Competition between replication potential and predictable secondary structure formation sets the alphabet size of RNA

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Abstract

The simplest possible informational heteropolymer requires just two alphabets to be able to store information. The evolutionary choice of four monomers in the informational biomolecules DNA and RNA is intriguing, given the inherent difficulties in the simultaneous and localized prebiotic synthesis of all four monomers of progenitors of DNA from common precursors on early Earth. Excluding the scenario where a binary-alphabet genome eventually expanded to include two more alphabets to code for more amino acids on teleological grounds, we show that an evolutionarily successful heteropolymer sequence in the RNA-world needed to be composed of at least four alphabets in order to predictably fold into a secondary structure. Using a model that we previously used to demonstrate the evolutionary advantages of unidirectional replication and anti-parallel strand orientation of duplex DNA, we show that the dual constraints of maximum replicative potential and predictable secondary structure formation can be simultaneously satisfied only by RNA sequences composed of a minimum of four alphabets, within the premise of the presence of sequence-dependent asymmetric cooperativity in RNA. We illustrate this conflict between replicative potential and predictable secondary structure formation, the latter being a metric of information stored in primordial self-replicators, using tRNA, a possible remnant of the RNA world.

Background

The information in the biological heteropolymers DNA and RNA is encoded in a script of four alphabets. DNA and RNA being products of evolution, it is reasonable to investigate the evolutionary significance of the choice of four alphabets. What was the evolutionary advantage of prebiotic informational heteropolymers composed of four alphabets, over heteropolymers composed of lesser number of alphabets? \[1, 2\]. Does the evolutionary force that selected four-alphabet heteropolymers still persist in extant genomes? The prevailing reasoning is that at least four alphabets are needed to code for the twenty amino acids, since, with
the three-nucleotide genetic code, one can have $4^3 = 64$ possible amino acid alphabets, enough to code for twenty amino acids with degeneracies (three alphabets would not accommodate heteromolecular base-pairing between nucleotides of DNA/RNA). The presumed sequence of events leading to a four-alphabet heteropolymer possibly involved beginning with a binary-alphabet heteropolymer, and later acquiring two more alphabets (monomers) that enabled expansion of the genetic code to include more amino acids. However, this explanation does not hold up to a closer scrutiny, since the acquisition of monomers corresponding to four alphabets by prebiotic heteropolymers must have come before the eventual expansion of the genetic code by evolution, which would have rendered those heteropolymers at a severe evolutionary disadvantage in the period between alphabet expansion and the genetic code expansion. The evolutionary disadvantage of those heteropolymers would stem from the need to maintain a sufficient feed of all four monomers for the heteropolymer self-replication, which would have required the presence of more complex geochemical processes, compared to the requirements for self-replication of a binary-alphabet heteropolymer, without any concomitant accrual of evolutionary benefits to the former. In addition, binary-alphabet heteropolymers would have dominated the evolutionary race due to the lower error rates, and hence a slower decay of information stored in them. This brings us back to the initial question about the evolutionary significance of four-alphabet heteropolymers, within an “RNA-world”-like scenario, where the information stored in the heteropolymer sequences was not about protein construction, but about the secondary and tertiary folded structures of the heteropolymers that were capable of catalysis of processes involved in their own replication. Our arguments below satisfy the constraint of immediate accrual of benefit to the replicator upon expansion of the alphabet space, thereby avoiding teleological explanations.

Within our premise described below, both the replicative potential of a self-replicating RNA strand and the specificity of its secondary structure depend on the RNA sequence. We show that, in binary-alphabet RNA strands, the sequences that maximize the replicative potential of RNA strands lack secondary structure specificity, i.e., cannot fold into a specific, predictable secondary structure, due to the availability of many possible secondary structure configurations. Conversely, binary-alphabet sequences that promote specificity/predictability of secondary structures cannot have maximal replicative potential. These two conflicting demands are met simultaneously by the introduction of another pair of monomers, resulting in four-alphabet RNA strands, that can both replicate efficiently and are able to form predictable secondary structures capable of catalyzing reactions associated with their self-replication. The evolutionary advantage stemming from the superior replicative and catalytic capabilities of four-alphabet RNA possibly offset the disadvantages described in the previous paragraph, leading to its preference over the binary-alphabet RNA.

The Premise: Sequence-Dependent Asymmetric Cooperativity in RNA

In our earlier investigations exploring the evolutionary advantages of the unidirectional 5’ to 3’ replica strand construction of DNA single strand and the anti-parallel strand orientation of duplex DNA, our assumption of the presence of asymmetric cooperativity in DNA helped us demonstrate that both the aforementioned properties are outcomes of evolutionary maximization of replicative
potential of progenitors of DNA. We also supplied substantial literature-based experimental support for the presence of asymmetric cooperativity in DNA in our earlier articles [8, 9]. Although the brief exposition below has already been covered in greater detail in our earlier article [9], we include it here to keep this article self-contained, and delay the introduction of ideas original to this article until the Results section.

We define asymmetric cooperativity as unequal and non-reciprocal kinetic influence of an interstrand hydrogen bond on its left and right neighboring hydrogen bonds, as illustrated in fig[1]. More explicitly, when an interstrand hydrogen bond in DNA lowers the kinetic barrier for formation and dissociation of its left neighboring hydrogen bond, and raises the kinetic barrier of its right neighboring bond, the strand is said to be in the left asymmetrically cooperative mode. Strands in right asymmetrically cooperative mode can be similarly defined. The presence of asymmetric cooperativity in DNA single strands increases their replicative potential by simultaneously satisfying two competing requirements for their self-replication: low kinetic barrier for interstrand hydrogen bond formation to easily induct monomers, and high kinetic barrier to retain the monomers already hydrogen-bonded to the template, enabling intrastrand covalent bond formation and replica strand elongation.

Our central premise in this article is the presence of asymmetric cooperativity in RNA, the genetic material of the RNA-world. Earlier [9], we theoretically factorized asymmetric cooperativity into a stronger sequence-independent part operative in DNA single strands, and a weaker sequence-dependent part operative in the DNA double strands, in order to demonstrate the evolutionary advantage of anti-parallel DNA duplex strands and to justify the counter-intuitive evolutionary choice of the complicated, piecemeal lagging strand replication mechanism. The sequence-independent asymmetric cooperativity dictates the direction of construction of replica strands, by decreasing the kinetic barrier for new hydrogen bond formation towards one direction (the 5′-end) of the single-stranded template strand, and raising it for the already-formed bonds behind the growth front, to stabilize them. The evolutionary advantage provided by sequence-independent asymmetric cooperativity to the single strands of self-replicating progenitors of DNA rendered them (and their monomers) directional [8], which is reflected in the non-equivalence of 3′ and 5′ ends of DNA and its constituent nucleotides, within our model. The effect of the presence of sequence-independent asymmetric cooperativity on the kinetic barriers of hydrogen bonds between the template and replica strands of a self-replicating heteropolymer is illustrated in fig[2]. It has to be reiterated that the mode of the sequence-independent asymmetric cooperativity (left or right) is dictated by the 3′ – 5′ directionality of the template strands themselves and is thus sequence-independent. In this paper, we assume that sequence-independent asymmetric cooperativity is present in RNA as well.

The weaker sequence-dependent asymmetric cooperativity dominates in DNA double strands, since the stronger sequence-independent part, dictated by the 3′ – 5′ directionality of the single strands, stands cancelled due to the anti-parallel orientation of the two strands in the duplex. The sequence-dependence of asymmetric cooperativity in duplex strands arises from the dependence of the mode of asymmetric cooperativity on the “orientation” of the hydrogen-bonding base-pairs, which differentiates, for example, the base-pair 5′-G-3′/3′-C-5′ from that of its 180°-rotated counterpart, 5′-C-3′/3′-G-5′. Thus, the base-pair 5′-G-3′/3′-C-5′
Figure 1: Illustration of replica strand construction process of a circular autocatalytic polymer in the presence of (a) asymmetric and (b) symmetric nearest-neighbor hydrogen bond cooperativities (figure and caption reprinted from [8] with permission from Elsevier). The circles represent monomers and the thick vertical lines connecting a pair represent the inter-strand hydrogen bonds. Horizontal lines connecting the monomers represent covalent bonds between them. The color of the hydrogen bonds represent the height of the kinetic barrier separating bonded and unbonded configurations, higher the barrier, darker the color. Hydrogen bonding energy diagram is shown above for both cases of cooperativity. The bottom line of the three lines in the energy diagram corresponds to the energy of the bonded configuration, and the middle line corresponds to that of the unbonded configuration. Only a section of the circular strand is shown here for convenience. (a) In a strand with asymmetric cooperativity, the catalytic influence on and from a hydrogen bond’s left and right neighbors are unequal, simplified here to be catalysis and inhibition from left and right, respectively. Catalysis from a neighboring hydrogen bond is denoted by an arrow from the neighboring bond, and inhibition, with a bar-headed arrow. Such non-reciprocal, asymmetric catalytic influence among a pair of neighboring hydrogen bonds leads to low kinetic barrier for hydrogen bond formation/dissociation near the growth front, enabling faster monomer utilization for the replica strand construction. This also prevents the pre-existing hydrogen bonds behind the growth front from dissociation and provides them longer lifetime to facilitate covalent bond formation between the replica strand monomers. This asymmetrically cooperative hydrogen bonding behavior solves the conundrum of simultaneously requiring both low and high kinetic barriers for hydrogen bond formation/dissociation stemming from the conflicting needs of high monomer utilization and high covalent bond formation probability, but renders the strands directional. (b) In strands with symmetric cooperativity, two hydrogen bonds mutually catalyze each other’s formation/dissociation, by reducing the kinetic barriers symmetrically. In this case, already formed hydrogen bonds in regions away from the growth front (second bond from left) have smaller kinetic barriers than the bonds at or near the growth front (fourth bond from left). This reduces the template’s ability to attract monomers for replica strand elongation, and also its ability to keep the monomers bonded long enough to facilitate covalent bond formation, thereby reducing its replicative potential relative to templates with asymmetric cooperativity. Please note that the scales of energies in (a) and (b) are not the same.
Figure 2: Illustration of sequence-independent asymmetric cooperativity in DNA single strands. The asymmetric cooperativity mode of a single template strand is dictated by the 3’−5’ directionality of the strand (figure and caption adapted from [9] under a Creative Commons Attribution 4.0 International License). (a) A hydrogen bond between a lone nucleotide \( C \) and the template strand catalyzes the formation of another hydrogen bond to its right by reducing its kinetic barrier, while inhibiting the formation of its left neighbor by raising its barrier. The strength and the mode of sequence-independent asymmetric cooperativity dictated by the template strand is denoted by the thick black arrow below the template strand, pointing to the right. The thin arrows attached to the hydrogen bond denote the weaker sequence-dependent asymmetric cooperativity strength and mode. (b) Irrespective of the type of nucleotides occupying the template strand, the directionality of the template strand alone dictates the mode of sequence-independent asymmetric cooperativity. The thinner arrows on the hydrogen bond, denoting sequence-dependent part, though pointing in the opposite direction, does not alter the overall mode of asymmetric cooperativity due to the relative strength of sequence-independent part. This can be seen in the kinetic barrier diagrams above the bonds, where the barrier on the right of the hydrogen bond is lower in both (a) and (b), and the barrier on the left is higher. This assumption ensures that the daughter strand is always constructed from the 5’ end of the daughter strand to its 3’ end.
would lower the kinetic barrier for the formation/dissociation of its left hydrogen bond neighbor and raises the barrier for its right neighbor, whereas, the 180°-rotated base-pair $5'-C-3'/3'-G-5'$ catalyzes its right neighbor and inhibits its left neighbor. The effect of the presence of sequence-dependent asymmetric cooperativity on the kinetic barriers of hydrogen bonds in a DNA duplex is shown in fig. 3. The presence of sequence-dependent asymmetric cooperativity in DNA duplex strands renders the rate and the direction of unzipping of the duplex sequence-dependent, thereby providing a crucial, additional degree of freedom to control the kinetics of DNA replication. We have argued [9] that this additional degree of freedom is exploited by evolution to create sequences that allow for simultaneous replication of multiple segments of DNA, called replicores in Biology literature, thereby increasing the rate of replication substantially. This is done by creating segments with alternating modes of asymmetric cooperativity, by loading the top strand in one segment with more G's, and loading it in the next segment with more C's, and so on, i.e., through asymmetric nucleotide composition (GC and AT skews). The two types of interfaces between such segments function as origins and termini of replication, resulting in replication being carried out bidirectionally from the origins, to the left and right, enabled by the alternating permissive orientations for unzipping of the replicores. The cumulative skew diagram arising from GC skews is illustrated in fig. 4. Such skews are found in nearly all genomes, both prokaryotes and eukaryotes, studied thus far, and are widely used as a bioinformatic tool to find origins of replication [10]. By selecting appropriate sequences, the number of replication origins and hence the replication rate of the DNA can be regulated at evolutionary timescales to suit the environmental constraints such as the availability of monomers [11]. Within our model, due to this evolutionary advantage provided by sequence-dependent asymmetric cooperativity, RNA and DNA's evolutionary progenitors have evolved molecular structures for monomers that supported the incorporation of sequence-dependent asymmetric cooperativity, and later bequeathed them to the former. It has to be remembered that, while the unzipping rate of the DNA duplex depends on the sequence, daughter strand construction on the single-strand template is relatively sequence-independent, due to our assumption of the strength of sequence-independent asymmetric cooperativity in relation to its sequence-dependent counterpart.

Heteromolecular base-pairing, the base-pairing through hydrogen bonding of distinct molecular species (purines with pyrimidines), is necessary for incorporation of sequence-dependent asymmetric cooperativity in duplex DNA. Homomolecular base-pairing cannot be used to incorporate asymmetric cooperativity due to the left-right symmetry of the homomolecular base-pairs. For example, the homomolecular base-pair $5'-G-3'/3'-G-5'$, and its 180°-rotated counterpart, $5'-G-3'/3'-G-5'$ are one and the same molecule, does not have left-right asymmetry, and hence cannot incorporate asymmetric cooperativity, which requires distinguishing between left and right directions [9]. Hence a primordial heteropolymer with anti-parallel double strands supporting sequence-dependent asymmetric cooperativity can only be composed of even number of monomer species.

From the foregoing, it should be clear that the location of the replication origins of DNA duplex and the rate and direction of its unzipping, which is the first step in the DNA replication process, is sequence-dependent, instantiated by GC and/or AT skews. This sequence-dependence of unzipping rates and
Figure 3: Illustration of sequence-dependent asymmetric cooperativity (figure and caption adapted from [9] under a Creative Commons Attribution 4.0 International License). In a DNA double strand, the anti-parallel orientations of the two strands result in the cancellation of their respective opposing asymmetric cooperativity modes. If the nucleotides on both the strands are of the same type, the cancellation would be complete, due to symmetry. When the types of nucleotides on the two strands are different, say, with C on the 5′-3′ strand and G on the 3′-5′, the cancellation is not complete and the residual asymmetric cooperativity is dictated by the types of nucleotides, making the asymmetric cooperativity sequence-dependent. The thick arrows denote the sequence-independent asymmetric cooperativity dictated by the individual strands’ directionality, whereas the thinner arrows attached to the hydrogen bonds denote the sequence-dependent asymmetric cooperativity that changes its mode depending on the orientation of the base-pair. 5′-C-3′/3′-G-5′ base-pair orientation of the hydrogen bonds instantiates right asymmetric cooperativity, as shown in (a), whereas the 180°-rotated 5′-G-3′/3′-C-5′ instantiates left asymmetric cooperativity, as the last three bonds of (b) illustrates. Note that this definition is opposite to that of the one we used in [9]. The kinetic barrier diagrams above the strands in (a) and (b) are significantly different, illustrating the sequence-dependence of the unzipping behavior of DNA double strands. The unzipping of the strand in (a) would proceed sequentially from the rightmost end, whereas the middle two bonds would break and the strand will simultaneously unzip in both the directions in (b). This is proposed to lead to simultaneous construction of daughter strand on multiple segments of the single strand template in anti-parallel strands.
Figure 4: Schematic diagram illustrating the experimental observations related to GC skew in various genomes (figure and caption adapted from [9] under a Creative Commons Attribution 4.0 International License). The genome is composed of independently replicating subunits, usually referred to in biological literature as “replichores”, three of which are shown here colored in blue and green. The replichore that is enriched in C is denoted in blue, and in G, green. The sign of GC skew has been observed to correlate with the direction of replication along a template strand. Leading strands, the segments where replication and unzipping machineries travel in the same direction, have been observed to be enriched in the nucleotide G. Lagging strands, where these two machineries travel in opposite direction, must then be enriched in C. Note again that the asymmetric cooperativity mode defined here is opposite to that of the figure in [9]. It has also been observed that only one of the boundaries between the replichores function as origin of replication, whereas the other, as replication terminus. The schematic graph above the strands illustrate the cumulative GC skew, calculated in running windows of appropriate size over the entire genome. It is representative of skews observed in genomes of multiple species, and clearly shows the boundaries between replichores. The GC skew has traditionally been attributed to the difference in replication mechanisms between the leading and lagging strands. All these observations are understandable within our picture of sequence-dependent asymmetric cooperativity, where GC skew is treated as the cause of unzipping directionality. The arrows between the two strands, labeled RAC and LAC, denote the right and left modes of sequence-dependent asymmetric cooperativity respectively. At the origin of replication, pointed by a red dot on the graph above, the asymmetric cooperativities reduce the barrier from both left and right, rendering the bonds at the interface weaker and thereby allowing the interface to function as the origin. At the terminus, the barrier height for the hydrogen bonds are raised from both directions and can be broken only when the neighboring bonds are broken.
origin locations, and hence of the replicative potential of DNA double strands, makes certain DNA sequences evolutionarily dominant, among a pool of self-replicating DNA sequences, thereby selecting them. In the following, we extend our premise of the presence of asymmetric cooperativity in DNA to RNA as well, and will apply the above model to show that the evolutionarily dominant early circular RNA sequences are palindromes with maximal skews, and are thus predisposed to form hairpin or stem-loop secondary structures, one of the fundamental secondary structures of early ribozymes such as tRNA [12]. We will then show that the evolutionarily dominant, highly skewed, binary-alphabet RNA palindromic sequences can fold into many possible, nonspecific hairpin structures, whereas, the evolutionarily dominant, highly skewed, quadruplet-alphabet palindromic sequences will fold into a single, specific hairpin secondary structure.

Results

Palindromic sequences with high skews have higher replicative potential

Consider an RNA double-strand with a sequence of length \( N \). We will assume that this RNA sequence is composed entirely of only two nucleotides, \( G \) and \( C \). To be concrete, we assume that the base-pair \( 5'\text{G}-3'/3'\text{C}-5' \) is left asymmetrically cooperative, which reduces the kinetic barrier of its left neighboring hydrogen bond and raises the barrier of its right neighbor. It is obvious that the base-pair \( 5'\text{C}-3'/3'\text{G}-5' \) would be right asymmetrically cooperative. Please note that this definition is opposite to that of the one used in our earlier paper [9], to align our arguments more closely with experimental observations. A random RNA single strand sequence composed of a mixture of \( G \)'s and \( C \)'s cannot have maximal unzipping rate of all sequences, due to the presence of the mutually stabilizing dinucleotides \( 5'\text{G}-3'/3'\text{C}-5' \) and \( 3'\text{C}-5'/5'\text{G}-3' \), which presents a higher kinetic barrier for unzipping. This is demonstrated in fig. 5 (b), where the fourth and the fifth nucleotides from the left mutually stabilize each other, thereby presenting a kinetic barrier. The linear sequence \( 5'\text{C}-3'/3'\text{G}-5' \) do not have the above dinucleotide that impedes rapid unzipping, and will unzip from its right-most end, thereby allowing replication to proceed sequentially from the right-most end to left-most end, as illustrated in fig. 5 (a). The above homopolymeric sequence is still replicatively inferior to the palindromic sequence \( 5'-\text{C}_{N/2}\text{G}_{N/2}-3'/3'\text{G}_{N/2}\text{C}_{N/2}-5' \), which initially unzips at its center, due to the reduced kinetic barrier at the central \( 5'\text{C}-3'/3'\text{G}-5' \) dinucleotide, and proceeds to unzip bidirectionally towards left and right, due to the permissive right and left asymmetric cooperativity modes of \( 5'-\text{C}_{N/2}\text{G}_{N/2}-3'/3'\text{G}_{N/2}\text{C}_{N/2}-5' \) and \( 5'-\text{G}_{N/2}\text{C}_{N/2}-3'/3'\text{C}_{N/2}\text{G}_{N/2}-5' \) replichores, respectively. We call the above sequence a maximally-skewed palindrome, since the nucleotide skew, defined as percentage of \( (G-C)/(G+C) \) over running windows of fixed length, of the two strands of the duplex RNA on each replichore, is maximal in magnitude. Fig 5 (b) shows one such maximally-skewed palindrome. As can be seen in this figure, the two middle bonds have lower kinetic barriers, are weaker, and hence will be the first ones to break, thereby initiating the cooperative unzipping of the entire replichores on either side. Thus the rate of unzipping of the entire palindromic sequence
would be twice that of the homopolymeric sequence above, thereby increasing its replicative potential. Moreover, if we confine our attention to circular genomes, the choice of genome topology of primitive organisms (prokaryotes), homopolymeric sequences such as 5'-Cₙ-3'→3'-Gₙ-5' are at a further disadvantage, because of the impossibility of formation of sequence-dependent origin of replication, since the asymmetric cooperativity mode would be the same for the entire length of the genome, with constant kinetic barriers. In the following, we restrict ourselves to this evolutionarily earlier and simpler circular topology for RNA self-replicator [13], thereby avoiding the inherent problems associated with replicating the ends of linear replicators of recent evolutionary history [14].

In order to improve the rate of unzipping of sequences of fixed length $N$, we can proceed as above to divide the self-replicator into shorter replichores, such as 5'-C$_{4N/4}$G$_{4N/4}$C$_{4N/4}$G$_{4N/4}$-3'→3'-G$_{4N/4}$C$_{4N/4}$G$_{4N/4}$C$_{4N/4}$-5', which would introduce two origins of replication and hence would be expected to replicate nearly twice as fast as the palindrome 5'-C$_{N/2}$G$_{N/2}$-3'→3'-G$_{N/2}$C$_{N/2}$-5'. However, the former would consume monomers at a rate twice that of the latter, for self-replication, and hence, in environments where monomer supply is short, would not be favored. Sequence-dependent asymmetric cooperativity thus enables sequences to adapt to different environments that differ in their monomer supply rates. In any case, the rate-limiting step during self-replication of RNA in the primordial oceans would have been the acquisition of monomers for daughter strand construction, due to the initial exponential rise in the number of template strands. This is the regime where asymmetric cooperativity would have been very useful, by helping in both acquisition and retention of monomers. A relatively small, circular RNA self-replicator composed of a single palindrome and a single replication origin would have been replicatively more successful in this regime of limited monomer supply, when compared to both replicators with more than one replication origin and homogeneous-sequence replicators with no replication origin. The former are at, or soon would be at, a disadvantage due to low monomer supply, resulting in multiple replichores of the same replicator competing against each other for monomers. The latter, with no replication origin, cannot easily unzip from a predictable origin, due to high kinetic barriers across the length of the circular polymer. The foregoing is supported by the observation that the single-replication-origin genomes are the choice of most prokaryotes, that face the fiercest competition for monomeric resources, due to their numerosity.

Let us then concentrate on the single-replication-origin palindrome 5'-C$_{N}$G$_{N}$-3'→3'-G$_{N}$C$_{N}$-5', of length 2$N$ of the order of tens of nucleotides, the size of an average stem-loop inverted-repeat sequence found in tRNA molecules, one of the earliest ribozymes known [15]. Any modification of the sequence of this palindrome will adversely affect its smooth unzipping, due to switching of the sequence-dependent asymmetric cooperativity mode near the modified sections of the sequence. This mode-switching introduces high kinetic barriers, as illustrated in fig. 5, which in turn delays unzipping of sections of template for daughter strand construction, making such sequences replicatively inferior to the maximally skewed palindromic sequences. The latter do not have such high kinetic barriers, and hence will be able to attract more monomers to form inter-strand hydrogen bonds with the exposed templates, and hence will be replicatively superior. From the foregoing, it becomes obvious that a circular RNA self-replicator composed of only two nucleotide alphabet can maximize its replicative potential only if its
Figure 5: Illustration of high kinetic barriers presented by binary-alphabet RNA with an arbitrary sequence. (a) A homogeneous sequence composed entirely of a single nucleotide in a binary-alphabet RNA double-strand $5'\text{C}_6-3'/3'-\text{G}_6-5'$ is shown. This sequence presents smaller kinetic barrier for right-to-left unzipping and hence for the movement of the replisome through it from the right side. The kinetic barriers for individual hydrogen bonds are shown above the sequence. Although the kinetic barrier of the left-most bond is higher in the diagram, as the unzipping proceeds from the right, the barriers of bonds at the unzipping front become smaller. For example, the kinetic barrier of the second-to-last bond to the right is reduced when the last bond to the right is broken, due to the lack of stabilizing influence on the former by the latter. (b) An arbitrary sequence of a binary-alphabet RNA molecule presents a high kinetic barrier for the movement of the replisome through it, irrespective of the direction of the replisome movement. The high kinetic barrier arises from the mutually stabilizing influence of the $/5'\text{GC}-3'/3'-\text{CG}-5'$ base-pairs, located at the fourth and fifth positions from the left of the sequence. This mutual stabilization makes breaking of the hydrogen bonds of the two base-pairs kinetically more unfavorable, irrespective of the direction of unzipping, compared to the barriers presented by the homogeneous sequence in (a). This renders such non-homogeneous binary-alphabet sequences replicatively inferior when compared with homogeneous sequences of same length. This demonstrates the necessity of homogeneous sequences for rapid unzipping in binary-alphabet RNA sequences.
sequence is a maximally-skewed palindrome, within our premise of the presence of sequence-dependent asymmetric cooperativity in RNA.

It has to be remembered that, in our model, we have restricted asymmetric cooperativity to operate only between nearest-neighbor inter-strand hydrogen bonds, for ease of analysis and illustration, whereas, in reality, it probably extends over a few inter-strand hydrogen bonds on either side \cite{16, 17, 18, 19}. These longer-range interactions make the effect of high barriers resulting from any deviations from the maximally skewed palindromic sequence to be felt farther than nearest-neighbors, and hence can even reduce the unzipping potential of the origin of replication itself, rendering such sequences replications more inferior. Due to the longer range of asymmetrically cooperative interactions between base-pairs, the effective kinetic barrier reduction/enhancement at any single base-pair would be a weighted average of the effect of asymmetric cooperativity from an extended region, perhaps of the order of ten base-pairs. Such averaging can both dilute the deleterious effect of a single misoriented (left asymmetric cooperativity -moded in an otherwise right-moded sequence or vice versa) base-pair, and spread the deleterious effect over the entire extended region. A corollary is that, if a slim majority of base-pairs in an extended region have the same asymmetric cooperativity mode, the entire region will unzip cooperatively, albeit at a lower rate.

Our thesis above, that asymmetric cooperativity helps in speeding up the rate of self-replication of genomic sequences with highly asymmetric nucleotide distribution around the origins of replication, is corroborated by the observation of the extreme prevalence of GC skew or asymmetric nucleotide composition around replication origins in nearly all genomes studied thus far. In fact, most genomic analysis software programs use the switching of the sign of GC skew, defined as the running average of \((G - C)/(G + C)\) over large-enough windows of sequences, to identify locations of replication origins \cite{10}. There is also some evidence that the magnitude of GC skew is related to the speed of replication of genomes \cite{20}. In addition, it has been shown \cite{21, 22} that mammalian cells have profound G/C and A/T nucleotide skew switches near their replication origins, and that the magnitudes of skews correspond to the firing potential of the origins. The significant difference between the observed mutation rates of 5'-GC-3' (GpC) and 5'-CG-3' (CpG) dinucleotides, where the latter undergoes substantially more single-strand-induced deamination and consequent transition from C to T, has been explained \cite{23, 24} as due to differences in the two dinucleotides’ melting propensity. Our model provides a simple explanation for the difference in the melting propensity of the two dinucleotides, arising from the mutual stabilization of 5'-GC-3' (GpC) dinucleotides due to asymmetric cooperativity, resulting in higher kinetic barriers and reduced melting, and the mutual weakening of kinetic barriers of the 5'-CG-3' (CpG) dinucleotide. Our claim that the hydrogen bonds at the center of certain high-skew palindromic sequences have lower kinetic barriers due to sequence-dependent asymmetric cooperativity, resulting in them functioning as replication origins, is supported by experimental observations of palindromic instabilities, which render them susceptible to unwind locally, leading to cruciform extrusions \cite{25}. This local instability is also connected to these sequences functioning as recombination hotspots \cite{25}. Another line of evidence for our claim that high skews result in higher replicative potential comes from an in vitro selection experiment \cite{26} where self-priming oligonucleotide replicators with different sequences were al-
owed to compete for limited monomer resources. The experiment showed that all the selected replicators showed maximum skews, with the template strand of the most fit sequences composed entirely of pyrimidines and the daughter strands, made of purines. However, because the employed sequences in this experiment were self-priming and initiated replication at the ends, these sequences were not palindromic. More evidence for our claims above come from experiments on asymmetric primer extension kinetics, polar inhibition of replication forks, and asynchronous replication of mammalian mitochondrial genomes, which have been elaborated in our earlier paper [9].

**Binary alphabet maximally-skewed palindromes fold into nonspecific hairpin structures**

Single-stranded maximally skewed palindromic sequences, such as 5′-CN3′, can form stem-loop secondary structures. However, due to the homogeneous distribution of a single type of nucleotide within a single replichore of the above sequence, no information about specific secondary structures can be stored in the sequence, resulting in possibility of formation of multiple nonspecific secondary structures, leading to conformational plasticity. This is illustrated in fig 6. The 5′-CN-3′ replichore arm of the palindrome can bind with the 3′-GN-5′ arm, to form a double strand in many possible ways, due to the homogeneous nature of the sequence. For example, the palindrome can form an incomplete stem-loop structure, with a double-stranded stem segment of length s amidst a loop segment of length l and an overhang of length h. The lengths s, l and h are variable, within the constraint 2s + l + h = 2N, quantifying the variety of secondary structures that can form from a binary-alphabet maximally-skewed palindromic single strand. We have excluded the possibility of bulges within the dsRNA for simplicity, above. Fig. 6 shows three of the possible secondary structures. These nonspecific secondary structures will show variable catalytic activity due to the sequence variability in their loops, with some even inhibiting self-replication reactions. It has been known that secondary structures formed by RNA sequences with low information content, such as trinucleotide repeats, are nonspecific and form several alternative hairpin loop structures [27]. A roughly similar argument has been made theoretically elsewhere, without invoking asymmetric cooperativity [28, 29]. In [29], it was theoretically shown that long, random RNA sequences can form secondary structures only when the number of alphabets are in the range between 2 and 4.

The foregoing illustrates the conflict between high replicative potential and information-storing capability in a binary-alphabet self-replicator. While the replicative potential is maximized by a low-entropy sequence in a binary-alphabet RNA, the ability to store information requires a relatively large sequence space with a relatively flat fitness landscape, thereby allowing the evolutionary selection process to operate. Introduction of another pair of nucleotides in the RNA self-replicator system simultaneously satisfies both the above constraints.

**Secondary structure specificity requires quadruplet alphabet sequences**

We have laid out above the conflict between information-storing capability and the replicative potential of binary-alphabet RNA sequences, within the premise of the presence of sequence-dependent asymmetric cooperativity. Since the
Figure 6: This illustration shows a few possible secondary structures formed by a section of a circular, binary-alphabet, maximally skewed, palindromic RNA sequence. Due to the homogeneity of the sequences on either side of the origin of replication, multiple secondary structures are possible, leading to structural non-specificity or conformational plasticity. Bulges in the stem are excluded for simplicity. The capability of such non-specific secondary structures to catalyze the self-replication of their RNA templates is variable and sequence-independent, when compared to a quadruplet-alphabet, structurally specific, secondary structures, leaving the former at a selective disadvantage. Binary alphabet, maximally skewed, palindromic sequences cannot encode information about specific secondary structures that help catalyze their or their hypercyclic partners’ self-replication, due to the sequence homogeneity within each replicore. Quadruplet alphabet, maximally skewed sequences can simultaneously satisfy the unzipping requirements of replicative potential maximization and the secondary structure specificity for catalysis of self-replication.
replicative advantage provided by high-skew palindromic sequences cannot be foregone without jeopardizing evolutionary superiority over other sequences, evolution must have invented a way around the limit imposed by the near-homogeneous, evolutionarily superior, binary-alphabet sequences on information storage, by increasing the number of alphabets. The number of alphabets must increase by two, in order to satisfy the constraint of left-right asymmetry, required to incorporate sequence-dependent asymmetric cooperativity, as explained above, and also in [9]; i.e., the base-pair formation must be between two distinct monomers, in order to incorporate sequence-dependent asymmetric cooperativity. Thus the minimal number of alphabets needed to simultaneously satisfy the requirements of superior replicative potential and information-storing potential becomes four. With four alphabets, all sequences composed only of, for e.g., A and G nucleotides (purines) on one single-strand arm of the palindrome, i.e., on one replichore, and U and C (pyrimidines) on the other, would retain their high-skew character and asymmetric cooperativity mode, and hence would still have high replicative potential, assuming similar sequence-dependent asymmetric cooperativities for both the GC and AT base-pairs. It has to be noted that, with the introduction of two more alphabets, the constraint that maximally skewed sequences should be palindromic can also be relaxed, for instantiation of replication origins. This increase in the number of alphabets enlarges the subset of the maximum-skew sequences whose replicative potential is maximum and nearly constant across the subset, and allows evolution to select sequences with superior catalytic ability within this subset.

For example, the five-nucleotide double-stranded sequences $5'-UCUUC-3'/3'-AGAAG-5'$ and $5'-CUCUU3'/3'-GAGAA-5'$ will have similar unzipping kinetics due to the same mode of sequence-dependent asymmetric cooperativity in both, with all base-pairs catalyzing their right-neighboring hydrogen bonds. On the other hand, the sequence $5'-UGCUU-3'/3'-ACGAA-5'$ will have significantly lower unzipping rate compared to the former due to the mutually stabilizing influence of the $5'\text{-GC}-3'$ base-pairs [23, 24], arising from the switching of the local sequence-dependent asymmetric cooperativity mode, which also reduces the GC skew magnitude of the sequence (see fig.5(b)). Whereas $5'-GGGGG-3'$ is the only possible sequence that is maximally skewed within the subset of all binary-alphabet five-nucleotide long RNA sequences, a quadruplet alphabet five-nucleotide long RNA can have 32 possible maximally-skewed sequences, where each of the five positions can take either a G or an A (a purine), while preserving the sequence-dependent asymmetric cooperativity mode and hence the unzipping kinetics, and consequently, the replicative potential. This enlarged subset of sequences, with a nearly-flat replicative potential, allows evolution to explore sequences with catalytic abilities that enhances the rate of self-replication of themselves and/or its hypercyclic partners, without paying any fitness penalty.

With four alphabets, the replicatively successful maximally skewed RNA sequences can also fold into catalytically active, specific secondary structures, such as stem-loops. This is illustrated in the schematic fig. 7. Whereas the secondary structures formed by single-stranded maximally-skewed binary-alphabet sequences, such as $5'-C_NG_N-3'$, are constrained to be nonspecific due to sequence homogeneity, the secondary structures that can be formed by maximally-skewed quadruplet alphabet sequences are highly sequence-specific, due to the specificity of base-pairing between G and C, and between A and U (and, to a certain extent, between G and U). Unlike in the binary alphabet case, the lengths...
Figure 7: Illustration of the specificity of the secondary structure formed by a section of a circular, quadruplet-alphabet, maximally skewed, palindromic RNA sequence. With four alphabets, the sequence of a section of the RNA self-replicator completely specifies its secondary structure. Evolution can modify the sequence for an appropriate secondary structure to enhance its catalytic potential, without jeopardising the sequence’s replicative potential, assuming that both the base-pairs have similar asymmetric cooperativities. Such an evolutionary modification is not possible in a binary-alphabet, maximally-skewed sequence. Placing purines on one and the pyrimidines on the other replichore of a quadruplet alphabet sequence, with the two replichores separated by the origin of replication, satisfies the maximal-skew constraint required for easy bi-directional unzipping from the origin, and also provides two nucleotides on each replichore to constrain the possible secondary structures that the sequence can adopt. This evolutionary advantage provided the quadruplet-alphabet sequences superiority over binary-alphabet sequences, and possibly offset the disadvantages of the complex production mechanisms of all the four monomers required by the former, within our model.

of the loop \(l\), double-stranded region \(s\) and of the single-stranded overhang \(h\) of a stem-loop secondary structure can be specified completely by the quadruplet alphabet RNA sequence, allowing evolution to select the sequences that produce catalytically active secondary structures that are useful for self-replication.

Why aren’t the extant genomes maximally skewed in nucleotide composition?

We have argued above that the evolutionarily successful sequences of the RNA world that were simultaneously self-replicating and autocatalytic must have been maximally-skewed quadruplet alphabet sequences that were capable of forming structurally-specific hairpin loop secondary structures, within our premise that asymmetric cooperativity is present in RNA. Why don’t we find such sequences in extant RNA/DNA genomes? What mitigates the need for high skew in extant genomes?

In case of free-floating RNA strands, replicative potential is the only input to calculate the evolutionary fitness function, and maximal-skew sequences are beneficial, as argued above. However, the fitness function of a more evolved organism depend on traits beyond replication rate, and includes information in its genome about extracting energetic and material resources from its environment, which manifests through transcription. Since high GC or AT skew will reduce
the number of nucleotides of a specific type (say, pyrimidines) on the coding strand, the codons with those nucleotides cannot be used to encode for amino acids, resulting in reduced ability to store information in the genome. Thus, an increased pressure to store information in extant organisms may have exerted a downward pressure on the magnitude of nucleotide skews in extant genomes. Indirect evidence for this claim comes from the observation that the skews in the coding sequences of various genomes is higher in the third codon position, when compared to the first two positions, where the evolutionary pressure from information storage is higher [30]. Transcribed ribozyme sequences without an internal replication origin, that need to form stem-loop secondary structures to perform their catalytic functions, cannot support high skews, due to the base-pairing constraint between the two arms of the stem sequence, which requires equal numbers of purines and pyrimidines in the sections coding for stems. The evolutionary pressure arising from this constraint also reduces the magnitude of the overall skew in tRNA- and rRNA-coding sequences, and might have operated even in the RNA-world scenario. This downward pressure on the nucleotide skews arising from the need for information storage, together with an upward pressure to provide directional signals for transcription and replication machineries, possibly sets the effective skews in extant genomes. The effects of conflicting evolutionary pressures for both information storage and rapid replication, on RNA sequences, is explicated below using the case of tRNA.

0.1 A case study: tRNA stem-loops

tRNA molecules are ancient ribozymes that most probably evolved during the RNA-world epoch. Below, we illustrate, using tRNA stem-loop sequences, how the conflicting demands on nucleotide skews, with replicative potential requiring it to be high, and information storage requiring it to be low, are resolved within the genome. tRNA is an appropriate choice to demonstrate this conflict, because the information stored in the sequence pertains to the known secondary structure of tRNA, which makes the constraints posed by the information on the sequence, explicit.

It has been convincingly argued that the modern tRNA originated from a direct/indirect duplication of an earlier inverted-repeat sequence (composed of the acceptor stem and the \( T\psi \)-loop of modern tRNA) that was capable of forming a simple stem-loop secondary structure and also could be aminoacylated with some specificity [31, 32, 33, 34, 35]. The self-alignment or nucleotide matches between two halves of the current tRNA, one half containing the acceptor and the \( T\psi \) stem-loops, and the other half containing the anticodon and D- stem-loops, is found to be much better than what chance events would predict. Moreover, these stem-loop forming motifs seem to also have led to the origination of rRNA through multiple recombination events among such motifs [15].

We argue that the presence or absence of substantial nucleotide skews in tRNA stem sequences depend entirely on whether the sequence is replicated from an origin of replication within the tRNA-coding sequence or from without. As demonstrated above and elaborated below, positioning an origin of replication within the sequence requires the presence of a substantial nucleotide skew with opposite signs on either side of the origin. On the other hand, if the tRNA-coding sequence is replicated from an origin outside its coding region, then the presence of such a sign-switching nucleotide skew within the tRNA-coding
sequence renders its replication kinetically unfavorable.

**Nucleotide skews in tRNA stem-loop structures without an internal origin:** If the earliest ribozyme was a simple, circular, stem-loop structure that also replicated independently, it must have exhibited maximal nucleotide skews, with skew-sign-switching at the replication origin, within our model above. However, the stem-loop structures that are part of extant tRNA molecules do not exhibit such maximal skews. Obviously, the overall skew of the entire tRNA-coding sequence cannot be altered by the skew in the sequence coding for stem part of the tRNA, due to its double-stranded secondary structure. This is because a skew towards purines on one arm of the stem sequence would require a skew towards pyrimidines on the other arm, due to the base-pairing requirement between the two arms of the stem. Nevertheless, maximal skews are generally not seen even in sequences coding for single arms of the double-stranded tRNA stem. The reason for this skew-reduction stems from the fact that maximal skews in DNA sequences that code for tRNA stem-loop structures would render a section of the DNA sequence to have asymmetric cooperativity mode opposing the motion of the replisome (and the transcription machinery) through it, within our model. For example, if the DNA sequence coding for one arm of the stem has left-asymmetric cooperative mode, it would inhibit the movement of replisome moving right-to-left, whereas, the sequence coding for the other arm of the stem would have to have right-asymmetric cooperative mode, and would inhibit left-to-right moving replisome. Therefore, such maximal-skew stem sequences would impose high kinetic barriers on the replisomes originating from outside the tRNA-coding sequence, irrespective of the direction of the latter’s movement. This barrier for the replisome movement is illustrated in fig. 8(a). Such high kinetic barriers are minimized in tRNA by utilizing the averaging influence of asymmetric cooperativity from next-nearest neighbor hydrogen bonds and beyond. If such longer-range interaction between hydrogen bonds are included, then it can be deduced that the kinetic barriers of sequences such as 5′-CCCCCCGGGGGG-3′ would be stronger than that of sequences such as 5′-CGCGCGCGCGCG-3′. This is because of the cooperative reinforcing of the kinetic barriers from the next-nearest neighbor interactions in the former, near-homogeneous, sequence. This skew reduction due to beyond-nearest-neighbor interactions in a low-skew sequence and its effect on the kinetic barrier for replisome movement is illustrated in fig. 8. Nevertheless, kinetic barriers in stem-loop-forming sequences, due to residual skews on single arms of the stem, can result in replication fork barriers at these genetic loci, and such barriers have been observed at the stem-loop-forming tRNA and rRNA genes in various genomes. Thus, within our model, the lack of significant nucleotide skews in the single arms of the stem-coding sequences of extant tRNA molecules is the result of selection to avoid stalling of replicative machinery through tRNA-coding sections.

From the foregoing, it is also clear that long palindromic sequences such as 5′-CCCCCCGGGGGGG-3′ will not be tolerated by genomes, due to kinetic barrier they present to the replisome movement. This deduction accords well with experimental observations of length-dependent instability of palindromes (reviewed in [25]), and of strong under-representation of long inverted repeats and palindromes in stable genomes [39, 40]. Studies that relate nucleotide skews
in palindromic sequences and their occurrence probability in various genomes would provide a more direct evidence for or against our thesis. However, such studies are currently lacking, as far as we know.

Incidentally, the above argument can also be applied to explain the experimental observations of pausing or stalling of replication forks when they traverse unidirectionally through dormant replication origins, that are replicated from without, resulting in local genomic fragility, in genomes of various eukaryotes [41, 42, 43]. Within our model, this replication fork barrier results from the presence of different asymmetric cooperativity modes on either side of the dormant replication origin, instantiated by skew sign switching, with one side opposing the movement of the replication fork through it, while the other side is permissive. Thus, the replication fork barriers at stem-loop-forming tRNA and rRNA genes and at dormant replication origins have a common origin, that of asymmetric cooperativity-imposed kinetic barriers.

The loss of directionality signal due to the reduction of skew in stem sequences is mitigated by introducing skews in the sequences coding for the unpaired sections of tRNA molecule, such as loops, bulges and junctions, through a preference for purines [44, 45]. The skew in the unpaired sections of tRNA guides the replisome in the correct direction through longer range beyond-nearest-neighbor asymmetric cooperativity interactions, within our model. Apart from tRNA, this purine excess in loops, bulges and junctions of ribozymes is also seen in rRNA, and has been shown to be present in tRNA and rRNA codes of all kingdoms of life [46, 47]. Thus, the selective force underlying this convergent evolution towards nucleotide skews in sections of tRNA and rRNA genes that code for single-stranded secondary structures, seen in genomes across all kingdoms, could be the need for directionality signal for the replisomes, provided by the left/right asymmetric cooperativity modes instantiated by the skews.

Nucleotide skews in tRNA-like stem-loops with an internal origin:
The origin of replication of the light strands of mammalian mitochondrial genomes has been found to lie within a region encoding mitochondrial tRNA genes, and has been located at the loop of a tRNA-like stem-loop sequence [48, 49, 50]. This stem-loop sequence has been found to display a strong nucleotide skew, with one arm of the stem sequence containing a near-maximal number of purines, and the other arm, pyrimidines. This tRNA minihelix has been shown to function as a substrate for aminoacylation in vivo as well [51]. An interesting experiment performed by S Wanrooij et al [48] showed that reducing the amount of skew in each of the arms by swapping purines and pyrimidines across the two arms of the stem sequence reduced the origin activity. Moreover, surprisingly, albeit entirely consistent with our model, they observed that the origin activity is completely abolished when the entire arms of the stem sequence are swapped (figure 3 in their paper, construct e). These observations can be explained in a straightforward manner within our model of sequence-dependent asymmetric cooperativity. When a stem sequence’s asymmetric cooperativity modes are such that the central bonds of the loop sequence between the two stem sequences are kinetically weakened (as shown in fig 3(b)), the stem-loop sequence can function as an origin of replication. These opposing asymmetric cooperativity modes on either side of the loop sequence are instantiated by nucleotide skews, with purines on the 3′ end and pyrimidines on the 5′ end. Right asymmetric co-
Figure 8: Illustration of reduction of average nucleotide skew through beyond-nearest-neighbor asymmetric cooperativity interactions. Here R refers to purines and Y, to pyrimidines. (a) A maximally skewed stem-loop sequence such as $5'\text{-}\text{CCCCCCC-CCCC-GGGGGGG-3'}$ imposes high kinetic barriers for the movement of a replisome originating outside the sequence, irrespective of the latter’s direction. For a right-to-left moving replisome, the right-side stem imposes high barriers, due to its left asymmetric cooperativity mode, where the barriers of hydrogen bonds to the right are raised. The kinetic barriers corresponding to the two stems and the loop are shown above those segments. The high barriers are due to cooperative reinforcing of the barriers of each of the hydrogen bonds by all other bonds of the entire stem section, due to sequence homogeneity. (b) The high kinetic barriers imposed by homogeneous stem sequences can be reduced by using low-skew sequences such as $5'\text{-}\text{CCGCGCG-CCCC-CGCCGCG-3'}$. Due to longer range beyond-nearest-neighbor interactions, the effective kinetic barriers of each of the hydrogen bonds is smaller in the low-skew sequence, as shown in the barrier diagram above the strand. The directionality signal for the movement of replisome, reduced due to reduction in skews in the stems, is restored by incorporating skews in the loop section of the stem-loop sequence, which, through longer range interactions, guides the replisome in the correct direction, within our model. The enhanced nucleotide skews of loop sections of tRNA and rRNA coding sequences have been observed in genomes of all kingdoms of life.
Figure 9: Illustration of abolishment of origin activity by swapping the purine and pyrimidine stretches of the two arms of a maximally-skewed stem-loop sequence. In fig.3(b), purines to the 3′ and pyrimidines to the 5′ instantiated an origin of replication within a palindromic sequence. This figure demonstrates that swapping of the purines and pyrimidines, by sending purines to the 5′ and pyrimidines to the 3′, results in a replication termination region, with high kinetic barriers in the center of the palindrome. This is due to the reversed directions of asymmetric cooperativity, with both the arms of the palindrome raising the barriers of the two central base-pairs, as shown in the barrier diagram above. This explains the abolishment of origin activity observed by [48] at the mitochondrial light strand origin of vertebrate genomes. Sequences with skew switches opposite in sign to that of origins are found at the replication termination regions of prokaryotic genomes.

Operativity mode on the left side and left asymmetric cooperativity mode on the right of the central loop sequence renders the latter region’s kinetic barriers smaller, priming the region to function as an origin, as seen in fig.3(b). When the sequences of the two arms of the stem region are swapped, the purines to the 5’ and pyrimidines to the 3’ instantiate left asymmetric cooperativity mode to the left and right asymmetric cooperativity mode to the right, as shown in fig.9. This sequence arrangement raises the kinetic barriers of the central loop sequence, thereby abolishing the origin activity. Sequences with such skew switches are found at the replication termination regions of prokaryotic genomes [10], to stop the replisome from moving into and replicating another replicochrome, within our model. This experiment suggests that the formation of stem-loop secondary structure at the light strand origin might not be the cause of its origin functionality, but the nucleotide skew of the stem sequence, through asymmetric distribution of purines and pyrimidines across the two arms of the stem, results in the origin functionality. This observation, taken together with other, more general observations of profound skew switches around origins of replication in other genomes [21, 22, 52], suggest that nucleotide skews are essential to creating origins of replication.

The importance of the skew to the light strand origin functionality can also be seen from the observation that the stem (particularly the pyrimidine stretch) of the tRNA-like origin sequence of mitochondrial genomes is highly and universally conserved across vertebrates, while the loop sequence is highly variable [18]. This has to be contrasted with conservation of tRNA sequences that are replicated from origins outside their coding regions, where, the loop sequences are highly conserved, to preserve their catalytic functionality, while the stems are variable [53, 54].

The above example, that of a near-maximally-skewed tRNA-like stem-loop
sequence, possesses the desired qualities of a replicatively superior, primordial self-replicator that also possibly employed its secondary structure for catalytic purposes during the RNA-world epoch. We can imagine the counterfactual that if this near-maximally skewed sequence is composed of only two monomers, we would have to conclude that the secondary structure formed by this sequence would be non-specific, and will not be competent to be aminoacylated with equal affinity among all its isomeric folded forms. This again demonstrates, in a concrete fashion, the need for four-nucleotide alphabet. The foregoing also demonstrates the sequence adaptations that resolve the conflicting demands imposed by replication and information storage on tRNA nucleotide skews, by segregating the skews to unpaired loop sections of the molecule.

1 Summary and Discussion

We have shown above that the replicatively superior, binary-alphabet self-replicators in the RNA-world were maximally-skewed, circular, palindromic or inverted-repeat sequences, capable of forming stem-loop secondary structures, within our premise of the presence of sequence-dependent asymmetric cooperativity in RNA. This superiority arises from the reduced kinetic barrier in the central region of the sequence, due to opposing modes of asymmetric cooperativity from either side, thereby creating an origin of replication there. This origin allows for simultaneous replication of the two arms of the inverted repeat sequence, resulting in reduced replication time. On the other hand, the constraint of maximal nucleotide skew with opposite signs in the two arms of the inverted repeat sequence, required for replicative potential maximization, severely reduces the information-storing capacity of the binary-alphabet inverted repeat sequences. This maximal skew constraint forces each arm of the binary alphabet inverted repeat to use just one nucleotide, thereby nullifying its information storage potential. We showed that the conflicting sequence requirements for both the maximization of replicative potential and information-storing capacity, the latter measured in terms of specificity of secondary structures that such RNA sequences can form, can be simultaneously satisfied only by sequences composed of a minimum of four-letter code. This might explain the choice of quadruplet alphabet DNA and RNA in extant organisms.

We have used tRNA-like stem-loop-forming sequences as a model system to illustrate this conflict between replication and information storage requirements, and outlined the nuances in applying our model to extant genomes. We explained why stem sequences cannot have high skews when the entire sequence is replicated from an origin outside the stem-loop sequence, and why maximal skews with opposite signs around the origin are beneficial for stem-loop sequences that are replicated from an internal origin at the loop. Due to their sequence complementarity, sequences coding for the two arms of the stem of the tRNA-like stem-loop structure will have opposing asymmetric cooperativity modes within dsDNA. When these arms have high nucleotide skews, a replisome from an origin external to the tRNA gene sequence will face a high kinetic barrier when traversing through the sequence of one of the two arms. The replication fork barrier that results from such high kinetic barriers can be avoided by choosing stem sequences with low skews. The stalling effect of conflicting directionality signal for the replisome, due to the opposing residual skews of the two
arms of the stem sequences, is mitigated by introducing skews in the sequences coding for unpaired sections, such as loops, bulges and junctions of the tRNA and rRNA secondary structures, as observed universally in genomes of all kingdoms. The need for maximal skews of opposite signs around an origin within the tRNA stem-loop sequence can similarly be explained using asymmetric cooperativity modes. The origin is instantiated within the loop sequence between the two arms of the stem sequence through the opposing asymmetric cooperativity modes of the two arms, that, together, reduce the kinetic barriers at the central loop sequence. In the primordial scenario, simultaneous replication of the two arms of the stem sequence from an origin at the central loop resulted in reduced replication time for the entire stem-loop sequence, making such sequences replicatively superior to other sequences lacking the dyadic symmetry, within our model.

Our model above can be verified or falsified through various means. It can be tested by unzipping dsDNA with high skews from both ends and measuring the corresponding force signatures, taking care to perform the experiment near equilibrium and at conditions resembling in vivo environment. Polar replication fork barriers can be artificially created by inserting high skew sequences within genomes and the permissive and blocking orientations can be explored by switching the skews of the inserted sequence. Variations in replication fork barrier activity of inserted sequences with varying skew magnitudes would suggest the involvement of skews, and hence, asymmetric cooperativity, in instantiating such polar barriers. Palindromic sequences with high skews can be inserted into various genomes and examined for origin/terminus activity. Hydrogen bond lifetimes of high-skew dsDNA sequences carrying an origin can be measured using NMR, taking care to include the helicity and topology of the molecule as confounding variables. Bioinformatic searches for palindromic and inverted repeat sequences with high skews in various genomes might provide clues regarding their replicative potential, and hence, their transposability. Correlation between the magnitude of skew and transposability of palindromic sequences would support our thesis above.

From a broader perspective, the model above synthesizes data from disparate modes of analysis of genomic information content, collated from multiple, independent sources, to provide a unifying picture that causally connects observations such as nucleotide skew or asymmetric nucleotide composition, replication fork polarity and unidirectional replication, sequence motifs near origins and termini of replication, palindromic instabilities that serve as recombination hotspots, and purine loading of transcribed sequences, apart from the evolutionary advantage of quadruplet alphabet, the central idea of this article. The common denominator that causally connects all these observations above is our assumption of presence of asymmetric cooperativity in DNA and RNA, a property we arrived at earlier by maximizing the replication potential of generic primordial self-replicating heteropolymers. This then suggests that various sequence profiles of genomes, such as the polar high-skew sequences, palindromes and inverted repeats, skew-avoiding stems and skewed loops of stem-loop-forming sequences, skew-sign-switching origin and terminus sequences and so on, are deeply connected to the genomic replication kinetics, whose importance to the genome has remained nearly invariant over evolutionary time. This model also accords primacy to the DNA sequence, which determines the kinetics of replication within our model, over the DNA’s interactions with various DNA-binding
proteins, in causing its various functions, listed above, and demotes proteins as being mere modulators of these functions. Additionally, such a view disentangles the circular dependence of proteins and nucleic acids on each other for replication, and provides a plausible sequence of increasingly fitter evolutionary steps, beginning with a DNA/RNA-like progenitor molecule causing its own replicative superiority through evolutionary modification of its sequence from early on, in arriving at today’s complex and interdependent choreography of RNA, DNA, proteins and phospholipids in creating life as we know it. Crucially, if experimentally proven and quantified, asymmetric cooperativity in DNA will provide us with the ability to rationally design DNA sequences with predictable, direction-dependent replication kinetics, and will provide a theoretical basis to explore and alter the evolutionary trajectories of DNA and RNA genomes by rationally modulating their sequences.

**Declarations**

**Consent for publication**

I approve the manuscript for submission. I also confirm that the content of the manuscript has not been published, or submitted for publication elsewhere.

**Competing interests**

I declare no competing interests.

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