

TITLE

C-DNA may facilitate homologous DNA pairing

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ABSTRACT

In many organisms, homologous chromosomal loci can specifically associate in the apparent absence of DNA breakage and ensuing recombination. The direct pairing of intact DNA molecules with similar nucleotide sequences has long been pondered as an attractive underlying mechanism, yet until recently this hypothesis lacked theoretical and experimental support. Studies in *Neurospora crassa* yielded the first genetic evidence for the existence of this process and encouraged a computational search for all-atom models of DNA structures consistent with the *in vivo* results. The outcome of this effort suggests that the B-DNA conformation of the paired double-helices should be strongly shifted towards C-DNA. Coincidentally, C-DNA features a very shallow major groove, which could permit initial homologous inter-helical contacts without atom-atom clashes. Overall, the hereby conjectured role of C-DNA in homologous pairing is expected to revive the search of its biological functions as well as to clarify the mechanism of recombination-independent DNA homology recognition.

Recombination-independent homologous pairing: its existence and implications

In many organisms, homologous (or repetitive) chromosomal regions can engage in physical pairing or/and undergo concerted epigenetic changes in the absence of DNA breakage and recombination [1]. A diverse group of such homology-directed phenomena was described in mammals [1,2], including the transient pairing of the Oct4 alleles at the onset of lineage specification [3] and the co-localization of the X chromosomes prior to random X-chromosome inactivation [4]. Moreover, mammalian genomes contain large amounts of repetitive DNA silenced in the form of constitutive heterochromatin [5]. In mammals, the initiation of heterochromatin formation can occur at newly integrated repeat arrays in the absence of RNA interference [6,7]. Misregulation of this process was implicated in several types of cancer [8] and other disease (*i.e.*, Type I Facioscapulohumeral muscular dystrophy [9]). The basis of homology (or repeat) recognition in all of these situations remains unknown.

A classical instance of recombination-independent pairing was discovered in *Drosophila melanogaster* and other Diptera insects [10]. In these animals, homologous chromosomes remain spatially associated in the majority of cell types during development and in adult life. This paired state is dynamic [11] and plays a critical role in transvection, a situation in which the two alleles, due to their physical proximity, comprise one expression unit [10]. Recent work using haplotype-resolved chromosome conformation capture coupled to high-throughput sequencing (Hi-C) [12,13] and super-resolution microscopy [14] yielded two important insights. First, it was found that the level of pairing could fluctuate substantially along the chromosomes, having tightly-paired regions interrupted by loosely-paired regions. Second, within the tightly-paired regions, homologous segments could not be distinguished from one another even at the highest resolution, hinting at a possibility that they are held together by the direct dsDNA-dsDNA contacts.

The widespread occurrence of recombination-independent homology-directed phenomena contrasts with the limited understanding of their basis, for at least two reasons. First, such processes normally involve large and functionally important genomic regions, which are hard to manipulate and analyze experimentally. Second, the only generally accepted mechanism of DNA homology recognition relies on the Watson-Crick base pairing during cross-hybridization of complementary single strands, which occurs normally after strand breaking and base-pair opening by enzymes involved in recombination.

Elucidating the mechanism of recombination-independent pairing, therefore, requires uniquely adapted *in vivo* model systems as well as deeper knowledge of the biophysical properties of DNA.

This article is aimed at summarizing the progress towards understanding the basis of recombination-independent pairing. First, it highlights the contributions of fungal genetic systems to uncovering the existence of recombination-independent DNA homology recognition. Second, it proposes the role of the transition from B-DNA to C-DNA in facilitating the direct DNA pairing. Third, it overviews some key experiments supporting or rebutting the existence of C-DNA *in vitro* and *in vivo* and suggests that those contradictions need to be reexamined in the light of newly obtained results.

Meiosis as a dedicated stage for homologous chromosome pairing

Homologous pairing becomes very prominent in meiosis, a special type of cell division that halves the number of chromosomes to make gametes [15]. In meiosis, homologous chromosomes initiate pairing in early prophase I, after they start to compact and individualize; and by late prophase I they are found closely co-aligned in a configuration known as synapsis [16].

Canonically, the synapsis of homologous chromosomes requires the break-making function of Spo11 that initiates meiotic recombination [17]. This program is documented in mammals and also in some popular model organisms such as *S. cerevisiae* [18]. Alternatively, homologous chromosomes can pair and fully synapse in the absence of Spo11 [18]. Such recombination-independent program is observed in *D. melanogaster* and the roundworm *Caenorhabditis elegans* [18].

Several ideas were put forward to explain the phenomenon of recombination-independent pairing. For example, it was proposed to occur directly at the DNA level via G-quadruplexes [19] and other four-stranded DNA structures [20,21], or as a global co-alignment by long-range electrostatic interactions [22]. Alternatively, the pairing could be specified indirectly at the chromosomal level by proteins [23], RNAs [24], landmark genomic features (centromeres and telomeres) [25,26], and the regions of active transcription [27].

The question concerning the nature of the meiotic recombination-independent pairing proved hard to answer in part because the dynamics of meiotic chromosomes is typically studied by tracing their axes

and the synaptonemal complex [28], which are rather static features, and which may only appear in the wake of the transient recombination-independent pairing.

Meiotic silencing as a sensitive readout of the early recombination-independent meiotic pairing

Meiotic silencing by unpaired DNA (MSUD) is a process in which pairs of allelic loci lacking mutual homology begin to produce small interfering RNAs in prophase I [29–31]. MSUD was discovered in the fungus *Neurospora crassa* and found subsequently in other filamentous fungi [32]. Three lines of evidence attest to the high efficiency of MSUD. First, in *N. crassa* and related fungi, haploid parental nuclei undergo the last round of DNA replication and then fuse immediately prior to meiosis [28,33], thus precluding all forms of premeiotic pairing (which could have allowed homologous chromosomes to identify one another prior to prophase I). Second, the first signs of silencing can be seen soon after karyogamy, even before the two parental nucleoli had a chance to fuse [31]. Third, the reporter allele becomes silenced consistently in multiple independent meiotic lineages [31], suggesting that MSUD is not only fast but also very accurate. Yet MSUD operates normally without Spo11 and Rad51/Dmc1, in a situation where almost all meiotic recombination has been eliminated [34].

To better understand the mechanism of DNA homology recognition for MSUD, a genetic system was developed, which related programmable interactions between the allelic DNA segments to a silencing effect [34] (Fig. 1B). The sequence of the first (“reporter”) segment was obtained from the *Rsp+* gene, which is involved in determining the ascospore shape in *N. crassa* [31]. Because this sequence always remained the same, the homology relationship between the two segments could only be set by varying the sequence of the second (“test”) segment (Fig. 1B). To accentuate the silencing effect, the segments were placed next to a region of heterology (Fig. 1B). If the segments were detected as pairable, MSUD would not be induced, the endogenous *Rsp+* alleles would be expressed normally, and spindle-shaped (more eccentric) ascospores would be produced. However, if these segments were unpairable, MSUD would be activated, the *Rsp+* alleles would be silenced *in trans* by siRNAs expressed from the reporter segment, and oval-shaped (less eccentric) ascospores would be produced [34].

Using the above system, it was discovered that the presence of very short islands of homology spaced every 11 (or 21-22) base-pairs was sufficient to designate the segments as pairable [34]. Some pairable configurations corresponded to the overall sequence similarity less than 40% (*e.g.*, pattern “4H-7N”, in

which 4 matching base-pairs alternated with 7 mismatching base-pairs), thus arguing against the cross-hybridization mechanism as the basis for homology recognition in this situation.

Curiously, while the detection of unpairable DNA does not require meiotic recombination, the synapsis of homologous chromosomes in *N. crassa* actually depends on Spo11 [35,36]. This conundrum hints at a possibility that the transient recombination-independent pairing preceding DNA breakage can be a part of the canonical meiotic program. Indeed, early Spo11-independent pairing was described in mice [23,37] and budding yeast [38,39], two model organisms in which DNA breakage and recombination are required for normal synapsis.

MSUD shares its basis of homology recognition with repeat-induced point mutation (RIP)

In some filamentous fungi, premeiotic nuclei undergo two closely-related processes known as “repeat-induced point mutation” (RIP) and “methylation induced premeiotically” (MIP) [40,41]. During RIP and MIP, gene-sized repeats of genomic DNA become subject to strong cytosine-to-thymine mutation (RIP) or cytosine methylation (MIP). The well-documented ability of RIP to detect repeats irrespective of their particular sequence composition and genomic localization (although evidently demonstrating higher potency on closely-positioned repeats) suggests that an efficient genome-wide homology search must be involved [41]. Yet, RIP, similarly to MSUD, does not require Spo11 and Rad51/Dmc1; thus it is also recombination-independent [42].

Studies of the homology requirements of RIP used the “pairing-propagation” assay [42], in which two closely-positioned direct repeats (which triggered little RIP on their own) were extended by changing one adjacent segment, designated as “test”, while the corresponding “reference” segment was constant (Fig. 1A). When those segments were made identical, a very strong increase in mutation was observed (Fig. 1A). By systematically varying the test sequence, it was found that the segments related by the patterns “4H-7N” and “3H-8N” could promote RIP [42]. Further work showed that pattern “4H-7N” activated RIP also without adjacent perfect repeats, suggesting that it could satisfy the requirements of the underlying homology sensing process [43].

The above results suggest that RIP and MSUD may engage the same mechanism of DNA homology recognition: both processes are recombination-independent and able to detect the same type of weak

interspersed homology (*e.g.*, pattern “4H-7N”). Furthermore, both processes exhibit high fidelity, *i.e.*, the capacity to induce mutations (RIP) or siRNA expression (MSUD) strictly within the boundaries of their target regions. This latter property suggests that the matching dsDNAs must be made available for each other over distances of several hundred base-pairs, thus existing in a state that is incompatible with their wrapping in nucleosomes.

In principle, co-aligned dsDNAs can be paired directly or indirectly. While the indirect pairing cannot be ruled out, our findings [34,42] make this hypothesis less likely. For example, homology recognition by matching arbitrary tetraplets requires 256 bivalent adaptors with unprecedented properties, including high selectivity and the capacity to rapidly assemble in a long linear scaffold on one side of the DNA double helix. In addition, high concentrations of such adaptors would be necessary to support the high fidelity of RIP and MSUD.

Quadruplex-based model of direct homologous dsDNA-dsDNA pairing

It has long been appreciated that the major-groove edges of the four Watson-Crick (WC) base-pairs are self-complementary in their shapes and hydrogen bond valences [44]. This curious property provided the basis for some early theories of DNA replication postulating the pairing between the matrix and the nascent dsDNAs [45], as well as for the model of the infinite four-stranded complex [46] considered as an intermediate step in homologous recombination [21,47,48].

The current model of homologous DNA pairing also relies on the self-complementarity principle [49]. This model is based on quantum mechanics and molecular dynamics computations, and it was created to rationalize the results of experiments on *Neurospora* RIP. According to the model, one major-groove contact between two homologous DNA duplexes constitutes a stack of 3-4 planar quartets formed by identical WC base-pairs [49]. To enable pairing of long dsDNAs, such contacts must be spaced with a periodicity matching that of the double helices [49]. The model also suggested that the pairing should be more efficient if the participating DNAs were coiled in a right-handed **plectoneme** (see Glossary) [49]. Subsequently, by setting the length between the contacts to the one observed for RIP and MSUD, it was discovered [34] that conformations of the intervening dsDNA segments were strongly shifted towards a C-form conformation [50–52], thus implicating C-DNA in homologous pairing.

Direct pairing of homologous double helices may involve the B-to-C DNA transition

C-DNA is one of the three right-handed double-helical forms (along with A- and B-DNA) discovered in 1950s by molecular modeling based on several types of data, including X-ray diffraction patterns of crystalline DNA fibers [52–54]. While the B-form had been observed in biological samples first [55], and the A-form was later found in protein-DNA complexes [56], bacterial spores and virions [57–59], the biological significance of the C-form remained elusive. Interestingly, although the C-form appears visually different from the B-form (Fig. 2A), its valence and torsion angles do not go beyond the well-populated ranges of the B-DNA family, suggesting that the two forms are similar. Therefore, C-DNA has been considered as a deformed B-DNA or its close relative [52,60].

Two structural properties of C-DNA support its role in homologous DNA pairing. First, the low helical pitch of approximately 9 bp allows two C-DNAs folded as a right-handed plectoneme to have their major grooves facing one another every 22 bp, which coincides with the optimal spacing of interspersed homologous units for RIP and MSUD [34,42]. Second, the major groove of C-DNA is very shallow (while being as wide as that of B-DNA), permitting almost perfect initial quartet contacts without atom-atom clashing (Fig. 2B). Thus, it is reasonable to hypothesize that the B-to-C transition may facilitate homologous pairing by providing as a low energy pathway for the formation of quartet stacks. The mechanism and the driving forces of this transition remain to be elucidated.

C-DNA: a brief history of the field

Originally, C-DNA fulfilled an important role in corroborating the Watson-Crick model, based on the fact that the X-ray **fiber diffraction** patterns and the reversible transformations between those patterns could be explained by the conformational transitions between the A, B, and C-forms of the same DNA molecule with unperturbed topology of hydrogen bonding [55].

Until 1990s, C-DNA remained a subject of active investigations *in vitro* and *in vivo*, and claims of its existence under several conditions were made. For example, it was proposed that the C-form occurred in complexes of DNA with common biological cations [50]. On the other hand, when DNA fibers were neutralized with charged peptides, only the C- and the B-forms (but not the A-form) could be observed [61].

The in-solution B-to-C transitions are typically monitored by spectroscopic methods, in particular by circular dichroism (CD) [62–65]. The CD method measures the optical activity of chiral substances, thus being suitable for studying DNA, which is chiral both chemically and topologically. Due to the chiral environment of electrons in stacked bases, the CD spectrum of DNA features a characteristic mid-UV (180-300 nm) band that is very sensitive to transitions between different forms. Because this band does not overlap with the spectra of proteins, the CD method can be used to follow changes in DNA structure *in situ* and *in vivo*. As CD spectra cannot be calculated *ab initio*, their interpretation is always based on assigning specific spectral features to known structures [66]. Based on this approach, partial B-to-C transitions were reported for DNA in viral particles [62,63], in complexes with lysine-rich proteins or polylysine [67,68] and in chromatin [64,65]. In the case of chromatin, it was further shown that its CD spectrum could be very accurately reproduced by a weighted sum of the reference B- and C-form spectra, with the fraction of the B-form amounting to 30-50% [69].

These early findings were challenged by experiments on pure DNA in aqueous solutions. In particular, CD analysis suggested that a gradual and non-cooperative B-to-C-DNA transition was induced by high concentrations of common salts [64,65,70] or dehydrating co-solvents such as polyethylene glycol and methanol [70,71]. However, it soon became clear that under similar conditions, concentrated aqueous DNA solutions also produced cholesteric liquid crystals known as “polymer- and salt-induced DNA” (ψ - or Ψ -DNA) [72–74]. Ψ -DNA features CD spectra in the same mid-UV range, probably because of the resonance between DNA molecules arranged as periodic arrays. Such spectra may have different shapes and abnormally high amplitudes, regardless of which particular DNA form is present [75,76].

In addition, subsequent X-ray experiments showed that in well-hydrated fibers DNA remained in the B-form at concentrations of salts and co-solvents that allegedly induced the C-form in solution [77]. Similarly, particular covalent modifications that, according to CD, supported the C-form in solution, failed to do so in well-hydrated fibers [78]. Yet the same modified DNA assumed the C- and not the A-form under reduced hydration [78], implying that its propensity to undergo the B-to-C transition was opposed in well-hydrated fibers. Nevertheless, because the X-ray diffraction analysis was considered definitive, it was concluded that the large changes in CD spectra, originally attributed to the C-form, actually corresponded to minor conformational changes in B-DNA. In agreement with this notion, the

increase of the helical twist of DNA in high-salt aqueous solutions was estimated to be much smaller than the one attributed to the B-to-C transition [79].

Once the above conclusions became accepted, the biological interest in C-DNA waned; however, data continued to accumulate in other fields, shedding new light on the old contradictions. One such insight was the fact that the X-ray method was not always suitable for verifying in-solution experiments. For example, while the Z form was first observed in solution by CD spectroscopy [80,81], and its structure subsequently determined by X-ray diffraction in fibers and in crystals [82,83], the salt-induced B-to-Z transition (similarly to the B-to-C transition) in well-hydrated fibers was not reproduced, whereas such transitions were observed under reduced hydration [81,84]. Overall, the transitions from B-DNA to the other forms were never observed in well-hydrated fibers because, apparently, in these conditions, the crystalline order (which is required to obtain a diffraction pattern) is lost before the medium becomes similar to aqueous solutions.

On the other hand, the helical twist of putative C-DNA in high-salt solutions was measured [79] by an indirect approach developed and validated under nearly physiological conditions [85,86]. This method is based on measuring the number of ethidium bromide molecules incorporated into DNA during the unfolding of natively-supercoiled circular plasmids to planar circles. As a result, it depends on several assumptions and calibrated parameters that cannot be easily transferred to the high-salt conditions. For example, as the salt concentration increases, natively-supercoiled plasmids may undergo a transition to a “dense plectoneme” state, in which two coiled double helices become tightly associated along their lengths [87,88]. Unfolding such structures by the addition of ethidium bromide may involve a series of steps that can not be all described by a single set of parameters.

The above considerations suggest that the search for the possible biological role of C-DNA had been interrupted prematurely and thus warrant further investigations. In particular, recent work points to the B-to-C transition as a process that may facilitate homologous dsDNA-dsDNA pairing [34], endowing C-DNA with a function that may be involved in a large repertoire of genetic and epigenetic processes. The conditions favoring the transition are yet poorly understood, but may include the combined action of proteins, ions, cosolvents, and supercoiling.

Generalized implications for meiotic pairing in other organisms

While MSUD features a very efficient DNA homology search, it may represent an extreme instance of a more general process. Indeed, the chromosome-wide pairing can be accomplished by using a small number of dedicated sites in meiosis [89] and in somatic cells [12,13]. Thus, the apparently exhaustive sequence matching for MSUD may be restricted, in other organisms, to a handful of specialized loci.

Meiotic DSBs are normally made by the Top6-like complex containing Spo11 (the Top6A subunit) and the associated Top6B-like subunit [90]. While a reconstituted Spo11/Top6BL complex is unable to cut dsDNA *in vitro* [91], it has long been appreciated that its activity *in vivo* is controlled by many factors [92], including interactions between the allelic DSB hotspots (summarized in [92]). Two types of such interactions were reported. First, the probability of a given hotspot getting cleaved can be affected by the state of its allelic partner [93,94]. Second, even at a strong hotspot, only one of the four chromatids is cleaved normally [95]. These results support a hypothesis that transient recombination-independent pairing may guide the activity of the Top6-like complex in meiosis [39].

The all-atom pairing model features a right-handed plectoneme formed by two homologous dsDNAs with their major grooves facing each other every 11 bp [34]. The formation of a quadruplex contact can occur at any such position, but the spacing between any two consecutive contacts cannot be less than 22 bp [34]. In thermal equilibrium, alternative distributions of contacts will compete, and their probabilities will depend, among other factors, on the sequence composition of the contact sites [49]. As a result, various periodical patterns of the inter-contact spacing may be observed, but all of them should have a minimum at about 22 bp and a principal periodicity of about 11 bp. Such patterns were detected previously for RIP [42] and MSUD [34], and a strikingly similar pattern was reported for the size distribution of the concerted cleavage products made by Spo11 cutting at two positions within the same hotspot [96,97]. These results advance the idea that the Spo11/Top6BL complex may recognize the specific shapes of helix-helix crossings within the quadruplex complexes and cleave according to the general properties of Top6 enzymes [98]. If correct, this idea would further support the notion that homologous DNA pairing not only precedes but also regulates the initiation of meiotic recombination.

Concluding remarks

While the recombination-mediated search represents the only accepted mechanism of DNA homology recognition, accumulating data support the existence of the recombination-independent process, which can pair intact DNA molecules directly, possibly by forming interspersed quadruplex contacts. It has also become apparent that the energy barrier for the quadruplex formation can be reduced by having the participating double helices in the C-form rather than the canonical B-form. Yet many outstanding questions remain. For example, it is unclear if the B-to-C transition only occur concomitantly with the pairing, or C-DNA can exist in the absence of the inter-helical contacts? If C-DNA can indeed exist by itself, the factors that promote the B-to-C DNA transition need to be determined. Moreover, given that B-DNA and C-DNA are structurally similar, new approaches need to be developed to detect C-DNA specifically *in vitro* and *in vivo*. Further research in these directions is expected to yield insights into the biological role of C-DNA as well as the mechanism of recombination-independent DNA homology recognition.

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DECLARATION OF INTERESTS

None are declared by the authors

FIGURE LEGENDS

Figure 1. Genetic assays to understand homology requirements of RIP and MSUD.

(A) Homology requirements of RIP were examined using the pairing propagation assay. Interspersed homology pattern 4H-7N represents a situation when two DNA sequences share 4 matching base-pairs followed by 7 mismatching base-pairs, which occur periodically over the total length of 200 bp. RIP is induced by subjecting each repeat-carrying strain to sexual reproduction. RIP mutations are identified by sequencing the construct in a sample of cross progeny (germinated ascospores).

(B) The detection of unpaired DNA for meiotic silencing was examined using the *Roundspore* assay, in which the *Rsp*⁺ alleles can be silenced *in trans* by siRNAs expressed from the Reporter segment. Ascospores were imaged, segmented and automatically classified based on their eccentricity values.

Figure 2. The possible role of C-DNA in direct homologous dsDNA-dsDNA pairing.

(A) B- and C-DNA models are based on X-ray fiber diffraction and solid state NMR data [51,99,100]. Rigid double helices with juxtaposed major grooves were superimposed by interactive manual docking with restraints to inter-helix hydrogen bonds of one central quartet according to the quadruplex model [49]. The atom-atom clashes were avoided by imposing a minimal inter-phosphate separation of 5.5 Å.

(B) The orientation of bases in the central quartet of the theoretically predicted quadruplex [49] (left) versus that between docked C-DNAs (right).

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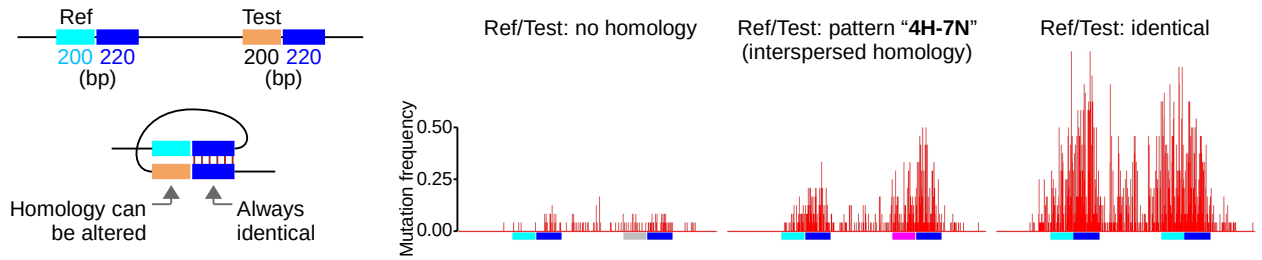
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A "Pairing propagation" assay for testing homology requirements of RIP

Two closely-positioned direct repeats can be extended by changing the sequence of "Test"



B Roundspore silencing assay for testing homology requirements of MSUD

siRNAs expressed from unpaired "Reporter" can silence *Rsp+* alleles *in trans*, resulting in ascospores that are less eccentric

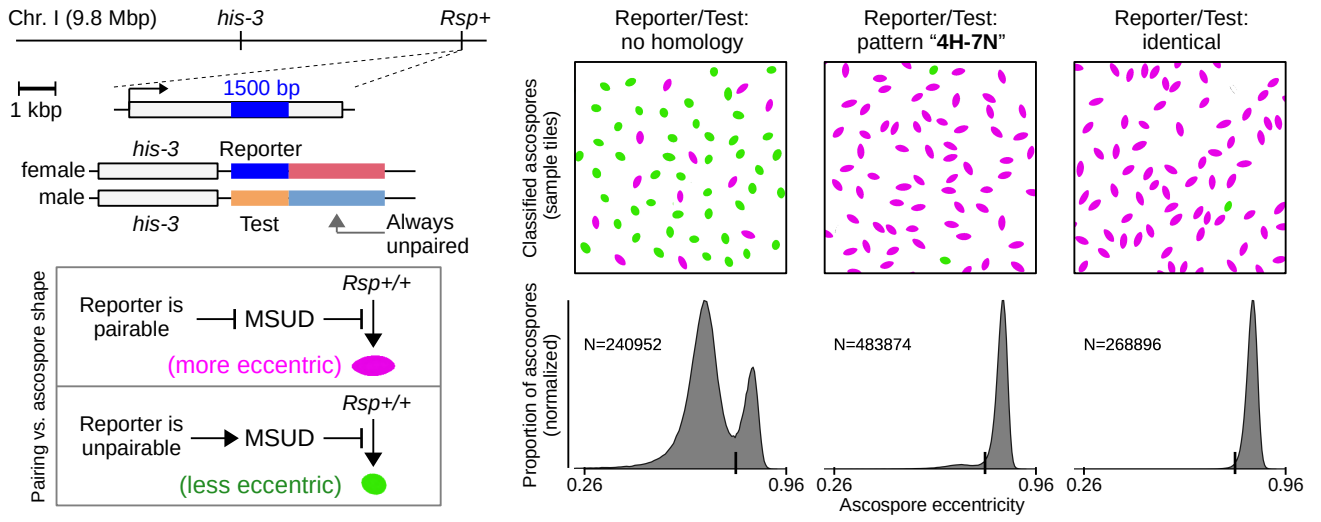


Figure 1

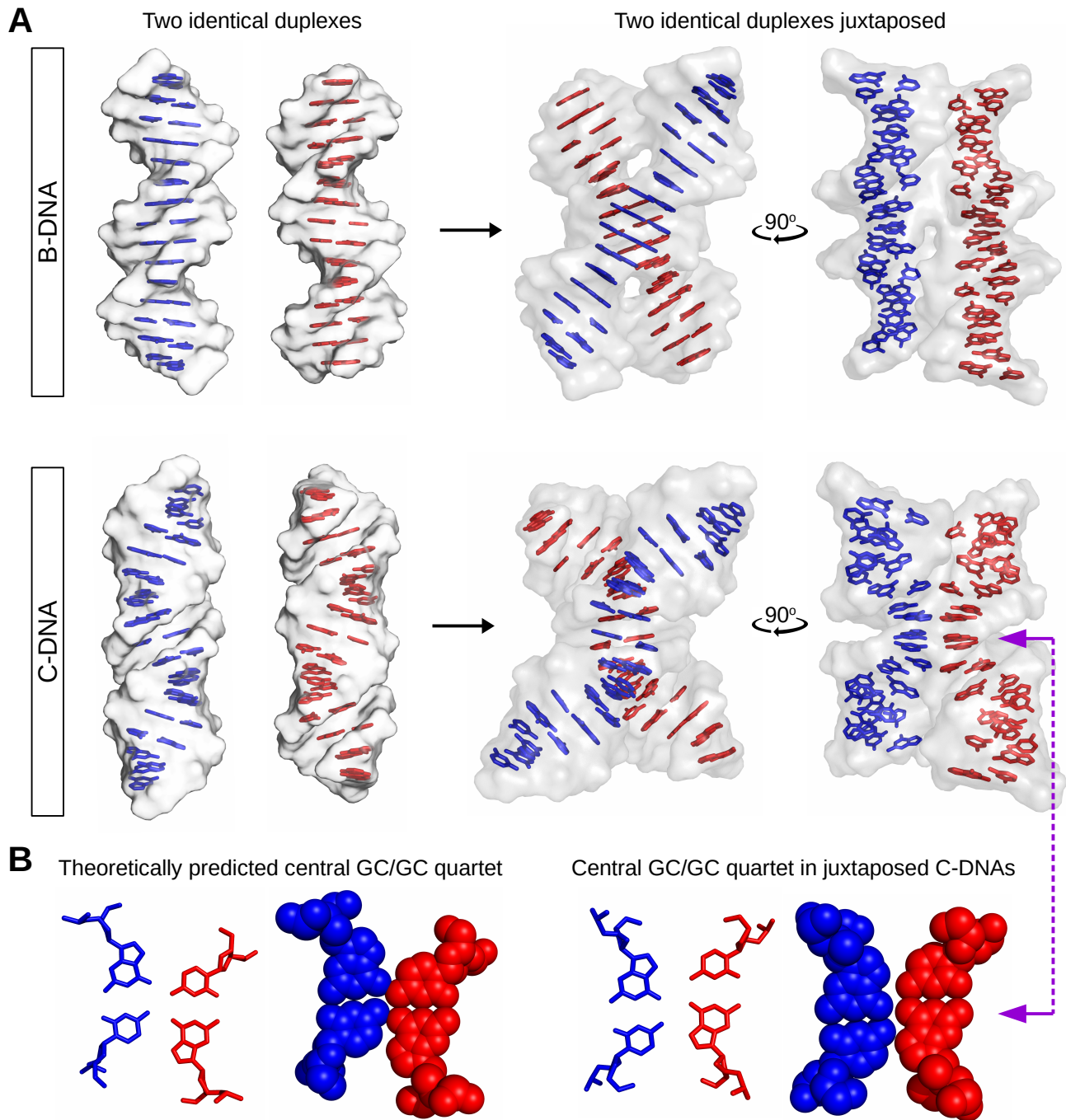


Figure 2