Chem Rep, 2025, 5(1): 298-307
DOI: 10.25082/CR.2024.01.005

RESEARCH ARTICLE

An Easy Method to Non-Destructively Separate the Strands of a Circular Duplex Plasmid Chromosome: A Preliminary Report

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Received: May 30, 2025;
Accepted: July 15, 2025;
Published: July 25, 2025.

Citation: Biegeleisen K. An Easy Method to Non-Destructively Separate the Strands of a Circular Duplex Plasmid Chromosome: A Preliminary Report. *Chem Rep*, 2025, **5**(1): 298-307.
https://doi.org/10.25082/CR.2024.01.005

Abstract: A straightforward and cost-effective method has been developed for the non-destructive separation of the two strands of a circular duplex plasmid chromosome. This method was adapted from the original protocol reported by Wu and Wu (1996), who achieved strand separation using prolonged low-current electrophoresis. In contrast, the method described here utilizes a two-step denaturation process: initial alkali denaturation to pH 13 to generate "Form IV" DNA, followed by denaturation in 90% formamide at 80°C under low-salt conditions. The resulting single strands were observed to migrate as two discrete bands on agarose gel electrophoresis, indicating successful strand separation without degradation. This approach offers a reproducible, rapid, and inexpensive alternative for investigating the topology and helicity of circular DNA molecules.

Keywords: DNA topology, plasmid, Form IV, strand separation, non-helical DNA, circular DNA

1 Introduction

A biological theory posits that the structure of DNA within living chromosomes has never been definitively determined, despite existing substantial support for this notion. This theory, however, has been overlooked for many decades. The Watson-Crick double-helix structure of DNA was determined using X-ray crystallography. However, this method cannot be applied directly to chromosomes. To perform X-ray crystallography on DNA, several preparatory steps are necessary:

The chromosome must be removed from the cell nucleus, which results in its fragmentation into numerous pieces, thereby destroying any aspect of DNA structure that requires chromosomal integrity. Histones or protamines, which provide numerous positive charges that interact with the negative charges on DNA phosphate groups, must be removed. The proposition that the removal of these proteins does not affect the DNA structure is implausible. Most of the water must be removed from the DNA, as crystals cannot form under the high humidity conditions within the cell nucleus. Given these limitations, it is presumed that fully intact, unperturbed chromosomal DNA adopts the double-helix structure. However, since the publication of the Watson and Crick Nature article in 1953, only two studies have specifically addressed the question of helicity in native, unperturbed chromosomes: Stettler et al. (1979) [1] and Crick et al. (1979) [2]. The Stettler study reported an uncontrolled experiment and drew conclusions that were not supported by the presented data. The Crick publication was a review paper that argued for the Watson-Crick helical structure in topoisomerase experiment bands but did not discuss the topology of the native structure prior to topoisomerase treatment, which was presumed a priori to be helical without justification.

The only reliable evidence for topological net helicity in unperturbed living systems is the difficulty in non-destructively separating the strands of small circular plasmid or viral chromosomes. Notably, both non-destructive separation and reannealing of small circular DNA have been reported [3,4], yet these findings have been largely ignored by the molecular biology community.

Further evidence against helicity in unperturbed chromosomal DNA is the inability to solve the structure of the complex between protamine and DNA in sperm cells if DNA is presumed helical. To date, only one published atomic model for the protamine-DNA complex exists,

which was obtained by starting with a non-helical DNA structure [5]. The molecular biology establishment has not proposed a hypothesis for protamine structure under the assumption of helical DNA due to modeling impossibilities.

In summary, despite the widespread acceptance of the Watson-Crick double-helix structure, the structure of DNA within living chromosomes remains uncertain due to methodological limitations and inconsistencies in existing studies.

2 Materials and Methods

ΦX174 and pBR322 plasmid DNA were obtained from New England Biolabs, both supplied at a concentration of 1000 μg/mL (*i.e.*, 1000 ng/μL). Sybr Green I, Tris-acetate-EDTA (TAE) buffer, and other reagents were acquired from ThermoFisher. Reaction tubes (0.5 mL) were obtained from Eppendorf.

2.1 Terminology

The terminology used to describe structural forms of small circular duplex DNA follows the classification originally proposed by Vinograd (1965) [6]. Although alternative naming systems have been introduced over the years, the Vinograd system remains the most widely recognized. The relevant forms are defined as follows:

Form I: The native plasmid form, characterized as circular and double-stranded. This form typically exhibits topological supercoiling, often appearing as a right-handed (negatively supercoiled) structure.

Form II: An open-circular, relaxed structure generated from Form I by introducing a single-strand nick in the sugar-phosphate backbone. This nick acts as a swivel point for the release of superhelical tension, rendering the molecule similar in behavior to linear DNA.

Form III: The fully linearized duplex DNA form, produced by complete cleavage of both strands in Form I. Although included here for completeness, Form III is less relevant to the present study.

Form IV: The denatured form resulting from exposure of Form I DNA to alkali conditions, typically at pH 13.

The name "Form IV" was not actually coined by Vinograd, but is an extension of his terminology, coined by William Strider, a graduate student in biochemistry at the New York University School of Medicine (hereafter, "NYU"). Strider, and his advisor Robert Warner (who chaired the biochemistry department at U.C. Irvine for many years), did the lion's share of the published work on denaturation-renaturation of Form IV, precisely defining the parameters for both processes [7–9]. In Strider's PhD thesis he stated:

> *"This denatured form has been variously designated by different workers, e.g. 'denatured double- stranded ring', and 'double-stranded cyclic coil'. To avoid the repeated use of these rather cumbersome descriptions, and in recognition of the fact that the denatured molecule is a discreet and stable species under many conditions, it seems appropriate to extend the terminology used by Vinograd..."* (Strider, 1971, p.2) [8].

Form IV has certain extraordinary properties. The property which caused people to take interest in it, in the 1970s, was its great density, giving rise to a sedimentation coefficient, in velocity centrifugation studies, of nearly three times that of native Form I (Figure 1) [10]. This suggested that alkali denaturation might be a very useful way to separate viral DNA from cellular DNA in crude cell lysates, and that proved to be the case. But then a serious problem arose. The problem was that it seemed, at first, that it was impossible to re-nature the Form IV back to Form I. Form IV was happily stable over a broad range of pH, temperature and ionic strength, and none of the conditions suitable for renaturation of denatured linear DNA seemed to have any renaturing effect on Form IV.

In the process of time, however, conditions for the re-naturation of Form IV were eventually found. An illustrative example of the creation of Form IV by denaturation of Form I, in a pH *vs* sedimentation coefficient titration, is shown in Figure 1. An illustrative example of the re-naturation of Form IV back to Form I is shown in Figure 2.

The data in Figure 1 is largely based upon the study of Rush and Warner (1970) [10], which employed the duplex replicative form (RF) of the virus ϕx174, in a sodium concentration of 0.4 M, at room temperature. (Note that the graduations on the ordinate axis are not numbered,

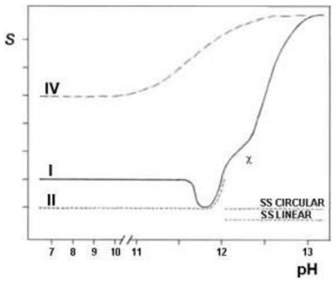


Figure 1 Idealized sedimentation coefficient (s) *vs.* pH for a typical duplex plasmid or viral chromosome.

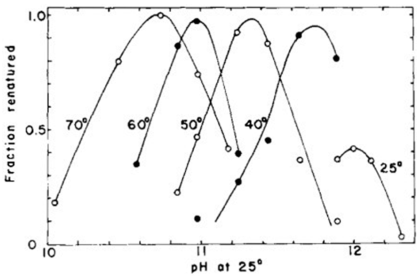


Figure 2 Form IV, percent renaturation *vs.* pH, in 1 M NaCl for 10 minutes, at the temperatures shown [7–9].

because the various species which have been studied each have their own characteristic s values). In the pH range 7-11, the sedimentation coefficient of the native chromosome (curve labeled "I") is constant; about 21 s in the case of ϕx174. In all species studied, there is a dip in s at around pH 11.5-12, reflecting the unwinding of right handed ("negative") supertwists. At higher pH, left handed ("positive") supertwists appear, causing s to increase again. There is always a shoulder at around pH 12.5 (χ), which is, however, never discussed. Beyond the χ shoulder, s greatly increases, up to about pH 13, at which s has increased nearly threefold (for ϕx174, about 56 s). This is Form IV. The conformational changes in Form IV are persistent, because when it is neutralized (upper curve – – –), Form I does not reappear, but rather s remains elevated, dropping only to about 40 s at pH 7. The present author has proposed a detailed model to account for the events in the high pH region of these data, as well as a theoretical atomic model for the structure of Form IV [11]. A more comprehensive and better-illustrated presentation than is possible on the printed page, may be found at https://NotAHelix.net, PowerPoint presentation: Form IV: the Final Puzzle Piece.

At any temperature, in Figure 2, there is only a single narrow range of pH within which renaturation is rapid and complete. If, at any of the temperatures shown, the pH of optimal renaturation is either increased or decreased by as little as 1/2 pH unit, percent renaturation is markedly diminished, and if altered by a whole pH unit, renaturation is totally abolished. It is also known (although not shown here) that if the pH and temperature are held constant, a similar set of curves can be obtained by varying the ionic strength, which curves also show remarkably narrow optimal ranges of ionic strength for renaturation (Strider (1971) [8], Figure 5; Strider, Camien and Warner (1981) [9], Figure 2). Similarly, if it is the pH and ionic strength that are held constant, another similar set of curves can be obtained by varying the temperature, which curves also reveal only narrow ranges of optimal temperature for renaturation (see https://NotAHelix.net, PowerPoint presentation: Form IV: the Final Puzzle Piece, slide 30).

Most of the work exemplified by Figure 2 was done by William Strider. I knew Strider personally, because I was a graduate student in that same NYU department, and our respective labs were only a few steps apart. I read his entire PhD thesis, and I therefore know a great deal about Form IV, which is a subject that nearly no one else cares about anymore.

In 1979 Charles Weissmann (co-founder of the biotech giant Biogen) and his associates, published a paper on what he alleged to be a "new DNA form", which he called "Form V" [1]. "Form V" was the name Weissmann appended to the DNA structure arising from the reannealing of previously-separated, fully-intact circular strands of a duplex plasmid or viral chromosome. This, according to Weissmann, should correspond to the so-called "side-by-side" topologically-non-helical structures of Rodley et al. (1976) [12] and Sasisekharan et al. (1976) [13] (see also Wu (1969) [14], Cyriax and Gäth (1978) [15], and Delmonte and Mann (2003) [16]), and the Weissmann experiment was quite deliberately intended to prove that those structures, which existed only in theory, could in fact be assembled in the laboratory, and that they would have abnormal physical properties, thus proving that they did not exist in native double-stranded circular DNA.

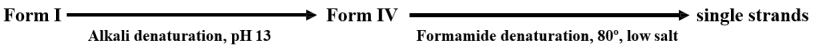
Until shortly before this publication, Weissmann had been a professor at NYU, and he knew of William Strider's work, wherefore he coined his "Form V" terminology as an extension of Strider's "Form IV".

The Weissmann experiment was done specifically in response to a verbal appeal by Francis Crick, at a New Zealand conference on the side-by-side DNA structure (Robert W. Chambers, chairman, NYU department of biochemistry, 1978, personal communication -– Chambers had

been "racing" with Weissmann to be the first to complete this work).

Weissmann believed that "Form V" was a novel base-paired structure, which I sincerely doubt, because I believe that what he unknowingly created was Form IV, which is not base-paired. My reasons for believing this have been previously explained in great detail (see https://NotAHelix.net, PowerPoint presentation: Form IV: the Final Puzzle Piece, slides 145-160). Among the various experimental results Weissmann reported, in his 1979 publication, was the finding that the strands of his "Form V" could be separated by incubation in 90% formamide at 80ºC and low ionic strength. See Stettler et al. 1979, p. 27, which reads: "Form V PβG DNA and ϕX174 DNA were heated in parallel to 80°C for ten minutes in 90% formamide, 10 mM Tris·HC1 at pH 8. This treatment led to the denaturation of form V PβG DNA".

It should be noted that the denaturing solution exhibits remarkably low ionic strength, as evidenced by the complete absence of sodium ions, including even trace amounts typically contributed by disodium EDTA. Since I believe that "Form V" is really Form IV, Weismann's report, which he intended to be a dagger in the back of non-helical DNA theory, may have now proven to be its salvation, since he inadvertently revealed the hitherto elusive secret of how to easily separate the strands of a duplex plasmid or viral chromosome, by a 2-step denaturation process:



The creation of Form IV by alkali denaturation is well-known; but the further formamide/low-salt denaturation is not known at all. It is, however, entirely consistent with my proposal for Form IV structure [11] (and once again, a more comprehensive and better-illustrated presentation than is possible on the printed page, may be found at https://NotAHelix.net, PowerPoint presentation: Form IV: the Final Puzzle Piece). My Form IV model, the only one in existence, is dependent upon salt bridges between phosphate groups, and is eerily reminiscent of Linus Pauling's pre-Watson-Crick theory for native DNA structure [17]. Although Pauling famously erred in connecting the DNA phosphate groups by hydrogen bonds, the general form of his structure lends itself perfectly to a similar one based upon sodium salt bridges. Because Watson and Crick considered Pauling to be the world's leading authority on the chemical bond, I have always maintained a keen interest in his DNA structural proposal, even though it clearly does not answer to the genetic requirements of chromosomal DNA. But there are no genetic requirements for Form IV.

The key to heat denaturation of Form IV, to single strands, therefore appears to be: formamide interference with base-base interactions, in an ultra-low-salt setting in which no confounding new structure, which is dependent upon sodium salt bridges between phosphate groups, can survive.

2.2 Experimental Protocol

The protocol described below is not intuitively obvious, but was the outcome of 52 years of deliberation. It should be followed exactly and precisely as written. Presumptuously changing anything, before confirming that it works as described here, only invites failure.

Six simple reagents were used. The perhaps-peculiar-looking reagent concentrations are for the express purpose of creating an easy and fast, room temperature denaturation protocol, in which each reagent is added in exactly the same volume, namely 10 μL:

(1) Buffer for all DNA stock solutions: 10 mM Tris, 1.0 mM EDTA, pH 8. The pH was adjusted without addition of any salt, by mixing approximately equal volumes of two stock solutions, one made with Tris·HCl, the other made with Tris base.

(2) NaCl, 0.81 M. The purpose of the NaCl was to provide part of the 0.4 M salt concentration used by Rush and Warner in their alkali denaturation experiment [10] (see Figure 1). The remainder of the 0.4 M salt concentration came from NaOH.

(3) NaOH, 0.39 M. Note that this ACS grade material is sold as being "97+% pure", wherefore the precise purity is unknown. For lack of any logical alternative, I weighed it out as if it was 100% pure. In this experiment, the attainment of a pH at or close to 13 was essential, to be absolutely certain of alkali denaturation of the DNA to Form IV (see Figure 1). The ability of the reaction mixture to bring the DNA all the way to pH 13 was therefore carefully ascertained, in advance. A mixture of equal parts of reagents (1), (2) and (3), in this list, had a pH of 12.9. This denaturing mixture had the correct final salt concentration of 0.4 M NaCl (see Figure 1), and if we assume there to be an "alkaline error" of about -0.33 pH units, the pH would then

have been clearly over 13, although 12.9 would have been entirely adequate.

(4) Tris·HCl, 0.94 M. This was for rapid neutralization of the alkali-denatured DNA (solely to prevent damage to the DNA from prolonged exposure to high pH). It was predetermined that addition of one volume of this reagent, to a mixture of one volume each of reagents (1), (2) and (3), resulted in a satisfactorily low pH of about 8.2.

(5) Ultra-low-salt buffer for dialysis: 10 mM Tris, pH 8. This was created by mixing Tris·HCl and Tris base. Note that this solution contains no EDTA. The dialysis buffer and the magnetic stirrer were therefore kept scrupulously at 4ºC, in the refrigerator. Do not, under any circumstances, add any salt to this buffer.

(6) Sybr Green I, 10,000X in DMSO For post-stained gels, a staining solution was made by adding 20 μL of Sybr Green I to 200 ml of TAE. In the key experiment described below, the TAE was supplemented with 0.1 M NaCl, since that was the salt concentration for the electrophoresis. The pH of the staining solutions was checked; it was about 8.0.

2.3 One-Day Denaturation Procedure

The following steps were all performed at room temperature, to match the conditions employed by Rush and Warner (Figure 1) for the creation of Form IV:

(1) 6 μL of the New England Biolabs pBR322 stock solution was diluted, from 1000 μg/ml to 600 μg/ml (600 ng/μL), by addition of 4 μL of reagent (1), *i.e.*, the diluent 10 mM Tris, 1.0 mM EDTA, pH 8. The total DNA amount was therefore 6000 ng, in a total volume of 10 μL.

(2) To the 10 μL of DNA solution, 10 μL of reagent (2), (0.81 M NaCl) was added.

(3) To the reaction mixture, 10 μL of reagent (3), (0.39 M NaOH) was added, with good stirring for about 1 minute.

(4) The reaction mixture was then neutralized by addition of 10 μL of reagent (4), (0.94 M Tris·HCl). The DNA was thusly converted to Form IV. The reaction mixture was subsequently kept in the cold, at 4ºC.

(5) 2 μL of the reaction mixture, containing 300 ng of Form IV, was mixed with 16 μL of cold reagent (1), (the diluent, 10 mM Tris, 1.0 mM EDTA, pH 8), and put aside as a Form IV marker for electrophoresis.

(6) The remaining 38 μL of the reaction mixture was put into a 0.1 ml Slide-A-Lyzer device (ThermoFisher), and dialyzed for a total of 2 hours, against 2 changes of low-ionic-strength buffer (5) (10 mM Tris, pH 8, no EDTA), 150 ml per change. Both the dialysis buffer and the magnetic stirring motor were kept in the refrigerator. After 2 hours, the DNA solution was transferred to a new 0.5 ml Eppendorf tube, and kept at 4ºC.

(7) 18 μL of formamide was put into a new 0.5 ml Eppendorf tube. 2 μL ($\sim$300 ng) of dialyzed DNA was added to the tube. The mixture was incubated at 80ºC for 10 minutes, then put aside at 4ºC pending electrophoresis. This was the primary experimental DNA, now doubly-denatured.

(8) 1 μL of New England Biolabs pBR322 stock solution (1000 ng/μL) was added to 66 μL of cold reagent (1), (the diluent, 10 mM Tris, 1.0 mM EDTA, pH 8). 18 μL of this ($\sim$270 ng) was put aside as a Form I marker for electrophoresis.

(9) A single-stranded DNA marker was made in exactly the same fashion as the Form I marker in the previous step, by the same dilution of New England Biolabs ϕx174 virion DNA (also sold at 1000 ng/μL). 18 μL of this ($\sim$270 ng) was put aside for electrophoresis. ϕx174 (length = 5386 bases) was used because single-stranded pBR322 (4361 bp) is not commercially available.

3 Results

In the preliminary electrophoresis experiments, only the first denaturation step (Form I $\rightarrow$ Form IV alkali denaturation), and not the second (low-salt formamide denaturation) were done, because at that time I was primarily concerned with learning the relative electrophoretic mobilities of the three species: Form I, Form IV and single-stranded. Consequently, those early gels were pre-cast with Sybr Green I (for real time monitoring), and were cast with ordinary TAE containing no added salt. I was dismayed to note that all three DNA forms co-migrated almost exactly (Figure 3). This was totally unexpected, and threatened to be the undoing of the

entire experimental design.

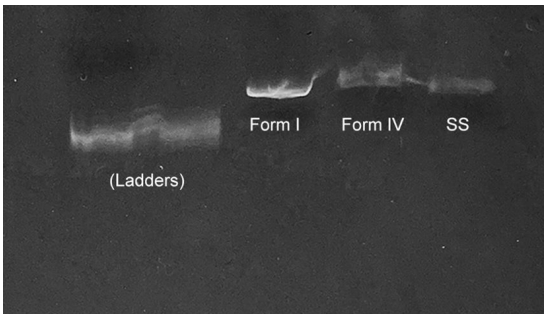


Figure 3 Initial electrophoresis of Forms I, IV and SS, showing that they all approximately co-migrate! 0.7% agarose gel (10.5 × 10 × 0.4 cm) (well capacity 22.4 μL), TAE with no added salt, 100 v. Running time 10 minutes. This gel was pre-cast with Sybr Green I, which produces suboptimal visualization; even at 20 minutes (not shown) the Form IV and SS bands were nearly invisible. These problems were solved by (a) post-staining, (b) adding salt to the TAE/agarose, and (c) decreasing the current to 10 mA, as described in the text.

A way to increase the electrophoretic mobility differences between the DNA forms therefore had to be found. First of all, it had been learned at that point that Sybr Green I is positively charged, and DNA is dragged backwards by it toward the anode in the electrophoresis chamber, wherefore it was realized that post-staining would have to be performed. More importantly, a rarely-cited 1968 paper [18] was remembered, which showed that the densities of both Form IV and SS DNA are markedly dependent upon ionic strength, whereas Form I showed little or no such dependence (Figure 4). Therefore, the possibility of adding salt to the TAE buffer was investigated.

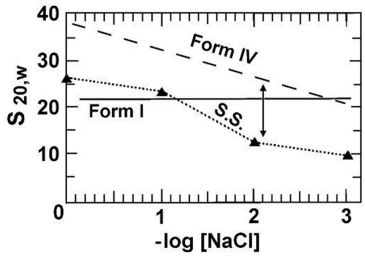


Figure 4 Dependence of sedimentation coefficient (s) of ϕx174 RF (*i.e.*, duplex) chromosome on salt concentration. The double arrow shows the typical salt concentration of an agarose gel. (Data adapted from Pouwels et al., 1968 [18])

In the experiment shown in Figure 4, the s values for Forms I, IV and SS were significantly different at the salt concentration of a typical agarose gel, but this is velocity sedimentation, not electrophoresis. In my early electrophoresis studies (Figure 3), all 3 forms essentially co-migrated! Note, however, that in the data shown here, at 0.1 M NaCl, the s values for Forms IV and SS increased substantially, whereas that of Form I remained constant at all salt concentrations. I therefore realized that I was going to have to add 0.1 M salt to my agarose gels.

Electrophoresis was performed with 0.1 M NaCl added to the TAE that was used to make both the agarose gels and the running buffer. A near-total absence of searchable literature on the subject of high-salt electrophoresis was found, which was dismaying, so learning had to be done by trial and error. It was quickly discovered that at common operating voltages (35-100 v), the electrophoresis chamber would quickly overheat or even boil over with a salt concentration of 0.1 M.

The salt concentration was reduced to 0.03 M. At 100v, the electrophoresis chamber quickly became too hot to touch. When the voltage was lowered to 35v, electrophoresis could be performed without boiling or near-boiling temperatures, but the electrophoretic mobilities of the three species, although slightly different in 0.03 M salt, were still too close together to persuasively distinguish between them (data not shown). Therefore, it was known that more salt would be needed.

The electrophoresis conditions used by Wu & Wu (1996) [3] were thereafter employed, namely 10 mA constant current, 0.8% agarose and pH 8.5. At 10 mA, the addition of 0.1 M NaCl

to the TAE caused no overheating of the electrophoresis chamber at all, but the electrophoresis had to be run for 20 hours. These, therefore, were the conditions of electrophoresis that were used in the key experiment below:

(1) TAE for agarose and running buffer, both with added 0.1 M NaCl, pH 8.5;
(2) 0.8% agarose gel (10.5 × 10 × 0.4 cm) (well capacity 22.4 μL);
(3) 10 mA constant current;
(4) Running time 20 hours.

At the end of the 20 hour run, the strands of Form IV (Figure 5, lane B) had clearly separated into two bands (Figure 5, lane C).

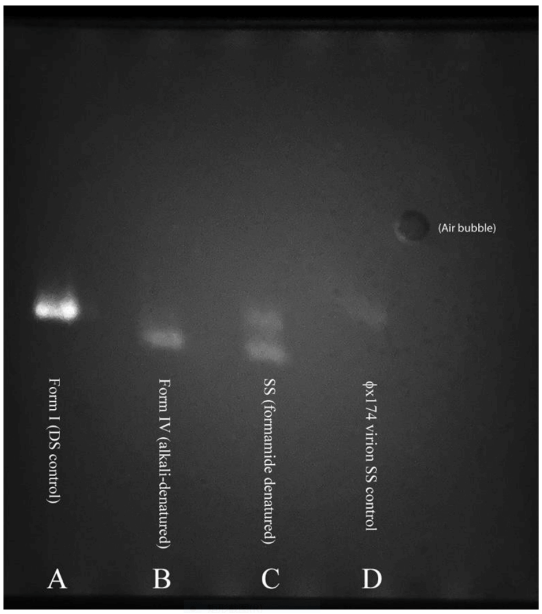


Figure 5 Electrophoresis of pBR322. 0.8% agarose with added 0.1 M NaCl, pH 8.5, 10 mA constant current. Running time 20 hours. Sybr Green I post-stained. (A) Form I double-stranded control. (B) Form IV, the product of alkali denaturation of the material in lane (A). (C) The experimental DNA, *i.e.*, the Form IV from lane (B) after a second denaturation in 90% formamide, 80ºC, at low ionic strength. (D) ϕx174 single-stranded control. It is clear that the experimental DNA (C) is migrating as two separate bands.

This result was entirely expected, if not for which the time, energy and expense would never have been expended on this research for so many years. However, what is being shown here will be cognitively dissonant for the majority of molecular biologists to even grasp, much less to be believed. Before this result is reflexively rejected, however, it should first be considered that it is entirely consistent with the previous publication by Wu and Wu (1996) [3], in which the same strand separation shown here in Figure 5 was reported, employing a plasmid named pHTB4 (Figure 6). pHTB4 is described by Wu and Wu as having been constructed from pBR322, *i.e.*, the same plasmid used in the present study, by insertion of the following sequence:

5' ttcgcccagcttcgctcagct ... aatatgcactgtacattcca 3'
3' aagcgggtcgaagcgagtcga ... ttatacgtgacatgtaaggt 5'

The purpose of the insert was to have a means of documenting that his two bands were the top and bottom strands respectively, of the pHTB4 chromosome (*i.e.*, of the slightly-modified pBR322 chromosome), by Southern blotting, that being one of three controls Wu and Wu employed in their rigorously-controlled experiment.

Between 12 and 36 hours of electrophoresis, the strands of Wu's native chromosome separated (Figure 6, yellow arrows). The similarity to the results reported here in Figure 5 is evident, other than that Wu's data is "prettier", because unlike me, he was not compelled to work with 6-12 month-old DNA and stain.

One could ask, and absolutely should ask: Even if we dare to presume that the strands of a duplex plasmid are not topologically linked, why should two strands from the same plasmid give two distinct bands on gel electrophoresis? Wu's answer is that one of the strands only, the sense strand, is "weighed down" by D-loops containing strongly-bound mRNA [3]. In accordance with this, I should note that Wu isolated plasmid DNA in his own lab, from E. coli

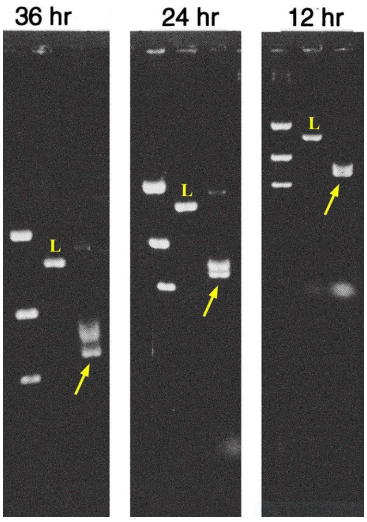


Figure 6 Electrophoresis of a pBR322-derived plasmid chromosome. The lanes on the right (yellow arrows) are the experimental DNA. At 12 and 24 hours the band is starting to split. At 36 hours the split is complete. The middle lanes ("L") are a control experiment, showing what happens if the experimental DNA is cleaved to linearity (thus the "L") with a restriction nuclease: the band does not split. The splitting of the bands in the experimental lanes on the right therefore cannot be cavalierly dismissed as having been due to accidental strand cleavage, because intentional strand cleavage actually prevented the bands from separating. The left lanes (unlabelled) are a ladder. Data adapted from Wu and Wu (1996) [3].

cells grown to log phase (*i.e.*, confluence in the roller bottles, after which DNA synthesis ceases, but transcription of mRNA continues). It has recently come to my attention that commercial preparations of pBR322 may routinely involve RNase treatment, which is apparently desirable for procedures involving cloning and PCR. Use of such plasmid DNA, however, will generate a negative result in this experiment, because the two strands, having had the mRNA enzymatically removed from the D-loops of the sense strand, will subsequently have the same electrophoretic mobility!

4 Discussion

Regarding the two bands in Figure 5C, how may they be explained, other than by the explanation offered here? Is there any other plausible explanation? It is self-evident that neither of those two bands can be either Form I or Form IV, since markers for those forms are present in the gel. Nor can either band be Form II (nicked Form I), because any Form II would have been converted to single strands by the formamide denaturation step.

The assertion could be made that these results are trivial, because one of the two bands in Figure 5C is SS linear, and the other SS circular. But how so? Such a result could not be explained as having arisen from the double-denaturation protocol presented here. The Form IV (Figure 5B) was created by subjecting the DNA to exactly 1 minute at pH 13, and it is inconceivable that after 1 minute at pH 13, exactly one nick would have been received by essentially every Form IV duplex molecule, if indeed any significant number of nicks were introduced at all by 1 minute at pH 13. If less than 1 nick per molecule had been caused by the alkali treatment, then a significant amount of Form IV would still be present in lane 5C, which is not observed. If more than 1 nick per molecule had been caused, then an assortment of random-length fragments would have appeared, causing lane 5C to be a blur, not a set of two fairly-distinct bands.

Likewise, the second denaturation step, 90% formamide at 80ºC for 10 minutes, should not, to the best of my knowledge, cause any significant nicking of DNA. It is therefore asserted that the best explanation for the two bands in Figure 5C is the explanation that has been proposed, namely that they represent the top and bottom strands of pBR322, non-destructively separated by this double-denaturation protocol.

Profound skepticism is strongly urged to be exercised by the readers of this manuscript, and this work should be repeated by themselves. The great majority of readers have infinitely better supplies and technical supports than are available here, and this work could be done in literally

a single day by any one of you. Nor is there any excuse not to do so, because it may be that a great deal of the future of molecular biology depends upon the outcome.

Merely repeating this work would not, of course, be the end. Next, the products of this double-denaturation would have to be characterized in at least three ways: (1) by being subjected to treatment by SS-specific and DS-specific nucleases, (2) by showing that they can be reannealed back to Form I, but only at the conditions of optimal reannealing shown in Figure 2 above, and (3) by showing that the reannealed Form I will, upon double-denaturation, once again yield two bands.

While the current study offers compelling preliminary evidence, full validation requires more comprehensive follow-up. Such work is best undertaken in well-equipped laboratories with access to high-quality reagents, consistent environmental controls, and modern imaging tools.

Funding Support

This report is dedicated to my late wife, Anita Shatz, who loved me during her life, and who funded this study in her death.

Conflicts of Interest

The author declares no conflict of interest.

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