Recent progress in double helix conjecture

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July 15, 2018

Abstract

It is generally assumed that DNA structure has been solved by Watson-Crick in 1953. However, the finding of zero linking number topoisomer indicated that the winding direction inside the double helix should be ambidextrous, rather than plectonemic. Hence, a double helix conjecture was proposed that in any kind of plasmid a zero linking number topoisomer, i.e., a non-linked plasmid，could be found. By denaturing and renaturing plasmid in various different ways, the topological transformation of the DNA was observed by agarose gel electrophoresis. We found that the two strands of a covalently closed circular DNA molecule can be completely dissociated under very mild conditions and this dissociation is reversible. The experimental phenomena indicated that two strands of DNA are unlikely winding right-handedly as in the canonical double helix model. It paves the way for the demonstration of the double helix conjecture which would provide solid evidence to amend the Watson-Crick Model.

Keywords: *Ambidextrous double helix: DNA replication; left-handed DNA; figure 8 structure; homeobox; double bi-helix*

Abbreviations: *DNA I, supercoiled DNA; DNA II, nicked DNA; DNA III, linear DNA; DNA IV, denatured DNA; ssc DNA, single stranded circular DNA; ssl DNA, single stranded linear DNA; EthBr, ethidium bromide.*

Introduction

The finding of the double helix [1] has been generally marked as the beginning of molecular biology. Although the original paper did not provide enough experimental data or new evidence, their suggested DNA molecular structure with the capacity of self-replication aroused great interest and curiosity. Many scientists rushed into the field of DNA investigation and lots afterwards evidences confirmed that most features of the double helix can be verified, i.e., the two complementary strands are coiled around each other in opposite directions with 10 base pairs per turn and they are held by hydrogen bonds between paired bases. The only exception is about the winding direction inside the double helix which is very difficult to get solid evidence even today.

It is clear that the model building of the double helix is based on the knowledge of chemical structure, base pairing, DNA fiber X-ray diffraction and the progress in biochemistry and biology at that time.

Alexander Rich once stated: "fiber diffraction could not 'prove' a structure" [2]. The finding of Z- DNA indicated that the two strands could wind left-handedly [3]. However, Z-DNA requires alternative purine and pyrimidine sequence; hence it cannot always be found in native DNA.

It is well documented that at each replication fork, the newly synthesized DNA advances at 1 k bp per second. If the double helix is really a right-handed DNA that would need the parental strand unwind at the rate of 100 rps or 6,000 rpm. Common sense tells us that it is almost impossible since the friction and viscosity inside the cell is a formidable handicap to keep extremely slender strands of DNA intact during this high speed unwinding. Besides, this supposed quick unwinding has never been proved by experiment and nobody knows if it really exists.

Nevertheless, common sense is not always reliable. It contributed many unscientific concepts, such as geocentric dogma from the observation of sun rising from the east, or spontaneous generation theory from the fact of maggots produced in rotten meat**.** Science needs reliable solid evidence that should be reproducible under strictly defined conditions. Scientific knowledge has to be objective, independent, universal and provisional. The topological difficulties encountered in DNA replication leads the proposal of a hypothesis that the two strands of DNA could be wound ambidextrously [4, 5]. And the hypothesis was supported by the finding of zero linking number topoisomer [6, 7].

According to the Watson-Crick Model, the two strands are always winding right-handedly with 10 base pairs per turn; so the linking number of any plasmid molecule, a covalently close circular DNA containing several thousands of base pairs,

must be a very big integer and cannot be zero. Apparently the finding of a zero linking number topoisomer breaks the traditional doctrine on the secondary structure of DNA.

It is widely accepted that all experimental sciences including physics, chemistry and biology, evidence is the foundation of any theory. Physicist Samuel C. C. Ting pointed out in his talk that if there is a conflict between theory and experiment, theory must be wrong. Hence, experiment can disprove or overturn a theory, but not the vice versa.

Evidently, the ambidextrous DNA model is greatly different from that of the Watson-Crick Model. In order to distinguish which model is closer to the truth, a double helix conjecture was proposed [7]. According to the ambidextrous double helix model, a non-linked plasmid molecule could be experimentally proved by the separation of its two ssc DNAs. Such kind of evidence is actually asked by Crick et al. [8] for a sufficient demonstration of the presence of alternative double helix structure**.** Once it was proved, that may provide a clear, sufficient and significant evidence for anyone who is not familiar with the DNA topology to understand the meaning of the ambidextrous double helix.

The first step of proving the double helix conjuncture is to denature the covalently closed circular DNA in such a way that is mild enough as to not cause any nicking or breakage of the back bone in the whole plasmid molecule and strong enough to destroy all the hydrogen bonds between the two complementary strands. According the Watson-Crick Model that the two circular strands are always tightly tangled with each other, this job is theoretically unreasonable and practically impossible. Whereas, according to our hypothesis, it is not only possible but also practically achievable.

Hence, the double helix conjecture, i.e., the speculation of the presence of a special plasmid with equal number of opposite winding strands inside the same covalently closed circular DNA molecule, raised a challenging question about the secondary structure of DNA. It serves as a touchstone to test which model is closer to the truth and calls people to prove or disprove its accuracy.

Someone may ask: why is it important to prove or disprove the double helix conjecture? The answer is that DNA is one of the most important macromolecules in biology and it closely related to many biological functions including growth, mutation, development, evolution, etc. Although it has been investigated in many laboratories by lots scientists all over the world for many years, many questions are still unanswered. Just as nobody predicted the outcome of the double helix when it was found in 1953, a better understanding of its secondary structure may bring us some unforeseen knowledge and surprising benefit.

The trouble is that the information inside the double helix is rather difficult to obtain. No reliable and trustable tool or instrument can be used to reveal the exact feature of the secondary structure of DNA. Limited by resolution, even the most advanced electron microscope today is still not good enough to help us to "see" the winding directions inside the double helix of a plasmid molecule clearly.

Most of us did not realize that our knowledge learned from textbooks or seminars on the DNA structure is incomplete. Many scientists believe that there is no problem in double helix and their attention is focused on other important problems.

After many years of investigation, some experimental results obtained which are supposed to be small but important progress in proving the double helix conjecture. These experiments seem rather simple but the implication of the results is worth for careful scrutiny and deliberation. This recently gained knowledge may be useful for interested scientist to continue the experimental exploration for the final demonstration of the double helix conjecture.

Material and methods

pBluescript DNA (CPG Biotech # 202205) or pBR322 DNA (New England Biolabs, # N3033S) was prepared with TianGen EndoFree Maxi Plasmid Kit (TIANGEN Biotech # DP117) according to their alkaline lysis protocol from over-night LB plus ampicillin (Beyotime # ST007) culture of a DH5α strain of *Escherichia Coli* (New England Biolabs, # C2 9941) harboring the corresponding plasmid. Finally, the plasmid sample is stored in 0.1 x TE buffer (1mM Tris-HCl / 0.1mM EDTA, pH 8.0) and kept at -20°C.

Relaxed plasmids were prepared from native plasmid by incubating the native DNA over night at 10°C with *E.coli* topoisomerase I (New England Biolabs # M0310S). After the relaxation, the relaxed DNA I' is precipitated with 2 volume of ethanol, washed with 75% ethanol and finally resuspend in 0.1 X TE.

A regular agarose G-10 is purchased from Biowest (WG111860), distributed by Gene Company Ltd. Chloroquine (# PHR1258), EthBr (# E7657-1G), and PEG 8000 (#8910-250G) are all purchased from Sigma-Aldrich. Dr. GenTLE precipitation carrier is purchased from TaKaRa Company (# 9094). 1 kb ladder comes from New England Biolabs (# N 3232L).

As reported before [6], the horizontal slab agarose gel electrophoresis (AGE) is carried at room temperature in TBE buffer (90mM Tris-HCl, 90mM Boric acid, 2.5 mM EDTA pH 8.0) with or without chloroquine indicated in each test. After

AGE, the gel is stained with 1μg EthBr/ml at room temperature 1 hr and washed with dH_2O and recorded with UV image detector (Tanon GIS 2500). The images were inverted with software Tanon MP for better presentation or reprinting.

TAE-Mg buffer contains 40 mM Tris/ 20 mM HAc/ 2mM EDTA/12.5 mM $MgAc_2$.

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plasmid heat stability test for Figure 1: 4 μl supercoiled or relaxed pBluescript DNA add 16 μl dH_2O and 20μ 1.8% NaCl was put in 1.5 ml Eppendorf tubes, adding 20μl paraffin (Sangon Biotech # A6018888) to avoid evaporation. The capped samples were put at boiling water bath for different times and chilled immediately on ice. Spin down the samples to the bottom of each tube and adding loading dye for AGE test

Alkaline denaturing test for Figure 2: 5μl relaxed pBluescript DNA mix with 5μl 0.5 N NaOH at room temperature, then add 30μl formamide together with 5 μl different reagents and treated differently as indicated in the Figure 2 legend. The concentration of PEG8000 is 5% (W/V) in deionized water.

Gentle denaturing test for Figure 3: *First group*: 5μl native pBluscript DNA adding 395μl dH_2O and 100μl paraffin in 6 Eppendorf tubes. Three of them were incubated in 65˚C for 15, 30 and 60 minutes respectively, shifted to 42˚C water bath for 10 minutes and kept at 4˚C 24 hours. The other 3 samples were also treated in the same procedures as the above three samples, the only difference is that after the 65˚C incubation, 40μl of 10x TAE-Mg buffer were added.

Second group: 5μl native pBluscript DNA add 195μl dH_2O, 200μl formamide and 100μl paraffin in 6 Eppendorf tubes. Then do the same operation as the first group.

After 12 samples were all kept at 4˚C 24 hr, the DNA samples were precipitated with 40μl 3M NaOAc, 8μl Dr. GenTLE precipitation carrier and 800 μ 100% ethanol, centrifuged at 4˚C, 12 k rpm for 15 minutes. Decant the supernatant carefully and completely. The invisible pellets were resuspend in 40μl TE, vortex 2 minutes, spin 1minute, adding 3 μl 6 x loading dye before put them into the dry agarose gel well (the TBE buffer is carefully added below the agarose gel surface lever). Turn on the power to let all samples enter into the gel. After 10 minutes add more buffer to cover the gel surface and the AGE is carried at room temperature with electric voltage gradient 2v/cm overnight.

Results

1. **Covalently closed circular DNA is stable at high temperatures.**

It is well known that due to topological constrain, covalently close circular DNA is stable at high temperatures. That was the basis of boiling method of plasmid preparation. As shown in Figure 1. Lane 4, 5 and 6**,** when examined by AGE, native supercoiled pBluescript DNA heating at 100°C in saline solution for 1, 5 or 20 minutes and quickly chilled to 0°C is mainly unchanged.

However, longer heating time may cause considerable denaturing or nicking (figure 1, lane 7). Moving faster than the supercoiled DNA, the denatured DNA IV appeared as a big spot which is supposed to be a set of random coils composed of two tangled ssc DNAs. A small spot is also visible that may be the ssc or ssl DNA.

The relaxed pBluescript DNA in saline after heating treatment appears in a similar way as shown in Figure 1. Lane 9, 10, 11, 12.

2. **Supercoiled or relaxed DNA can be denatured by alkaline and the denatured plasmids can be renatured easily.**

When relaxed pBluescript DNA is denatured in 0.25 N NaOH at room temperature, the hydrogen bonds between the two strands can be destroyed immediately. Since the denaturing process is very fast, the two strands do not have time to separate due to topological constrain, they are actually tangled loosely forming random coils in 0.25N NaOH solution. As soon as these random coils entered into the agarose gel for AGE, the sudden pH change provides renaturing possibility. It is likely that illegitimate intra or inter strand base pairing leads the whole molecule turning into a compact entity. That is why it moves faster than supercoiled DNA in the electric field as shown in Figure 2 lane 5.

It was found that these alkaline denatured pBluescript DNA can be renatured in the presence of 66% formamide at RT 10 hr. as shown in Figure 2, lane 6. The reason is probably due to the competing hydrogen bonding of formamide with nucleotide bases and less alkaline strength after the addition of formamide. In formamide solution, the ssc DNA become more rigid and stiff that has been proved by the formamide–basic protein technique for EM sample preparation [9]. It leads the two ssc DNAs situated as a pair of loosely linked circles (in three dimensions). Once entered into the agarose gel during AGE, the sudden pH change makes the perfect annealing of the original complementary strands possible. Since the two circles unlikely have relative axial rotating movement during alkaline denaturing, their renaturation is supposed to be

easier than that of the annealing process in PCR reactions. Because the topological constrain keeps the two ssc DNA always situated nearby, their annealing is easy and reasonable.

An interesting fact is that the renaturing of those denatured plasmids in formamide can be prevented effectively by shaking as shown in Figure 2, lane 7. When this disturbing factor is removed, the renaturation can be resumed as shown in Figure 2, lane 8.

It is also found that when the solution contains long molecules of PEG 8000, the denatured two complementary ssc DNA would unable to anneal as shown in Figure 2, lane 9.

3. **Supercoiled plasmid DNA can be completely denatured in low salt aqueous solution at appropriate temperatures.**

An interesting fact was found that the same plasmid in low salt solution behaves strangely. As shown in Figure 3 native supercoiled pBluescript DNA is almost invisible after heating in 0.001 x TE buffer at 65°C for 15, 30 or 60 minutes. (Lane 4, 5, 6).

The questions are — where the DNA goes and why the supercoiled DNA can be denatured at 65°C in 0.001 x TE buffer?

Since the plasmid is stable at 100°C, it is unlikely that the backbones of the plasmid are destroyed by heating in low salt solution at 65°C within 60 minutes. Presumably, the denatured plasmid turns into two ssc DNAs, practically a pair of random coils loosely tangled with each other that is inseparable due to the topological constrain. Since EthBr stained single-strand DNA is much less sensitive to be detected under UV than that of the double stranded DNA, and the tangled random coils may form numerous different forms spreading along the track of electric field, their disappearing to the naked eyes is plausible.

In the presence of annealing buffer, some denatured DNAs are reversible and can be detected at their original locations on the agarose gel (Figure 3, lane 10, 11)

However, another strange phenomenon is that the amount of the renatured DNA is closely related to the heating time. The plasmid that has been treated by heating at 65°C 15 minutes recovered the most (Figure 3, lane 10).. The plasmid that has been heated at 65°C for 30 minutes recovered less (Figure 3, lane 11). Whereas, most of the supercoiled DNA that has

been heated in 0.001 x TE solution at 65°C 60 minutes cannot retain their original form (Figure 3, lane 12). This experimental fact worries us very much. At first, we didn't understand why some random coils cannot renature in the presence of TAE-Mg buffer, which is supposed to be a good annealing buffer allowing many different pairs of complementary ssl DNAs annealed correctly in a same solution. [10]

We guess, this test indicates that these invisible denatured DNAs, treated in 0.001 x TE with different heating times are indeed different. Although they are apparently similar, composed of two tangled ssc DNAs, the way of the tangling is actually different. The two strands of denatured DNA with shorter heating treatment may tangled less complex or the two complementary ssc DNAs may deviated not far away from each other, so the two strands could found their partner easily when renaturing condition (at 42 °C 10 minutes than 4°C 24 hr with salt) is available. A reasonable deduction is that the two strands of denatured DNA with longer heating treatment are tangled in some complicated way or the two ssc DNA deviated far away causing them unable to renature under similar annealing conditions.

Such reason can also be used to explain the result of Figure 3, lane 7, 8, 9. It clearly shows that when supercoiled pBluescript DNA in 67 % formamide and 0.001 x TE at 65°C for different time can be denatured differently. Whereas, these differently denatured plasmids do not have same chance to renature until entering the agarose gel. It is possible that the less tangled single strands are less free to move around to anneal after entered the agarose gel. Probably, totally denatured plasmid in 67% formamide can be renatured in the agarose gel during electrophoresis. It explains why the renatured plasmid in lane 7 is less than that of lane 8 or lane 9.

It has been known for long time that NaCl stabilizes hydrogen bond [11]. The function of cation ions is to neutralize the negatively charged phosphate atoms between complementary strands. The shielding effect of cation ions could be reduced by lowering the salt concentration which may cause the repelling of two complementary strands. A remarkable presentation was reported by D. I. Cherny & T. M. Jovin [12]. They found supercoiled pPGM1 DNA in TE buffer can turn into apparently relaxed form with only one or two notes, i.e., the crossing number between different DNA locations of a plasmid molecule appeared on two dimensional EM picture. Some molecules even appeared as an ordinary ring carrying a loop that is formed from two single stranded DNA with the size of 200-300 nucleotides.

It was also found that the lower the temperature, longer time is needed for the complete dissociation of the plasmid in aqueous solution with low salt. For example, the same plasmid in 0.005 x TH buffer was incubated at 55˚C or 37˚C, when checked by AGE, the disappearing of the supercoiled DNA needs 4hr or 5 days respectively (refer to data).

It is likely that the hydrogen bonds between the two complementary strands of DNA can be very slowly dissociated at 10°C in pure water. It was happened that after several months of storage in 10°C refrigerator, our plasmid preparations in pure water was undetectable by AGE. That incident reminds us to avoid keeping plasmid preparations in pure water.

Discussion

A topological problem is involved in the double helix, which was quickly noticed and pointed out by Watson and Crick themselves shortly after their first paper [13]. It is noteworthy to point out that in a letter to Watson, Max Dalbrück wrote: *"I am willing to bet that the plectonemic coiling of the chains in your structure is radically wrong, because (1) the difficulties of untangling the chains do seem, after all, insuperable to me. (2) The X-ray data suggest only coiling but not specifically your kind of coiling.* " [14]

The argument around the winding direction inside the double helix is a long story. Trying to answer the topological questions around the double helix, Crick et al. published their review in 1979 and the side-by-side model proposed by Rodley and others [15] was refuted with the evidences available at that time. They wisely proposed a reservation, i.e., if a dramatic experimental demonstration is provided that the two circular strands of a plasmid can be physically separated, that may be strong enough to admit the presence of an alternative double helix. They suggested that that should and could be done by raising the temperature until the structure denatured.

Perhaps someone has tried the way suggested by Crick et al. but has never succeeded or has never been reported.

While searching the literature, we noticed that some facts had indirectly revealed the coexistence of bi-directional winding in DNA strand or DNA-RNA hybrid strand. For some reason, the authors of those papers did not aware or pointed it out. For example:

a) The two strands of λ DNA can be progressively separated by single strand DNA binding protein [16].

b) The λ DNA can be stretched to twice its normal length [17].

c) The kinetics chart of the T7 DNA sedimentation coefficient has a sudden s value drop at 54°C [18].

d) The exon region between the loops of introns on the EM pictures has to have oppositely winding DNA-RNA hybrid stretches [19].

We designed several experiments and found it is true that right-handed DNA and left-handed DNA do coexisted in the same molecule. These tests are:

e) The two strands of relaxed pBR322 can be dissociated and the two ssc DNA are loosely linked [6, 7].

f) The two complementary pBluescript ssc DNA can be associated and form a regular plasmid [6, 7].

g) The two strands of singly nicked plasmid can be quickly separated by alkaline [6, 7].

h) After inserted into two phagemid molecules, the two complementary 2kb λ Hind III ssl DNAs can anneal and form a 2kb double strand in a figure 8 structure [6, 7].

All these evidence are consistent with each other and cannot be explained by the Watson-Crick Model. They construct a chain of evidence showing the two strands of DNA are winding ambidextrously.

Notably, ambidextrous double helix is somewhat similar to the so called "side-by side" model; actually, they are somehow different. Differing from the "side-by-side model" [15]; the ambidextrous double helix model does not suppose the DNA has a repeated structure of "5 base pairs right-turn and 5 base pair left turn". It only emphases the winding direction inside the double helix is polymorphous and bidirectional, rather than plectonemic. Perhaps it is appropriate to give this model a new name, **double bi-helix,** to distinguish it from that of the side-by-side model and the Watson-Crick Model.

It seems necessary to introduce super helical index Si = W / (N /10.4) to substitute the super helical density σ for further discussion. The reason is that according to traditional double helix model, $\sigma = (L - L_0) / L_0$, there is no problem since it is assumed that L_0 is the linking number of the relaxed plasmid with N base pairs, i.e., $L_0 \approx N /10.4$. Whereas, in the double bi-helix model, the linking number of relaxed plasmid could be zero; it is no more reasonable to put a zero as denominator. On the other hand, Si is related only to two experimentally measurable data, i.e., W and N. Si becomes an objective datum unrelated to any theory or hypothesis. Therefore, Si can be used as an index to indicate and compare the supercoiling of different plasmid molecules in any DNA model. The reported σ value can still be used as Si value.

The Si can also be assumed as the extra turning numbers of a plasmid in such a way that in average 1 extra turning generated from every 10.4 bp is equivalent to 1 Si unit; in other words, one extra turning generated from 1040 bp is equivalent to 0.01 Si unit.

Based on canonical double helix model, the length of DNA is 3.4Å / 10 bp or about 1μm / 3kbp DNA and the σ of native plasmid is around -0.05 [11]. So, it means that in most native plasmid, the DNA lost about 5 turnings per 1kb (5 turns / 1kb). According to the Watson-Crick Model, it means every 1kb of native plasmid molecule has 95 right-handed turns (95 turns/1kb) or 95 turns / 0.33 μm. Whereas, according to the bi-helix model, every 1kb of native plasmid has 5 left handed net turns, (5 net turns/1kb) or 5 net turns/0.33 μm. The big differences on the expected average value of net turns per 1kb between two models should be reflected by plasmid denaturation tests.

According to the Watson-Crick Model, the linking number of a 3 kb relaxed plasmid should be around 300, i.e., its two strands coil around each other 300 times. When all the hydrogen bonds were destroyed, the two strands could not be separated. The difference between relaxed (Lk=300) or supercoiled DNA (Lk=285) should be negligible to the final appearance of random coil, since the two strands should keep L = T + W = 300 or 285, denaturation would turn them into a compact entity.

Plasmids denatured by alkaline was originally studied more than 50 years ago with ultracentrifugation [20, 21]. The sedimentation coefficient of alkaline denatured plasmid is much higher than that of native plasmid. It means the alkaline denatured plasmid (DNA IV) in solution is a very compact entity greatly different from that of their native counterparts (DNA I). Their result is consistent to the AGE tests for denatured plasmid (DNA IV)) as shown in Figure 2.

An interesting result of denatured plasmid with different supercoiling was reported earlier (Figure 7) [7]. When artificially prepared pBR322 DNA with higher negative supercoiling was denatured by alkaline and examined by AGE, the denatured samples appear migrating slower in the same electric field.

A notable phenomenon is that when extremely highly supercoiled plasmid ($W \geq 50$, $Si \geq 0.2$) was denatured in 0.25 N NaOH, some plasmids are unable to be denatured as shown in (Figure 7, lane 14, 15) [7]. The reason is that the two strands are so tightly tangled with each other that there is no space allowing the denatured two single strands to depart away enough distance.

This phenomenon can easily be explained by bi-helix model. In a hyper-supercoiled plasmid, there are more than 20 turns per 1040 bp at $|Si| \geq 0.2$. It is getting more difficult to let the two single strands (out of 0.33μm DNA) depart far more than 2 pm (the dimeter of double strand) and kept 20 times inter winding as shown in oval circle of Figure 4.

Whereas, according to the Watson-Crick Model, the more negatively supercoiled, the plasmid would have fewer turnings between the two strands and should easier be denatured, this is inconsistent with the experimental result. (Figure 7, lane 14, 15)[7]

Additionally, a thought test has suggested that it is not true that highest negatively supercoiled DNA could reach to a point leading the non-linkage of its two ssc DNAs [22].

On the other hand, if the two strands of a plasmid are always tightly tangled with each other as Watson-Crick Model suggested, it would be very difficult to know why the plasmid is undetectable in low salt solution above room temperatures.

When denaturing is proceeded slowly and gently it is easy to understand even the supercoiled DNA could be invisible on the agarose gel as shown in Figure 3, lane 4, 5, 6. Because the two strands are not tightly tangled with each other, the hydrogen bonds between the two strands can be removed step by step to form numerous different intermediates. The result is that when all these partially denatured plasmids were checked by AGE, they are dispersed in the agarose gel and cannot be detected. However, this phenomenon cannot be explained by traditional double helix model.

The fact that those denatured plasmids (Figure 3, lane 4, 5, 6) can be renatured differently as shown in Figure 3, lane 10, 11, provides additional clue that they should be somehow different. Without the help of formamide, the slightly denatured single strands can be renatured in the presence of salt. The completely denatured single strands would be more difficult to find its partner, and the result is that the annealing product is almost invisible under the same experimental conditions, as shown in Figure 3, lane 12.

Comparing the result of Figure 3, lane 12 and lane 15, the addition of formamide is helpful for the renaturing of those denatured ssc DNAs in the presence of salt. The reason is that formamide helps the ssc DNAs to be in more extended state that makes the two complementary strands closer, and leading the annealing of them easier.

It can also explain the EM pictures [12] observed on supercoiled plasmid pPGM1 that most of the plasmid turns into apparently relaxed form and some of it carries single stranded 200-300 nucleotides loop as shown in a schematic drawing of Figure 4 with red star. It provides additional evidence indicating what the shape a denatured supercoiled plasmid could be at the beginning of dissociation.

Whereas, if plasmid is quickly denatured by alkaline, even the two strands of relaxed plasmid could not be separated as shown in Figure 2 lane 5. Our explanation is that due to sudden pH change, the hydrogen bonds were destroyed immediately, the two complementary strands of the left-handed or right-handed DNA start to reject its partner and adjust their positions, but they have to follow the topological law keeping the linking number unchanged. However, in the alkaline solution, different parts of molecule are relatively independent; the closest neighboring oppositely winding parts can be greatly affected by this denaturing process. As soon as the linking number is unchanged, the alkaline denatured plasmid would be appeared as a random coil composed of two ssc DNA with partially left-handed and right-handed regions (not the same as hydrogen bonded double stranded DNA). When this random coil enters the agarose gel during AGE, the illegitimate base pairing between inter or intra strands would lead the formation of a rigid entity appeared as shown in Figure 2, lane 5. However, if the alkaline denatured plasmid is placed in 67% formamide, the situation would be changed (due to the competing hydrogen bonding of formamide with base pairs and lowering of alkaline concentration), the two ssc DNAs become rigid and stiff. It makes the annealing of the two originally complementary strands easy as shown in Figure 2, lane 6.

An interesting phenomenon is that when the denatured plasmid was kept shaking, the annealing is failed. It implied that the two strands of relaxed plasmid, after destroying all hydrogen bonds, still have enough space to move around and cause the annealing impossible, as shown in Figure 2, lane 8.

The addition of long molecules of PEG 8000 forbids the annealing of denatured plasmid as shown in figure 2, lane 9. Apparently, the long molecules of PEG 8000 have inserted into the space between the denatured strands and block the annealing of those complementary single-stranded DNAs. It is a comprehensible that the space between the two ssc DNA has to have enough space allowing the insertion of long PEG 8000 molecules. It is unlikely if the two ssc DNAs are tightly tangled with each other (as required by Watson-Crick Model) could provide such a chance for the insertion of PEG 8000（Refer to black circle of Figure 4）.

Although these tests reported here do not provide strong evidence on the winding directions inside the double helix, the various experimental results do provide some information that is favorable to the double bi-helix model. Those observed phenomena cannot be interpreted by the Watson-Crick Model, especially the disappearing of the supercoiled plasmid.

Scientists are easily convinced by the result of x-ray crystal analysis. However extrapolating the reliable result obtained from a small fragment of DNA to long strands of a plasmid may cause unreliable conclusion.

It may not important to know who get the escrowed $ 5 bet for a 1953 argument on how purely right-handed double helix is untangled between Crick and Delbrück. However, what matters is what the structure of DNA really is.

The finding of zero linking number topoisomer is a disproof of the plectonemic double helix, and a solid disproof of it is enough to refute the right-handed double helix! The power of disproof was clearly pointed out by psychologist Mihaly Csikszentmihalyi [23]:" *What I try to do occasionally is to disprove certain widespread assumptions. The advantage of disproof over proof in science is that whereas a single case can disprove a generalization, even all the cases in the world are not enough for a conclusive positive proof. If I could find just one white rave, that would be enough to disprove the statement: 'All ravens are black.' But I can point at millions of black ravens without confirming the statement that all ravens are black."* That was the claim of our previous articles and it is the starting point of this article.

In contrast to the evidence of x-ray crystal analysis from oligonucleotides, the combination of experimental result and topological knowledge can let us know the general feature of the long circular plasmid DNA, rather than a small section of DNA. The two results obtained from two different methods are not contradictory to each other, but synergistically contributed to a better understanding of the DNA with both macroscopic and microscopic images [3, 6, 7, 13]. Just as Einstein once pointed out: *"Creating a new theory is not like destroying an old barn and erecting a skyscraper in its place. It is rather like climbing a mountain, gaining new and wider views, discovering unexpected connections between our starting point and its rich environment. But the point from which we started out still exists and can be seen, although it appears smaller and forms a tiny part of our broad view gained by the mastery of the obstacles on our adventurous way up."* [24]

We are confident that the ambidextrous double helix is closer to the fact. Our dream is that when the double helix conjecture is proved, the test itself could be a classic textbook example for future students to understand what double helix really is. It is somehow like playing a Chinese linking ring magic with plasmid DNA. Different from the tricks of any

magician using shining metal rings, the zero linking number topoisomer has no nick and it can be performed by any student!

Nowadays, many people believe the finding of topoisomerases can solve the unwinding problems in DNA replication. After many years of investigation, the complicated mechanism of DNA replication in *E. coli* has been elucidated clearly [25]. Presently, there are many videos on the internet showing how various components and steps are working together to accomplish the semi-conservative bi-directional replication. Unfortunately, all of these videos have a same defect or unanswered question: how one topoisomerase and one helicase sitting at the front of each replicon can open the tightly winded double helix at the rate of 6000 rpm.

It is reported that the mechanism of gyrase, the cardinal enzyme in unwinding the double helix, is rather complicated [26]. Unlike many fast catalytic enzymes, the reaction rate of gyrase is only 6 times per minute [27]. Besides, the helicase is completely unable to break and reunion the DNA. Obviously, the DNA replication rate is much higher than that of gyrase can cope with. This dilemma seems can easily be solved by the bi- helix model.

Likewise, the bi-helix model can be used to solve the topological difficulties happened in epigenesis. Since DNA carries the message of all living creatures, there is no fundamental difference between prokaryotes and eukaryotes. It is likely the knowledge obtained from plasmid DNA is applicable to eukaryotes. It is well known that each organ cell comes from the same embryo cell with the same set of chromosome. Different organ cells differ greatly in shape, location and function. How the genetic message hidden in the chromatin can be recognized by similar homeobox products at special time and location[28] remains unanswered. According to the double helix model, the mechanism must be very complicated [29]. Whereas if the DNA is in the bi-helix model, that would make the situation much simpler.

Conclusion

Above room temperature the hydrogen bonds between the complementary strands of covalently closed circular DNA can be gently dissociated in aqueous solutions with very low salt or pure water. Higher temperature favors this dissociation. The experimental phenomena revealed that two strands of DNA are unlikely always winding right-handedly as in the canonical double helix Model. It paves the way for the demonstration of the double helix conjecture which would provide solid evidence to amend the Watson-Crick Model.

Acknowledgements

Thanks to Professor Q. L. Li for his critical reading and suggestion, to J. Q. Hu, A. M. Ni and J. F. Gu for their help in experiment preparation. This work is partially supported by the National Natural Science Fund (31671348, 31301064 and 8137245

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Fig-1

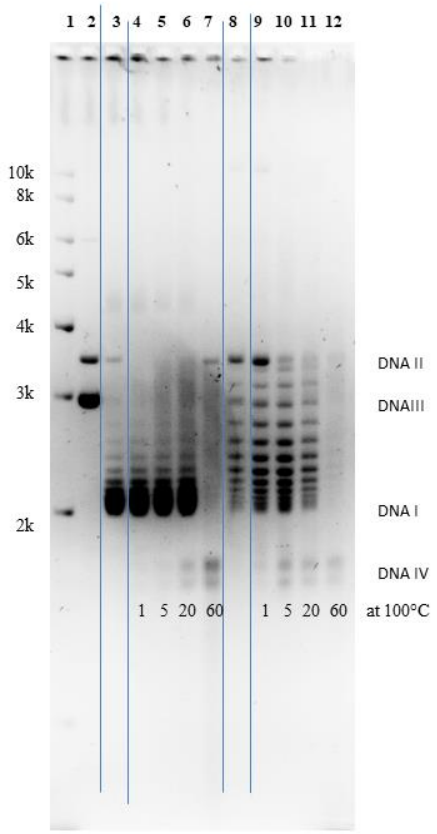

Figure 1. ***Supercoiled or relaxed pBluescript DNA heat stability test*** *(AGE with 1% agarose in TBE buffer is carried at RT with 2v/cm)*
Lane 1. 1 kb DNA Marker;
Lane.2. linear and nicked DNA;
Lane 3. 1.7 µg of supercoiled DNA in 40 µl saline solution add 20 µl paraffin; keep in 1.5 ml Eppendorf tube.
Lane 4, 5, 6, 7. Same sample as in lane 3 are heating at 100°C for 1, 5, 20 or 60 minutes respectively and quickly chilled at 0°C.
Lane 8. 2.0 µg relaxed pBluescript DNA in 40µl saline solution, add 20 µl paraffin, keep in 1.5 ml Eppendorf tube. (Relaxed pBluscript DNA is prepared from native plasmids with topoisomerase 1 in appropriate provided by New England biolabs at 10°C,)
Lane 9, 10, 11, 12 same sample as in lane 8 are heating at 100°C for 1, 5, 20 or 60 minutes respectively and quickly chilled at 0°C.

Figure 2

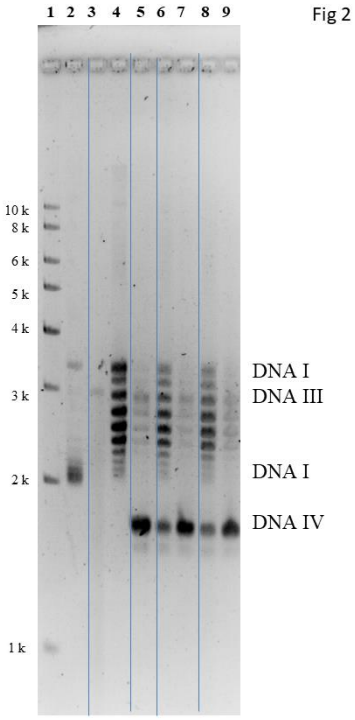

Figure 2. ***Denaturing and renaturing relaxed pBluescript DNA***
(AGE in 1% agarose is carried out in TBE buffer with 1μg chloroquine/ml at RT with 5v/cm.)
Lane 1. 1 kb DNA Marker;
Lane. 2. Supercoiled pBluscript DNA;
Lane 3. linear pBluscript DNA;
Lane 4. 160ng/μl relaxed pBluescript DNA in 5 μl;
Lane 5. Same sample as lane 4, add 5μl 25 N NaOH and immediately loaded into the well just before doing AGE;
Lane 6. Same sample as that of lane 5, adding 20μl formamide and was stay at RT for 10 hr;
Lane 7, The same sample as that in lane 6 was kept in a shaker shaking at the rate of 150 rpm at RT for 9 hr.
Lane 8. The same sample as that of lane7, after shaking 9hr was further incubated at 42°C for 1 hr.
Lane 9. The same sample as that of lane 8, but with addition of 5μl PEG 8000 before shaking, after 9 hr shaking, further incubated at 42°C for 1 hr.

Figure 3

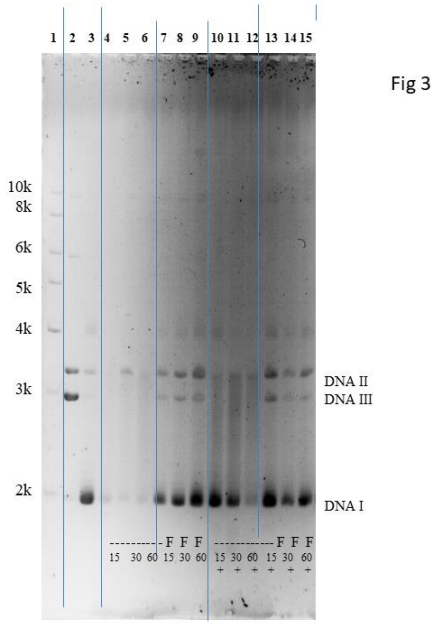

Figure 3. ***Denaturing and renaturing supercoiled pBluescript DNA*** *(AGE in 1% agarose is carried out in TBE buffer with 1μg chloroquine/ml at RT with 2v/cm.)*
Lane 1, 1kb DNA Marker;
Lane 2. Nicked and linear DNA;
Lane 3. 2.2μg supercoiled DNA in 5 μl 0.1 X TE;
Lane 4, same sample as in lane 3, add 395μl dH_2O and 100μl paraffin, heating at 65°C for 15 minutes, then keep at 42°C for 10 minutes and 4°C 24 hr;
Lane 5 and lane 6. Same treatment as lane 4, but heating at 65°C for 30 and 60 minutes respectively; then keep at 42°C for 10 minutes and 4°C 24 hr;
Lane 7, The sample is the same as in lane 3, add 195μl dH_2O and 200μl formamide and 100μl paraffin and heating at 65°C for 15 minutes, then keep at 42°C for 10 minutes and 4°C for24 hr;
Lane 8 and lane 9. Same treatment as in lane 7, but the heating at 65°C for 30 and 60 minutes respectively; then keep at 42°C for 10 minutes and 4°C 24 hr;
Lane 10, 11, 12. 13, 14, 15 are same as that of lane 4, 5, 6, 7, 8, 9, after 65°C heating, add 40 μl 10 x TAE-Mg buffer and then 42°C 10 minutes and 4°C 24 hr
After 4°C 24 hr, all the samples were precipitated as described in material and methods.

Figure 4

30.

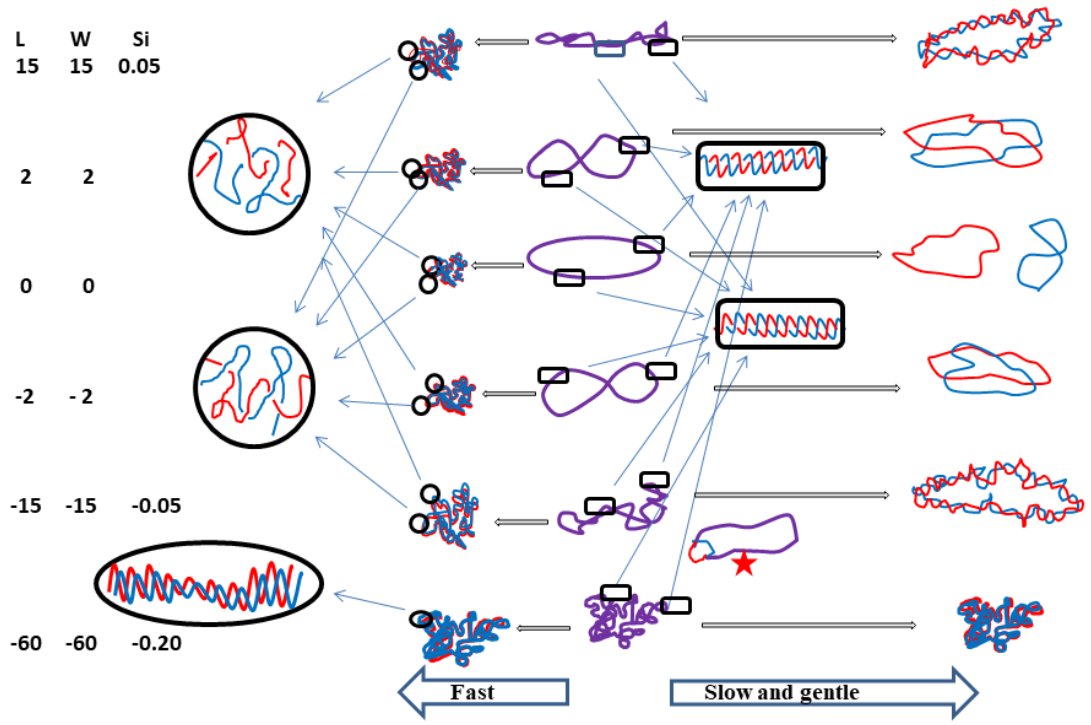

Figure 4. Schematic possible shapes of differently supercoiled plasmid before and after denaturing.

Appendices

It was stated above in the article that the lower the temperature, the longer time needed for the complete dissociation of the plasmid in aqueous solution with low salt. That is supported with the observation of two test results (2µl supercoiled pBluescript in 0.1 x TE mixed with 38µl dH_2O and incubated at indicated temperatures for different times) as shown in two presentations bellow: