammerheads are RNA motifs formed by three double-helix regions flanking a core of 15 highly conserved nucleotides critical for catalytic activity (5), which mediate self-cleavage of replicative intermediates and, hence, are essential for viroid replication. Hammerhead viroids showed elevated genetic variability (6), but this variability results from the combined action of mutation and selection and therefore cannot be used to directly estimate mutation rates.

To achieve this goal, we inoculated plants with an in vitro transcript of CChMVd (7), and at the onset of symptoms we screened for mutations at the 15 core nucleotides plus the nucleotide preceding the self-cleavage site in each of the two hammerheads (32 sites). Considering that these mutations are lethal for the viroid, their population frequency must equal the mutation rate. To determine the strength of selection against mutations elsewhere in the viroid genome, we determined the proportion of sites that were rapidly regenerated because of highly error-prone replication. Viroids can tolerate such elevated per-site mutation rates owing to their minimal genomes, whereas more complex genomes would accumulate an excessive mutational load (8). Given their genomic simplicity and autocatalytic activity, hammerhead viroids are reminiscent of the postulated RNA world replicons (9). These primitive replicons would also resemble hammerhead viroids in their extremely error-prone replication. Thus, our results support the notion that the emergence of replication fidelity mechanisms was central to the evolution of complexity in the early history of life.

References and Notes

9. This work was supported by grants from the Spanish Ministerio de Ciencia e Innovación: BFU2008-14839-CO2-01/BMC to S.F.E., BFU2008-03154/BMC to R.F., and BFU2008-03978/BMC to R.S. GenBank sequence accession numbers are FJ647228 to FJ647553.

Supporting Online Material

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Materials and Methods

Figs. S1 to S3

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Published 6 March 2009, Science 323, 1308 (2009)
DOI: 10.1126/science.1169202

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Material and Methods

Site-directed mutagenesis

A pUC18 derivative carrying a dimeric head-to-tail CChMVd wild-type sequence (CM20) (EMBL accession AJ878085) ($S1$), was used for site-directed mutagenesis as previously described ($S2$, $S3$).

In vitro transcription

Plasmid DNA was linearized and transcribed at 37ºC with T7 or T3 RNA polymerase to obtain dimeric CChMVd RNA of (+) or (−) polarity, respectively. For in vitro self-cleavage analysis, transcriptions were incubated 20 min in 2 mM MgCl$_2$, quenched with 3 volumes of denaturing solution (8 M urea, 0.25% bromophenol blue and xylene cyanol), heated at 95ºC for 1 min, and the resulting RNAs were separated by PAGE in 5% gels containing 8 M urea that were stained with silver. For plant inoculation the DNA template was digested with DNaseI following transcription and the RNA was recovered by phenol extraction and ethanol precipitation. For competition assays plasmid DNA of the 24 mutants and of the wild-type were quantified spectrophotometrically by triplicate, pooled at equal amounts, and transcribed, whereas for the infectivity assays of the hammerhead mutants, each mutant clone was treated separately.

Plant inoculation

Transcripts were diluted in borate buffer and mechanically inoculated to one-month old chrysanthemum plants ($Dendranthema grandiflora$ Tzvelev, cv. ‘Bonnie Jean’) ($S1$). In a preliminary titration assay, we determined that 10 ng per plant of the wild-type transcript elicited symptoms in all plants. For competition assays plants were inoculated with 200 ng of RNA to avoid random sampling of sequences, and for infectivity assays of hammerhead mutants, plants were inoculated with 25 ng per plant to minimize the probability of mutant reversion. Plants were maintained in a greenhouse at 20-22ºC with thermal oscillation between 25ºC and 15ºC and a photoperiod of 16 h.

RNA extraction

Apices and the first two expanding leaves were collected on ice and stored at −80ºC, ground in liquid nitrogen, and homogenized in extraction buffer (0.1 M Tris-HCl pH 9.0, 0.1 M NaCl, 10 mM EDTA, 0.1 M 2-mercaptoethanol, 5 M urea). Following centrifugation the supernatant was phenol-extracted, and the RNAs were recovered by isopropanol precipitation and resuspended in 0.5 mL of nuclease-free water.
RT-PCR, cloning and sequencing

RNA preparations were mixed with a specific complementary primer, heated for 2 min at 95°C, ice-cooled and used for reverse transcription (RT) with MMLV reverse transcriptase and PCR amplification with the high-fidelity Phusion™ DNA polymerase. Reverse/forward primers annealing at positions 377-399/1-25, respectively, were used for RT-PCR amplification of the full-length CChMVd sequence. For amplification of the hammerhead region, reverse/forward primers annealed at positions 149-170/346-366, respectively. PCR products were extracted from agarose gels, ligated into pUC19/SmaI, column-purified, and used for electroporation. Plasmid DNA was purified from individual colonies and molecular clones were sequenced using vector-based primers.

We performed control experiments in which we applied the same RT-PCR protocol to the in vitro transcript used for inoculations. We found a single C → A substitution in 45 clones of a 145-nt region encompassing the two hammerheads, yielding an experimental error of $1.5 \times 10^{-4}$.

Northern-blot analysis

Aliquots corresponding to 150 mg of fresh tissue per plant were mixed with 3 volumes of denaturing solution, heated at 95°C for 1 min and separated by PAGE in 5% gels containing 8 M urea. Gels were stained with ethidium bromide and RNAs were electrotransferred to nylon membranes and fixed by UV-irradiation. Membranes were hybridized overnight at 70°C in the presence of 50% formamide with a $^{32}$P-labeled riboprobe for detecting the CChMVd (+) strand, washed, and autoradiographed or quantitatively scanned with a bioimage analyzer.
**Fig. S1.** Schematic representation of the wild-type plus (a) and minus (b) CChMVd hammerheads according to crystallographic data from the *Schistosoma mansoni* hammerhead (S4). On the left of each panel are represented the two complete hammerheads with numbering of nucleotides, helices and loops following the standard convention. For each hammerhead, the arrow indicates the self-cleavage site (after nt 17 which is a C in most natural hammerheads) and boxes the 15 core sites, which are highly conserved in nature (S5). Ovals denote the proposed tertiary interactions between loops, and open square-triangles the Hoogsteen-sugar edge interaction. On the right side of each panel are represented the spontaneous mutants from the plus (M1-M7) and minus (M8-M14) hammerheads found in the 32 core or self-cleavage sites. M5 was found in two independent samples. The number of mutations per site did not significantly differ from the Poisson expectation ($\chi^2$ test, $P = 0.326$), indicating that mutations were distributed randomly across the 32 sites.
Fig. S2. a) Analysis by denaturing PAGE in 5% gels and Northern-blot hybridization of the infectivity of the 14 hammerhead mutants and the wild-type CChMVd. A radioactive riboprobe was used to detect (+) strands. For each mutant (upper panels), total RNA preparations from three inoculated plants were pooled, whereas for the wild-type (lower panels), leaves from each plant were processed separately. Lanes contain total RNAs corresponding to 750 and 250 mg of fresh tissue for each mutant and for the wild-type variant, respectively. RNAs from the pool of three mock-inoculated plants (H), and from a symptomatic (+) and a non-inoculated (−) control were also included. 4S RNAs stained with ethidium-bromide were used as loading controls. The prominent hybridization signal observed in the autoradiographies is generated by the linear CChMVd (+) RNA, which accumulates to higher levels than the circular form.

b) Self-cleavage during in vitro transcription of the 14 hammerhead mutants and the wild-type. Upper panels: schematic representation of the dimeric head-to-tail transcripts and their expected self-cleavage products. Vector and viroid sequences are denoted by black and grey bars, respectively, the self-cleavage sites are marked by arrows and their expected sizes are indicated by numbers on the right of the bars. Lower panels: analysis by denaturing PAGE in 5% gels stained with silver of the 14 hammerhead mutants and the wild-type. Numbers on the right indicate the size of the primary transcripts and of the products resulting from self-cleavage mediated by each hammerhead individually and by both hammerheads concurrently.
Fig. S3. Genetic variability of CChMVd recovered from competition assays mapped on the predicted secondary structure of the CChMVd wild-type sequence (5′). Plus and minus hammerheads are delimited by flags, the 15 core nucleotides are boxed, and the self-cleavage sites are marked by arrows. Solid and open symbols refer to plus and minus hammerheads, respectively. Dotted lines indicate a stabilizing kissing-loop interaction (S3). The 24 point mutants were obtained by site-directed mutagenesis, pooled together with the wild-type at a proportion of 1:25 each, and used for inoculation. Blue and red squares refer to mutations recovered (neutral or nearly neutral) and not recovered (deleterious or lethal), respectively, from the infected plants. Residues in magenta indicate spontaneously arisen polymorphisms. Inset. Symptoms induced by the wild-type CChMVd (bottom) compared with a healthy control (top).
Supplementary References