LETTER

REPLY TO HETTINGER:

Hydrophobic unnatural base pairs and the expansion of the genetic alphabet

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We have recently reported the successful creation of a semisynthetic organism that has an expanded genetic alphabet by virtue of the retention on a plasmid of either the d**NaM**-d**5SICS** or d**NaM**-d**TPT3** unnatural base pair (UBP), the latter of which can be retained at natural-like levels with the use of a Cas9 editing system (1, 2). Despite these demonstrations that a cell can retain a UBP in its DNA, Hettinger (3) now concludes that such hydrophobic UBPs are not suitable for the expansion of the genetic alphabet. Hettinger cites Hirao and Kimoto (4), who note that self-pairing may be a problem inherent to hydrophobic UBPs, and Hettinger (3) refers to their mode of pairing as a "default condition," meaning they simply exclude pairing with hydrophilic, natural nucleotides, and notes that even small levels of self-pairing can lead to UBP strand inversion. However, self-pairing is UBP-specific, and kinetic studies suggest that the d**NaM**, d**5SICS**, and d**TPT3** self-pairs are replicated over 1,000-fold less efficiently than the correct UBPs (5–7), and polymerase proofreading is likely to further reduce the formation of self-pairs (8). The kinetic data have consistently indicated that the most common mutations are those that involve a natural nucleotide. These mutations result in decreased UBP retention and are expected to be particularly problematic because they may impart a selective replication or growth advantage. It is only with the development of the reported Cas9 system that this concern has largely been eliminated (2), and our attention can turn to the possibility of self-pairing and the UBP inversion it might cause. If inversion turns out to be more significant than the kinetic data predict, once translation is established selection pressure may be used to prevent inversion, just as deleterious natural base mutations are eliminated by negative selection. Alternatively, if the default condition extends to transcription and translation, inversion would be eliminated as an issue, even if it occurs, because either nucleotide in a codon would selectively pair with either nucleotide in the anticodon, but neither would pair with a natural nucleotide.

Hettinger (3) also suggests that sequences with consecutive UBPs are too destabilizing, with the objection presumably being that they will not be replicated. Although this may be true, consecutive UBPs are not required for a massive expansion of the genetic code. Even restricting the UBP to only the second position of a codon (where it would always be flanked by at least two natural nucleotides) would provide more new codons than would ever be used.

Finally, only extensive in vivo evaluation of the UBPs from our laboratory, from the Benner laboratory, and from the Hirao laboratory, will reveal the most promising UBPs. It is our hope that all three strategies are ultimately successful, allowing semisynthetic organisms to be bestowed with the greatest capacity to store unnatural information. Although it is certainly true that we have many challenges left to overcome, the demonstration that d**NaM**-d**TPT3** can be propagated within the DNA of a living organism is a promising start.

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