A Journey in the World of DNA Rings and Beyond

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Abstract

I was born in China and would have remained there but for the tumultuous events that led many of my generation to the United States for graduate studies. Norman Davidson introduced me to DNA when I became a postdoctoral fellow in his group at the California Institute of Technology in 1964, and a fortuitous conversation there ignited my interest in DNA ring formation, which later led me to study different topological forms of DNA rings—catenanes, knots, and supercoils. In 1968, a chance observation led me to identify a new enzyme capable of converting one DNA ring form to another, an enzyme now known as a DNA topoisomerase. My interest in DNA rings and DNA topoisomerases continued throughout my years at the University of California, Berkeley, and Harvard. The fascinating ability of the topoisomerases in passing DNA strands or double helices through one another and their importance in cellular processes have kept me and many others excited in their studies.

Contents

EARLY YEARS

I was born in Jiangsu Province, China, the second and last child of an arranged marriage. Seven months after my birth, the Marco Polo Bridge incident propelled the long conflict between Japan and China into a full-fledged war, and my father left home to follow the move of the central government to Chongqing, the wartime capital. I did not see him again before I turned nine.

The eight-year duration of the war, and the civil war that followed it, fragmented my early schooling. When I was about five, my mother set up a school in the countryside of her hometown Liyang, which was then under Japanese occupation. I became a second grader in her school, but Mother soon became very ill. The school, which had probably consumed much of her dowry and inheritance, had to fold. Mother never recovered from her illness. When she passed away a couple of years later, my childhood ended. I was six and a half.

After the Sino-Japanese War, my remarried father returned to Nanjing, the capital, and I became a fourth grader there. By then my sister, who was five years older than I, had passed away; the war years had taken a heavy toll on her weak constitution. My schooling was again disrupted a year and a half later when the Red Army crossed the Yangtze River. In 1949, the family moved to Taipei, and I managed to squeeze through the entrance examination of a junior high school. Thereafter, my education was never again interrupted.

Looking back, during my early years, I benefited greatly from what Mother taught me about reading on my own, which allowed me to cope with the interruptions and deficiencies in my schooling. But formal education did have a huge influence on me. Among my fourth-grade classmates in Nanjing, there was one I met again in college in Taipei and later married in Denton, Texas, and many of my high school classmates became my closest and lifelong friends. Several of my high school teachers gave me much needed encouragement during my formative years, and a painting by my high school chemistry teacher, given to me during the last year of her life when she heard about my election to the National Academy, still graces the wall of my study.

COLLEGE AND GRADUATE STUDIES

I wanted to study medicine after high school, but my parents thought that I should go into engineering. I ended up as a chemical engineering major at the National Taiwan University because of my interest in chemistry. In those years, there were too many required but not very inspiring courses, and many of us skipped classes and studied on our own. There were a couple of bookstores close to campus, and the owners of the stores were very willing to print photocopied books for us. We surely owe many authors in the West apologies for encouraging, and sometimes instigating, book pirating. It is perhaps a small consolation to them that we could not possibly have afforded the originals.

After graduation, I remained at my alma mater for one year as an assistant lecturer—basically a full-time teaching assistant. In those years, many college graduates would go abroad to pursue graduate studies; the Taiwanese economy had yet to take off. With my miserly salary of about US$20 a month, however, I could not afford the application fees demanded by many of the universities in the United States. Thus, when a college classmate wrote from the University of South Dakota to ask whether I might be interested in a fellowship in the chemistry department there, I quickly jumped at the chance and was equally quickly offered the fellowship.

When I finally secured a visa from the U.S. Consulate in Taipei, the fall semester had already started. I looked on a map, found Omaha as the biggest circle close to Vermillion, South Dakota, and spent more than half of the $720 I had borrowed from three classmates for the one-way airfare to Omaha. An agent at the Omaha airport sold me a ticket to Yankton, South Dakota, which, he told me, had the airport closest to Vermillion.

I was truly lost one Sunday morning in early October 1960. Vermillion was less than an hour away by bus from the Yankton airport, but to my chagrin there was no bus on Sundays. Because I had arrived by the cheapest route, which took me through several Pacific islands—Okinawa, Guam, and Oahu—before landing in Oakland, California, for an overnight flight to Omaha, all on propeller-driven aircrafts, I was exhausted and felt a bit topsy-turvy by the time I got to Yankton. While I was contemplating what to do next, a young couple at the airport discovered my quandary and offered to drive me to Vermillion. In my dazed state of mind, I forgot to ask their names after they delivered me there, and I never properly thanked them. Their kindness at the very beginning of my life in a new country had, however, left an everlasting impression in my memory.

Studying turned out to be the least challenging aspect of my new life. I discovered that my fellowship would leave me very little after covering tuition charges and paying back part of my debt. I was able to manage, however, by moving out of the dormitory after the first semester and into a sparsely furnished room off campus. My MA thesis research was on telomer formation by free radical chain transfer. The term "telomer" had nothing to do with "telomere," which denotes distinctive structures at chromosome ends. The general idea of my assignment was to use a free radical initiator to start polymerization of a vinyl compound and to terminate the free radical chain reaction very early by the inclusion of a high concentration of a free radical scavenger in the reaction mixture. In this way, very short polymer fragments—telomers—could be obtained and characterized. My thesis advisor, Professor George P. Scott, suggested that I use styrene as the polymerizing agent and ethanethiol (ethyl mercaptan) as the free radical scavenger. The work proceeded smoothly, and I was able to complete my MA program by the following June. But working with ethyl mercaptan, one of the smelliest substances, had its consequences. One evening, I passed by the TV lounge of the dormitory after a long day in the laboratory and decided to watch the evening news for a few minutes. As soon as I stepped into the room, people started sniffing. I realized what was happening and beat a hasty retreat.

In 1961, I was admitted into the PhD program of the University of Missouri Chemistry Department. I applied there for two reasons. First, it did not charge an application fee. Second, the university was well known in Taiwan because quite a few people in the news media there had trained at its School of Journalism. When Christmas vacation came that year, I arranged a ride to Denton, Texas, where Sophia and I were married. I returned to Missouri after the vacation, but Sophia stayed in Denton for another semester to complete her master's program. She finally came in June 1962 and became a librarian at the university. The following April, our first daughter, Janice, was born.

I received a PhD in Physical Chemistry in 1964, but my thesis research with John E. Bauman, Jr. dealt with transition metal ion complexes. John was then freshly out of his own PhD studies, and I was his first student. He suggested that I try my hands on metal ion complexes of imidazole and its derivatives, a subject he had been pursuing. I was able to clarify a few issues about imidazole complexes, such as the roles of the pyridine and pyrrole nitrogens of the imidazole ring in complex formation and the formation of metal imidazolates following ionization of the pyrrole hydrogen. But I did not find the results particularly thought provoking. I was more intrigued by the results of a calorimetric study of the metal ion complexes of a family of α-amineoximes, carried out in collaboration with Professor R. Kent Murmann. Substitution of the amino hydrogen of an α-amineoxime by an alkyl group was found to significantly affect ΔH and ΔS, but it had very little effect on ΔG. I realized that this enthalpy-entropy compensation could best be attributed to solvation changes; this was the first time I became aware of the concept of hydrophobic effects. Another finding that made a strong impression in my memory was a striking thermochromism exhibited by the 2,2′-bi-2-imidazole complex of Cu(II). I was trying to crystallize the complex one afternoon, and I put the green solution in the refrigerator. When I returned after supper and took out the solution, I was puzzled by its blue color. As I was holding the small beaker containing the solution and thinking about whether I might have remembered its color incorrectly, the solution gradually turned green in front of my eyes! By then, however, I was about to receive my PhD and move on, and I never learned the exact mechanism of this color change.

CALIFORNIA INSTITUTE OF TECHNOLOGY AND MY INTRODUCTION TO DNA

Although I thought that I could and should have done much better in my graduate studies, my professors were apparently much kinder in their assessments. Thus, even before I got around to sending out application letters, I already had an outside offer of a postdoctoral fellowship as well as a research position in a chemical company. But this time I had decided to send out a few letters to inquire about other possibilities. An encouraging reply soon came from Professor Norman Davidson at California Institute of Technology (Caltech), in which he said that he would know in a couple of weeks about his funding situation, and he asked me to call him in case waiting would pose a problem. Two weeks later, he wrote again to offer me a postdoctoral fellowship. He indicated in his letter that he was no longer working on small ligands, but there had been some work in his laboratory on mercuric ion binding to DNA, which he thought I might work on.

It was a very smoggy afternoon in June 1964 when we drove into Pasadena. Both Sophia and I felt our eyes hurting, but fortunately, our one-year-old baby was sleeping soundly and did not seem to care. The next morning, I met Professor Davidson for the first time; I was in my only suit, and he was in his casual post-tennis outfit. After greeting me, he asked what I knew about DNA, and I confessed my ignorance other than a vague notion of its double helix structure. He pulled out a book from his bookshelf for me to read and then went on to describe the status of the various ongoing projects in his laboratory.

The book Norman gave me turned out to be terribly boring, and I was soon itching to start

in the laboratory. Because Norman's laboratory had already shown that mercuric ion binds preferentially to the base thymine in DNA and had succeeded in using this base preference to fractionate DNAs of different AT contents by density gradient centrifugation in Cs_2SO_4, I thought that perhaps what was missing was a more quantitative basis for these findings. I devised a spectrophotometric assay that monitors the competition between DNA and bromide ions for Hg(II). From these measurements and the known equilibrium constants for the formation of mercuric complexes with 1 to 6 bromide ions, I managed to place Hg(II) binding to different DNAs on a quantitative scale. I also did some pH-stat measurements, at Norman's suggestion, to quantify the number of hydrogen ions released when a mercuric ion binds a DNA. Norman was apparently pleased with these results, and he soon gave a departmental colloquium with the title "Crossing the Bridge." The title had a double meaning: The first concerned Hg(II) binding to DNA, as our results suggested that a mercuric ion often bridges the two complementary strands of a DNA by binding to a pair of cross-strand bases, in particular thymines. The second message was that he had crossed the bridge and would no longer dwell on metal ion studies. I too followed him and crossed that bridge; it was in December 1964, six months after my arrival at Caltech.

AFTERNOON COFFEE AT CALTECH

Norman sometimes went around the lab late in the afternoon to collect those of us free to go for coffee in the Caltech cafeteria. The conversation over coffee was usually about science rather than idle chatter, and Norman always had his notepad ready. It was during such a coffee break that Norman raised a question about the size of the "sticky ends" of phage λ DNA. A few years earlier, Alfred Hershey and his associates (1) had discovered that phage λ DNA possesses single-stranded ends with complementary sequences, which allow the ends to join intramolecularly to form rings, or intermolecularly to form linear oligomers. Various experiments suggested that these sticky ends were short, but how short was unclear. Remembering my calorimetry days, I ventured that one might be able to calculate the length by measuring the enthalpy change when the ends join. Norman quickly did some scribbling in his notepad and concluded that at a reasonable λ DNA concentration the enthalpy change would be too small for calorimetry.

I was not entirely convinced by his estimate and thought that the experimental difficulty was not insurmountable. It so happened, however, that a manuscript came the very next day from Dale Kaiser at Stanford University, in which he and Hans Strack (2) included data showing a steep temperature dependence of the infectivity of λ DNA that was kept in the refrigerator for a long time. In those days, the infectivity of purified λ DNA was measured by a "helper phage assay," and there was a strong indication that infectivity in this assay required the presence of sticky ends in their single-stranded state. It immediately occurred to me that if the enthalpy change for the joining of the sticky ends was very difficult to measure by calorimetry, then one could more easily deduce the same quantity from the temperature dependence of the reaction equilibrium. Assuming that the infectivity was strictly a measure of the fraction of λ DNA molecules with disjoined ends, then the enthalpy change could be readily calculated from the temperature dependence of infectivity. In five minutes, I had my answer, and I went into Norman's office to tell him that the length of a sticky end was about 10 nucleotides.

Norman saw right away that the validity of such an estimate would depend on whether the joining of the sticky ends could be represented by a two-state model, in which the nucleotides in a pair of sticky ends were either all in a single-stranded state or all paired up. After thinking for a few minutes, he concluded that the two-state approximation would hold, and he asked me whether I would write up a short note for publication. I was still very much a chemist then, and I was not yet comfortable with biological data

such as infectivity measurements. I suggested that perhaps it would be better to first do some direct equilibrium and kinetic measurements of the joining of the DNA ends.

The first thing I had to learn before any real experiment on the joining of λ DNA ends was how to grow phage λ. Norman sent me to see Jean Wiegle, the foremost expert on λ. Jean gave me the required recipes as well as the bacteria and phage stocks, and he showed me how to do phage plaque assays on agar plates. When I later made up the growth medium and autoclaved it, I saw a lot of precipitate. "Biology!" I mumbled to myself while filtering it. I showed Jean my plates the next day, and he was very puzzled. There was not a single phage plaque on them! The problem turned out to be a simple one. I had no idea that, in preparing the growth medium, several solutions must be separately autoclaved and then mixed together after cooling.

Equilibrium and kinetic measurement of λ DNA ring formation turned out to be straightforward because the joining of the sticky ends has a high activation energy, and therefore, the reaction mixtures could be quenched by cooling and then analyzed at leisure by ultracentrifugation. These data again gave the estimated length of the ends as about 10 nucleotides (3). Norman and I also had an apparatus made to pass a DNA solution repeatedly through a long glass capillary to hydrodynamically break a linear λ DNA into halves. By comparing the intramolecular joining of the ends of an intact λ DNA molecule and the intermolecular joining of the ends of two half molecules, we were able to analyze the molecular parameters of DNA ring closure. We showed that the key factor here is what we termed the Jacobson-Stockmayer factor or *j*-factor, which is the probability density or concentration of one end in the vicinity of the other end of the same DNA, a parameter closely related to the flexibility of the long molecule (4). In later years, when the recombinant DNA era made DNA rings a widely used cloning vehicle and when the importance of DNA loop formation in biology became apparent, the *j*-factor often popped up in various studies.

BERKELEY: DNA RINGS, SUPERCOILS, AND CATENANES

It was early 1965 when Norman walked in with Ignacio Tinoco from the Chemistry Department of the University of California (UC), Berkeley. Norman introduced us, and Professor Tinoco, Nacho to his friends and colleagues, said that the UC Berkeley Chemistry Department was looking for an assistant professor in the area of biophysical chemistry. Soon afterward, I was invited to give a seminar there. I talked about the mercury-DNA work I had just done with Norman, and a couple of months later, I received an offer.

The idea of starting at UC Berkeley in six months was frightening, however. True, I had learned a few things since my arrival at Caltech. When I attended a seminar on ribosomes during my first month there, I was probably the only one in the audience who did not know what a ribosome was. But when the UC Berkeley offer came six months later, my understanding of biology in general and DNA in particular was still primitive and marginal. Therefore, with Norman's consent, I asked for a postponement of one year, and UC Berkeley agreed.

The one-year delay was a good decision. It gave me time to start working on DNA ring formation, and it also gave me a unique opportunity to witness the discovery of supercoiled DNA in Jerry Vinograd's laboratory next door to Norman's (5). In addition, the postponement gave me ample time to apply for a National Institutes of Health (NIH) research grant to continue my work on DNA rings. Thus, when I became an assistant professor in June 1966, I already had an NIH award waiting for me—the beginning of NIH's uninterrupted funding over my entire research and teaching career. In those days, "start-up" research funds from a university were rare, and without the generosity of NIH and my new colleagues, especially Nacho and John Hearst, who allowed me complete access to their equipment, the start of my independent research career would have been much tougher.

I began with two lines of research in the newly constructed Hildebrand Hall at UC

Berkeley. The first was an extension of the work I started in Norman's laboratory. So far, physicochemical studies of DNA ring formation had only been done with phage λ DNA, and I thought that at least a second DNA with rather different sticky ends should be examined to ensure the generality of some of the principles distilled from the earlier measurements. The use of two DNAs with different sticky ends, which would prevent their joining to each other, would also allow me to examine the formation of interlocked rings (catenanes) between them.

The second line of research was based on Vinograd's pioneering work on DNA supercoils. I thought that it would be very nice if a nicked DNA ring—a double-stranded DNA ring with at least one single-stranded break in it—could be converted to the covalently closed form, namely a DNA ring with both strands intact. Upon covalent closure of a DNA ring under a fixed set of experimental conditions, the degree of topological linkage between the two complementary strands, that is, the linking number of the ring, could no longer change. Thus, any subsequent change in the helical structure of the DNA would lead to spatial supercoiling of the DNA ring. Because properties, such as the sedimentation velocity of a DNA ring, are very sensitive to its spatial supercoiling, the coupling between DNA helical structure and supercoiling would provide a very sensitive way of measuring changes in the DNA helical structure.

I soon completed a study of ring formation of phage 186 DNA, which showed a much faster rate of end-to-end joining. I also proceeded to study catenation between 186 and λ DNA by density gradient centrifugation, using 5-bromouracil-labeled λ DNA. Labeling with 5-bromouracil greatly increased the buoyant density of λ DNA, so that the unlabeled 186 DNA and the labeled λ DNA were well resolved by density gradient centrifugation. I was later joined by Harley Schwartz, my second graduate student, and we cyclized 186 DNA in the presence of a high concentration of labeled λ DNA rings to see if catenanes between the two would indeed form. A band with the expected buoyant density was soon identified between the light and heavy bands, and quantitation of the bands gave us a direct measurement of the probability of DNA catenation (6). I sent preprints of this work to several people, including Jerry Vinograd, who, with his coworker B. Hudson, was kind enough to cite our paper while it was still in press when he reported in *Nature* the finding of catenated HeLa mitochondrial DNA (7)—we sent our manuscript to a slow journal, and it took more than six months for it to appear in print (6). Soon afterward, Harley abandoned science. In Berkeley in the late 1960s, the Vietnam War had a strong effect on many students, and I failed to talk Harley out of his desire to do something more relevant to humanity than science.

For the second line of research, I chose to start with the covalent closure of cyclized λ DNA. I was convinced, from studies I carried out at Caltech and UC Berkeley, that cyclization of a linear λ DNA would juxtapose the 5′-phosphoryl group of one end and the 3′-hydroxyl group of the other end; thus, it should be possible to covalently close the DNA ring by splitting off a water molecule from each pair of these juxtaposed groups to form a phosphodiester bond. I started with the use of a carbodiimide to do the water removal.

For several weeks, all my trials with carbodiimides failed miserably. The project was saved, however, by a visit from B. M. (Toto) Olivera. Toto was a graduate student in Norman's group during my two years there, and upon receiving his PhD, he joined Bob Lehman's laboratory at Stanford. He and Bob had just succeeded in the identification of *Escherichia coli* DNA ligase and its cofactor NAD (8), and he thought that I should definitely try his enzyme preparation rather than playing with strange chemicals such as carbodiimides. The enzyme worked like a charm!

The ability to convert nicked DNA rings to the covalently closed form soon led to a number of findings. I found that the helical periodicity of DNA, that is, the number of base pairs per turn of the double helix, is dependent on temperature and counterions (9). More

importantly, the covalent closure of a nicked DNA also provided a very sensitive method for measuring changes in the DNA helical structure by other molecules. The insertion of an ethidium or actinomycin D in between two adjacent DNA base pairs, for example, was found to untwist the DNA double helix by about 26° (10, 11). At the suggestion of Mike Chamberlin in the Biochemistry Department, my first postdoctoral fellow Jean-Marie Saucier also examined the effect of *E. coli* RNA polymerase binding, and he observed in 1972 a readily measurable unwinding of the DNA double helix of ~1 turn per bound RNA polymerase (12). That result provided an early experimental support of a prevalent idea that the enzyme reads the DNA nucleotides encoding the genetic information by first disrupting a short stretch of the paired bases in a DNA double helix—a notion that was also supported by the finding of Tao-Shih Hsieh, a graduate student with me, who observed a hyperchromic shift of the DNA absorption spectrum around a wavelength of 260 nm upon RNA polymerase binding (13). Various experiments by others, especially high-resolution X-ray crystallographic studies, would later firmly establish that interpretation.

Covalent closure of nicked DNA rings in the presence of varying amounts of ethidium, followed by the removal of the intercalator, also provided a way of preparing DNA rings negatively supercoiled to different extents (9). Several series of experiments using such DNA samples were carried out in the 1970s to study the effects of DNA supercoiling on its interactions with other molecules. In collaboration with Suzanne Bourgeois and Mary Barkley, the association rate of *lac* repressor binding to its operator was found to increase upon negative supercoiling of the DNA ring bearing the operator, whereas the dissociation rate was found to decrease (14). These results illustrated the importance of supercoiling in processes involving DNA. Furthermore, the magnitudes of the measured rate changes were consistent with a small unwinding of the DNA double helix when a repressor binds to the operator, by about one quarter of a turn. An unwinding of that magnitude ruled out a model in which *lac* repressor binding involves the formation of a "cruciform" structure in which the twofold symmetric operator sequence extrudes a pair of short hairpins; it thus suggested that the recognition of a special DNA sequence by a protein requires no drastic change of the DNA helical structure. Transcription with *E. coli* RNA polymerase and nicking by single-strand-specific endonucleases were similarly studied, and supercoiling of a DNA was found to strongly affect its properties as an enzyme substrate: Negative supercoiling of the DNA template was found to stimulate total RNA synthesis by the polymerase but reduce its promoter specificity (15, 16), and the initial DNA nicking rates of the single-strand-specific *Neurospora crassa* and Mung bean endonucleases were found to show a steep increase when the DNA was negatively supercoiled beyond a certain level (16).

BERKELEY: PLUNGING INTO DNA ENZYMOLOGY

With hindsight, starting with carbodiimide in my first attempt to convert a nicked DNA ring to its covalently closed form was not a good idea. The conversion of λ DNA to a supercoiled form shortly after its injection into host cells was then already known, and this ready conversion all but ensured that a host enzyme must be joining the two pairs of juxtaposed 5′-phosphoryl and 3′-hydroxyl groups. But I was then simply too afraid of trying my hands on messy cell extracts. Once that fear factor was removed by my introduction to DNA ligase by Toto, I marched into DNA enzymology with the zeal of a new convert.

In 1968, I persuaded two graduate students to try two new ideas. The first was inspired by a 1963 paper I read, in which Mike Chamberlin and his associates reported the misincorporation of ribonucleosides by *E. coli* DNA polymerase I in the presence of manganese ions. I thought that this misincorporation could be utilized for DNA sequencing because RNA is labile at an alkaline pH, and thus occasional

misincorporation of one particular ribonucleotide at a time might be a way to generate four sets of DNA fragments after alkali hydrolysis, each terminating with a particular nucleotide.

The second idea dealt with joining any two DNA fragments together. I was then wondering what might be the simplest self-replicating unit. I thought that stitching sheared DNA fragments together to form a ring would provide a way of determining the essential components of a replicon. It seemed to me that the enzyme polynucleotide transferase could be used to add a stretch of As to the ends of one DNA fragment and a stretch of Ts to the ends of another, and these artificial sticky ends would allow the joining of two DNA fragments to form a ring, which could be introduced into *E. coli* spheroplasts to see whether it could replicate.

Alas, both ventures outlined above failed despite the hard work of the two students. The misincorporation turned out to be quite tricky, and the problem was compounded by our lack of a good way of monitoring the reaction. We dropped the project after several months of struggling. The second project did not fare much better. We started with the purification of the transferase from calf thymus, acquired from a local slaughterhouse, but the quality of the purified enzyme was never good enough to simply add single-stranded ends to a DNA. In 1970 while I was spending the second half of a sabbatical leave with Dale Kaiser at Stanford University, I learned that Peter Loban, a graduate student in his group, had also been working on adding artificial sticky ends to DNA, and by then Peter's work was already ahead of ours; soon afterward, I asked my student to switch to a different project.

BERKELEY: DISCOVERING A NEW ENZYME

Looking back, in the late 1960s, I was simply trying to do too many things too soon. My students and myself, with a strict chemical background and little hands-on experience in enzymology, were riding into the Wild West with no preparation. Perhaps, I also became too fond of my initial ideas and did not think more about other ways of achieving the same goals. My early adventure into DNA enzymology was not a total loss, however, and what I painfully learned became useful when an accidental finding led me to an entirely new line of inquiry that continued for decades.

Throughout my studies of supercoiled DNA rings, one question that constantly popped up in my mind was, Why are DNA rings isolated from natural sources negatively supercoiled? There were two types of models in those days: One postulated a difference in the helical structure of DNA inside and outside a cell, and the other involved the presence of a structurally unique region in an intracellular DNA, such as a "replication bubble" in which the two complementary strands are kept apart by bound proteins. I thought, in 1968, that it would be informative to examine the extent of supercoiling of DNAs of different sizes, all isolated from *E. coli* cells grown under identical conditions. Models of the first category would predict a proportional increase in the number of negative supercoils of a DNA with its size, and models of the second category would predict a constant number independent of size. I proceeded to examine the various DNA rings I could lay my hands on, in the size range of 2 to 50 kb, and I also examined the extents of supercoiling of phage λ DNA rings that had undergone replication and those that had never replicated. The results showed that, although the size of these DNA rings from *E. coli* cells spans a 25-fold range, their extents of supercoiling—the numbers of negative supercoils per unit length—were within a factor of 1.5. These results were duly published in 1969 (17).

But the most significant finding in that 1969 paper turned out to be one buried in Experimental Procedure. In a section with the heading "Enzyme activities in Brij lysate," Brij being the mild detergent used for cell lysis, I stated that, when lysis of cells bearing intracellular λ DNA rings was carried out in the presence of magnesium ions, a small fraction of rings with few

supercoils was also present in addition to the predominant negatively supercoiled form.

I would have missed the minor species but for an incident that occurred on June 28, 1968: A sample of intracellular λ DNA rings prepared on that day was found to contain almost entirely relaxed rather than negatively supercoiled rings! At that time, our daughter Janice was five, and our new baby Jessica was about 10 months old. Sophia called that afternoon for me to take a feverish Janice to a pediatrician. I had just finished assembling a set of centrifuge tubes containing a lysate of λ infected *E. coli* cells, so I placed the tubes in a rotor, started the centrifugation with the timer set to "hold," and rushed home for my fatherly duty. Normally, centrifugation time would have been 20 minutes, and the temperature of the centrifuge would be set to 0°C, but I did not return to the lab until two and a half hours later. Also, in my rush to leave, I did not adjust the temperature control of the preparative ultracentrifuge properly, and the temperature rose to ~20°C. The observation of mostly relaxed DNA rings in that very sample suggested to me that an activity in the lysate had converted the negatively supercoiled λ DNA to the relaxed form. I, therefore, made another preparation of the same DNA according to the standard procedure and carefully analyzed the sample for the presence of minor species by performing a series of sedimentation analyses in the presence of varying amounts of ethidium. The data showed that small amounts of DNA rings with fewer negative supercoils than the predominant species were present even when lysis was done according to the standard Brij lysis procedure in the presence of magnesium ions. I also found that DNA ligase activity was readily detectable in the lysate and suggested in the 1969 paper that a likely interpretation of the presence of small amounts of relaxed DNA rings was that an endonuclease was nicking the negatively supercoiled rings and that the nicks were rapidly sealed by DNA ligase (17).

The problem of uncoiling a pair of intertwined strands when a DNA replicates semiconservatively was well known since the proposal of the DNA double helix structure. With the discovery of covalently closed polyoma DNA rings in the Dulbecco (18) and Vinograd (19) laboratories in 1963, the problem of separating two topologically linked DNA strands during replication was screaming for an answer. Yet, since the mid-1950s, the problem had been largely overlooked. I thought that an endonuclease-DNA ligase pair would make a very nice "mobile swivel" (20); that is, the introduction of transient swivels at strategic locations in a replicating DNA could be an ideal way of allowing the uncoiling of the parental DNA strands. It occurred to me that such an *E. coli* endonuclease would have escaped detection because the presence of excess DNA ligase would mask its activity, but the use of a supercoiled DNA would now make it possible to characterize it. I therefore proceeded to purify this putative endonuclease activity.

I soon concluded, however, that the supercoiled removal activity had nothing to do with DNA ligase, as chromatography on a column of DEAE, a positively charged resin, readily separated these two activities. A series of experiments soon confirmed that a single enzymatic activity was responsible for the removal of the negative supercoils. I called this activity the "ω" protein—ω being the symbol for angular velocity because monitoring this activity had depended heavily on sedimentation velocity measurements of supercoiled DNA in the ultracentrifuge. I also found that the activity was quite robust in a dilute aqueous buffer, containing a few millimolar magnesium ions and nothing else. To me, this lack of a cofactor requirement ruled out any mechanism involving hydrolysis of a DNA backbone bond, for rejoining a pair of hydroxyl and phosphoryl groups in an aqueous solution would be endergonic and thus could not possibly occur in the absence of a cofactor such as ATP or NAD. I therefore postulated a bond swapping mechanism in which a hydroxyl group of an amino acid residue of the enzyme attacks a DNA phosphorus to form an enzyme-DNA O–P bond and breaks at the same time an O–P bond in the DNA backbone; the transiently broken DNA strand could

subsequently be rejoined by essentially the reversal of the first reaction. I also found that the ω protein could remove negative, but not positive, supercoils, and to account for this observation, I postulated that before breaking and rejoining a DNA backbone bond the *E. coli* enzyme must disrupt a short stretch of base pairs in a double-stranded DNA—a process that would be helped by negative supercoiling and opposed by positive supercoiling. All of these bewildering findings and postulates greatly worried the two referees of the paper after its submission to the *Journal of Molecular Biology* in July 1970, but after a few rounds of exchanges through the editorial office, the referees finally relented, and the paper was published in early 1971 (21). A decade and a half would pass before all postulates in the 1971 paper were experimentally proven true.

BERKELEY: KNOTTED DNA RINGS

In the fall of 1970, I started a sabbatical leave and taught two courses at my alma mater in Taipei, before returning to the United States to spend the second half of my leave at Stanford, during which time Dale Kaiser and I identified the gene *A* product of phage λ as a key player in the formation of the λ DNA sticky ends.

Work on the ω protein was left with two of my graduate students. The project again picked up steam when Leroy Liu joined my small group as a graduate student. I first met Leroy when he was a junior in the two courses I taught at the National Taiwan University, and in 1973, he became a graduate student in the UC Berkeley Chemistry Department after completing his military training in Taiwan. With the help of Dick Depew, a postdoctoral fellow in my laboratory, Leroy soon had a breakthrough in showing the presence of the postulated covalent intermediate between the *E. coli* enzyme and a DNA 5′-phosphoryl group (22).

Shortly afterward, he discovered knotted single-stranded DNA rings when he treated a single-stranded DNA ring with the ω protein (23). It so happened that he and I would also identify knotted double-stranded DNA rings a few years later. I was doing some experiments on phage P2 with Rich Calendar in the Molecular Biology Department, and Rich mentioned one day that during phage purification they would also obtain substantial amounts of tailless phage capsids that were of no use to them. I thought that these capsids would be just as useful to my group as intact phage particles because we would be extracting the DNA anyway. When I extracted a preparation of Rich's tailless phage particles with phenol, I immediately noticed that the viscosity of the DNA solution was much lower than expected, suggesting that there was something peculiar with the DNA. I asked Leroy to have a look at it by electron microscopy, and his micrographs showed monomeric P2 DNA rings with a knotted appearance. This knotted P2 structure was soon confirmed by additional experiments (24). Presumably, the joining of the cohesive ends of the compacted DNA inside a capsid is normally prevented by the attachment of one of the ends to the phage tail, but in the absence of the tail, the two ends readily join to form a complicated knot. A decade later, an MD–PhD student, Stanley Shaw, revisited the probability of knotting during DNA ring closure (25) and showed that the two chiral forms of a DNA trefoil could be resolved by gel electrophoresis (26). His high-resolution electrophoresis often ran for days, during which time he was busily attending patients.

HARVARD: DNA TOPOISOMERASES TAKE CENTER STAGE

I became a full professor in 1974, and work was progressing smoothly. But those of us in the Chemistry Department with a strong biochemical orientation were having a great deal of difficulty in persuading others of similar inclination to join us. The tradition and culture of a chemistry department are very different from those of a biochemistry or biology department, and the much heavier teaching load in chemistry was also making recruitment of the biologically

oriented a frustrating exercise. When our only successful biology recruit decided to move elsewhere after only one-and-a-half years of teaching introductory chemistry, I began to think in 1976 that perhaps it was time for me to leave.

I soon received two outside offers. The UC Berkeley Biochemistry Department also invited me to move there instead of across the continent, and the Dean of the College of Chemistry came by to convey my colleagues' wish that I stay. Torn by the different forces, I called up Norman for his advice, and he thought that I should perhaps take the Harvard offer. In the end, such decisions rarely rest on a strictly rational basis, and I accepted the offer from the Harvard Biochemistry and Molecular Biology Department (which decades later merged with its sister department to become the Department of Molecular and Cellular Biology). Some of my friends later put my leaving UC Berkeley in the category of a midlife crisis—I was 39 when the decision was made—but the move did make me very appreciative of all my friends and supporters, both within and outside the UC Berkeley campus.

In April 1977, five of my coworkers and I arrived in Cambridge. Leroy already received his PhD but was persuaded to stay on for another year to help the start of my new laboratory, and the newest member was Karla Kirkegaard, who joined my group during my last days at UC Berkeley, with the understanding that I might soon move to somewhere else. A month later, Sophia and the children arrived, and a few days after their arrival, a rare May snowstorm knocked out power for a week. I was very lucky that they did not go back to California right away.

By then, the study of enzymes that transiently break DNA backbone bonds was flourishing. In 1972, James Champoux and Renato Dulbecco (27) reported an activity in mouse cell extracts that can relax both negatively and positively supercoiled DNA in the presence or absence of magnesium ions. They called the activity the "nicking-closing enzyme." Four years later, Martin Gellert and his associates at NIH (28) discovered an amazing *E. coli* enzyme that catalyzes ATP-dependent DNA negative supercoiling, which they termed DNA gyrase. As evidence accumulated that these enzymes and the *E. coli* ω protein all share the common feature of breaking and rejoining of DNA phosphodiester bonds by a bond swapping mechanism—"transesterification" in the language of chemistry because it involves swapping an ester bond for another ester bond—the time seemed ripe to give them a formal name. Because all of these were characterized by their interconversion of topological isomers or "topoisomers" of DNA rings, I coined the term "DNA topoisomerase" in 1979 for such an activity (29). With the sanction of the Enzyme Commission, the *E. coli* ω protein became *E. coli* DNA topoisomerase I, the mouse nicking-closing enzyme became mouse DNA topoisomerase I, and *E. coli* DNA gyrase became *E. coli* DNA topoisomerase II—but the name gyrase remained popular.

Once the chaos of relocating the laboratory quieted down, research resumed its steady pace. Leroy became excited about DNA gyrase, and he proceeded with a large preparation of the enzyme from kilograms of lyophilized *Micrococcus luteus* cells that were commercially available. He found that purification often yielded very little active enzyme because its two subunits readily dissociated, but large amounts of it could be obtained by reconstitution of the fractions. He soon made a very significant finding that gyrase-DNA interaction involves the right-handed wrapping of a 140-bp-long DNA segment around the enzyme (30, 31), and later studies show that this wrapping is closely related to the unique ability of gyrase to negatively supercoil a DNA ring. His and Karla's experiments also showed that gyrase would make a pair of 5′-staggered breaks near the center of this 140-bp fragment and that the flanking regions of the 140 bp are wrapped outside the enzyme (31, 32). Lenny Klevan, a postdoctoral fellow, was able to purify particles each containing a dimeric gyrase and a 140-bp-long DNA fragment, which he fondly called a "gyrasome" (33).

On the *E. coli* DNA topoisomerase I front, Yuk-Ching Tse, a graduate student in the

Harvard Chemistry Department who chose to do her PhD thesis with me, showed that the enzyme-DNA covalent intermediate involves the formation of a phosphotyrosine bond (34). Her finding was very exciting to those of us interested in the reaction mechanisms of the DNA topoisomerases, but to many others, it was the phosphotyrosine she had to synthesize as a marker in her work that attracted their attention: Protein tyrosine phosphorylation was then a hot topic, and there was apparently some difficulty in obtaining the compound from commercial sources! A few years later David Horowitz, who joined my group after graduating from Caltech, devised a very nice method for determining which particular tyrosine of a topoisomerase is involved in forming the covalent link (35). Karla also made my day by showing that *E. coli* DNA topoisomerase I can relax a positively supercoiled DNA if a short single-stranded loop is engineered into the DNA (36), which proved what I had proposed a decade and half earlier, namely that the binding of the enzyme to a single-stranded stretch is responsible for its relaxation of negatively and not positively supercoiled DNAs (11).

My coworkers and I were also working on the identification of the *E. coli topA* gene encoding DNA topoisomerase I. Before my move from UC Berkeley, Dick Depew screened tens of thousands of colonies from a heavily mutagenized pool of *E. coli* to look for a thermal-sensitive mutant enzyme to guard against the possibility that the enzyme might be essential, but no good candidates were found. Dick and others in my laboratory also found that *E. coli*, *M. luteus*, and *Salmonella typhimurium* DNA topoisomerase I were readily distinguishable immunologically. By the late 1970s, recombinant DNA approaches were becoming popular, and I thought that we could screen for the structural genes encoding bacterial DNA topoisomerase I by examining *E. coli* cells expressing plasmids bearing *M. luteus* or *Salmonella typhimurium* DNA fragments or *Salmonella* cells expressing plasmids with cloned *E. coli* DNA fragments. The experiments were foiled, however, by the Cambridge City Council, which banned recombinant DNA work in Cambridge.

In collaboration with Rolf Sternglanz at Stony Brook, we decided to screen a collection of heavily mutagenized *E. coli* cells prepared by Yuki Hirota and his associates in Japan. Rather than the use of a sophisticated spectrophotometric assay, which Dick and I devised earlier for screening tens of thousands of samples, for this round, we just assayed the relaxation of a negatively supercoiled DNA by agarose gel electrophoresis for ~800 individual cell lysates. We found two *topA* mutants within this set, and the gene was duly mapped and studied (37). Kathy Becherer and I then went about cloning the gene (38), and its nucleotide sequence was determined by Yuk-Ching a few years later (39).

HARVARD: BUGLE CALL AND BACK TO DNA SUPERCOILING AND STRUCTURAL TRANSITIONS

About a year after my arrival at Harvard, Francis Crick contacted me about whether he might interest me in doing a simple experiment. In the late 1970s, several groups had challenged the double helix structure of DNA, and their views had sufficiently alarmed Francis. Francis thought that studies of supercoiled DNAs had already shown that DNA outside, as well as inside, living cells must be in a double helix form, and this supercoil-based argument would be watertight if a little control experiment could be added. In 1975, Walter Keller had shown that small DNA rings differing only in their linking numbers could be resolved by electrophoresis in an agarose gel (40), and during the same year, both Dick Depew and I and Vinograd and his associates had used Keller's method to study a population of DNA linking number topoisomers in thermal equilibrium (41, 42). Francis pointed out that in all such experiments we had taken for granted that the bands resolved by gel electrophoresis were linking number topoisomers and that the missing control experiment was one showing that the gel electrophoretic

mobility of such a band would be the same after a cycle of heating and cooling because a basic property of the linking number is that it cannot be altered by all changes short of transient breakage of DNA backbone bonds.

I had never taken the challengers of the double helix structure seriously, and there was not a shred of doubt in my mind that the topoisomers resolved by gel electrophoresis could only be linking number topoisomers. But the least anyone could do to pay his or her tribute to a living legend in the DNA world would be to rise to his bugle call, and so I did such an experiment and told Francis the result over the phone that there was no surprise. But a few months later, he called and said that he had also asked Bill Bauer at Stony Brook to try the same control, and he thought that perhaps both Bill and I could send him the actual experimental data for a joint publication (43). Alas, by that time I had already tossed my gel photographs, and I had to repeat that control again!

Around the same time, I was actually doing an experiment on the precise helical periodicity of DNA in solution. At a 1977 meeting at Cold Spring Harbor, Aaron Klug and Francis suggested that perhaps the number of base pairs per helical turn of a DNA in solution was not precisely 10.0, but no known experimental method could determine this quantity with sufficient precision. While I was at a meeting in Sicily in 1978, I thought of two ways of precisely determining the DNA helical periodicity; both were based on the topological properties of DNA rings. By 1979, I was able to show that the periodicity of a DNA of a typical sequence is very close to 10.5 bp per helical turn, with the precise number depending on the temperature and on ionic conditions (44). A couple of years later, by constructing plasmids with short inserts with different base sequences, a graduate student, Larry Peck, and an undergraduate student, Albert Shaw, showed that only DNA with As on one strand and Ts on the other has a periodicity very close to the integer 10.0, and all other sequences show a periodicity close to 10.5 bp per helical turn (45). Similar results were independently obtained in Aaron Klug's laboratory from the sites of nucleolytic cleavage of DNAs adsorbed to a flat surface (46).

In collaboration with Alex Rich and his associates at MIT, who discovered a left-handed Z-helical form of DNA in 1979, Larry Peck was able to confirm the expected result that negative supercoiling of a DNA could drive the flipping of a stretch of alternating CG sequence from the normal right-handed B-helical form to the left-handed Z-helical form; he also showed, by the use of two-dimensional gel electrophoresis, that this flipping could be revealed by a dramatic jump in the electrophoretic mobility of a DNA ring when its linking number is gradually reduced (47). Albert Courey, another graduate student in my lab, used the same approach to study the energetics and kinetics of extruding a pair of hairpins from a sequence with twofold symmetry (48). Albert and Sharon Plon, an MD-PhD student, went on to test how an enhancer sequence affects the expression of a far away human β-globin gene promoter. Their results argued against the involvement of DNA supercoiling and suggested that the enhancer-mediated gene transcription depended strongly on the structure of the DNA between the enhancer and the promoter (49, 50).

HARVARD: THE TWO-GATE CLAMP MODEL OF TYPE II DNA TOPOISOMERASES

In many ways, the year 1979 was a very exciting one in the study of DNA topology and DNA topoisomerases. By then, Leroy Liu was already busily working as a postdoctoral fellow in Bruce Alberts's laboratory at the University of California in San Francisco. He was supposed to study mRNA localization in *Drosophila* embryos, but his passion for DNA topoisomerases led him to play with, in his spare time, proteins that are involved in phage T4 DNA replication—a subject that had been extensively studied in Bruce's laboratory. Leroy soon made an amazing discovery that the T4 gene 39, 52, and 60 proteins constitute an ATP-dependent DNA topoisomerase that catalyzes the formation of reversible double-stranded breaks in

a DNA segment for the passage of another double-stranded DNA segment. Although the phage T4 enzyme is similar to gyrase in its ATP dependence, it differs from bacterial DNA gyrase in that the phage enzyme relaxes positively or negatively supercoiled DNA but does not supercoil DNA (51, 52). Independently, Patrick Brown and Nicholas Cozzarelli, at the University of Chicago, showed in an elegant experiment that DNA negative supercoiling by *E. coli* DNA gyrase also involves a double-stranded DNA breakage-rejoining mechanism (53). The DNA topoisomerases were hence divided into two types: the type I enzymes break and rejoin one DNA strand at a time and the type II enzymes break both strands in concert and then rejoin them after passing another double-stranded DNA through the transient break. Several laboratories soon found that the type II enzymes are widely present in bacteria, eukarya, and archaea, and it turns out that they can be further classified into the type IIA and IIB subfamilies; the latter was first found in archaea (54). Among the type I enzymes, those that fall in the *E. coli* DNA topoisomerase I category have been termed the type IA DNA topoisomerases, and those in the mouse DNA topoisomerase I category are now the type IB DNA topoisomerases (55).

In my laboratory, it was Tadaatsu Goto, a graduate student, who began our long quest to understand how a type II DNA topoisomerase works and what might be its cellular functions. Tad and I picked the budding yeast to get us started, and Tad was soon purifying the enzyme from kilograms of commercial yeast paste (56). He then prepared rabbit antibodies against the yeast enzyme and proceeded to identify the gene encoding the enzyme by reverse genetics. He was able to show that the *TOP2* gene encoding the enzyme is a single-copy essential gene (57). Then, in collaboration with Connie Holm and David Botstein at MIT, he showed that the essentiality of the sole type II DNA topoisomerase in the budding yeast lies in its separation of intertwined pairs of chromosomes during mitosis (58). His work and research independently carried out in the laboratories of Mitsuhiro Yanagida and Rolf Sternglanz (59, 60) laid the foundation of understanding the cellular roles of the type II enzymes. After receiving his PhD, Tad went on to study gene regulation in the fruit fly, first as a postdoctoral fellow with Tom Maniatis and then as a faculty member at Thomas Jefferson Medical School, but his career was tragically cut short when he succumbed to cancer.

It was in the early 1990s when I began to think about the molecular movements responsible for the transport of one DNA double helix through another by a single type II DNA topoisomerase. The diameter of a DNA double helix is about 20 Å; thus, for it to move through a transiently broken DNA, the enzyme domains covalently linked to the pair of broken DNA ends must move by at least 20 Å relative to each other. How might a single enzyme molecule manage such a large movement?

I came upon an idea that perhaps the enzyme might behave like an ATP-modulated molecular clamp: ATP binding to a pair of jaws in a protein dimer would trigger their coming together and clamp closure, and following ATP hydrolysis, the clamp could reopen. I persuaded Joaquim Roca, a postdoctoral fellow then frustrated by his experiments on probing the nucleoprotein structures inside yeast cells through the use of a photocross-linking agent psoralen, to test the protein clamp idea. Joaquim's experiments soon lent credence to such a model (61). But the question of how the second DNA double helix might exit the DNA-bound protein clamp after its passage through the DNA gate remained unanswered.

As far back as 1980, Mizuuchi and Gellert and coworkers, and my associates and I, had independently raised the possibility that a DNA-bound type II DNA topoisomerase might operate two separate protein gates, one for admitting a second DNA and the other for exit of the admitted DNA after its passage through the enzyme-bound DNA (62, 63). For many years I tried to persuade every new member of my group to link the two halves of a type II enzyme with a DNA tether, for example, by fusing a sequence-specific DNA binding

domain to the C terminus of yeast DNA topoisomerase II, because I thought that the two-gate model would predict that bridging the two halves in any way would prevent its relaxation or supercoiling of a DNA ring and that tethering the two halves with a short piece of DNA bearing a unique restriction endonuclease cleavage site could provide a way of locking and unlocking the putative exit gate. But there had been no takers. Joaquim came up with an alternative test, however. He thought that the two-gate and one-gate models might be distinguished by the one-step unlinking of two singly linked DNA rings. If a nonhydrolyzable ATP analog ADPNP is added to a type II DNA topoisomerase bound to one of the two rings in a singly linked catenane, the entrance gate would be permanently closed, and at the same time, there would be a certain probability for the unlinking of the component rings of the dimeric catenane. For the catenated rings that had undergone decatenation, the one-gate model would predict that both unlinked rings would remain locked inside the closed protein clamp, but the two-gate model would predict that one of the two unlinked rings would escape the protein clamp through its exit gate. His experiments very clearly showed that the type II DNA topoisomerase acts according to the two-gate model (64).

ACADEMIA SINICA: THE TWIN-SUPERCOILED-DOMAIN MODEL OF TRANSCRIPTION

In the mid 1980s, I became involved in setting up a molecular biology research institute at the Academia Sinica campus in Nankang, on the outskirts of Taipei. Several members of Academia Sinica, the highest government-funded research organization in Taiwan, thought that it was high time to emphasize molecular approaches in biomedical research on the island, and the government agreed to add two new research institutes, the Institute of Molecular Biology (IMB) and the Institute of Biomedical Sciences, to the 16 others in sciences and humanities at that time. Two members of the IMB planning committee came to my Lexington, Massachusetts, home one evening to persuade me to spearhead the task, and I surprised them by agreeing to commit one full year of my time to that mission, which later stretched to 14 months. I had always thought that I should contribute something to Taiwan to repay the free education I received there, and I could not really count my sabbatical semester there in 1970 because almost all of the students I taught there ended up in the United States.

I tried to persuade five young U.S. faculty members, who had received their early training in Taiwan (Tao-Shih Hsieh at Duke, Gloria Li at UC San Francisco, Leroy Liu at Johns Hopkins, James Shen at UC Davis, and David Tu at Penn State), to synchronize their sabbatical leaves and join me in this one-year expedition. Among the five, I had known Tao, Leroy, and James since my UC Berkeley days; Tao and Leroy did their graduate research with me; and I chaired James's PhD thesis committee while he was a student in John Hearst's group. I was greatly relieved when all five agreed, and Sophia also resigned her position at the Harvard Business School Library to accompany me. By the time we all arrived in Nankang to a brand new but empty building, we were joined by one senior and five tenure-track recruits.

All of us were awfully busy during our stay in Nankang, especially during the beginning months when many staff and technician positions had to be filled, new pieces of equipment unpacked and set up, supply lines established, and various reagents acquired. When it seemed that the quality of water might be responsible for our difficulty in culturing mammalian cells, many of our visitors from abroad would carry water they used for media preparation in their own laboratories. I could well imagine the puzzled and skeptical look of the custom service agents when they encountered these foreign scientists carrying strange looking containers, all claiming that it was just water inside!

But we also had ample time to discuss science when all the preparative work delayed the start of our experiments. One outcome of these discussions was the twin-supercoiled-domain

model of transcription that Leroy and I later published in 1987 (65). We were very curious about the results reported by Pruss & Drlica in their 1986 paper (66) that transcription of the *tetA* gene in a plasmid pBR322 was responsible for its extremely high degree of negative supercoiling in an *E. coli topA* mutant lacking DNA topoisomerase I. It was expected that inactivating a topoisomerase that removes negative supercoils would lead to hypernegative supercoiling of intracellular DNA, but why the specific dependence on the transcription of a particular gene? We knew about the suggestions that, as a transcription assembly R (including the RNA polymerase, the nascent RNA, and proteins associated with the RNA) moves along a DNA template, it would have to circle around the DNA because of the helical geometry of the DNA template. We also realized that if R is prevented from doing so then the DNA would be forced to turn around its helical axis to form positive supercoils ahead of R and negative supercoils behind R. Therefore, we suggested that in *E. coli* cells positive and negative supercoils are normally removed by different DNA topoisomerases, and thus, the lack of an enzyme that specifically removes negative supercoils in a *topA* strain would lead to the accumulation of negative supercoils in a DNA template.

The model has since been well substantiated, and the dependence on *tetA* transcription has largely been accounted for by the very efficient localization of the nascent tetA polypeptides to the cell membrane, which anchor the RNA polymerase to the membrane through the nascent mRNA tether (67, 68). The twin-supercoiled-domain model of transcription injected new insight into the interdependence between DNA supercoiling and transcription and other cellular processes involving the tracking of a macromolecular assembly on DNA, and I was particularly pleased that the paper reporting this model was among the very first that came out of the new institute in Nankang.

Several other findings were also made in my Nankang laboratory that year. Monika Pflugfelder, who received her PhD in Switzerland and flew directly to Nankang to work with me as a postdoctoral fellow, was able to clone and sequence a human gene encoding a human type II DNA topoisomerase (69). John Nitiss, another postdoctoral fellow who joined my laboratory in Nankang that year, developed a very powerful yeast genetic system for the study of the anticancer drugs targeting the DNA topoisomerases (70); by then, the DNA topoisomerases had been established as the targets of many antibiotics and anticancer drugs (71–75), thanks to the work of Marty Gellert and his associates shortly after the discovery of gyrase (71, 72) and to the work in Leroy's laboratory at Hopkins (73–75). John also had the not-so-enviable task of turning off the lights of my Nankang laboratory as the last one of my group to leave Nankang when our year there came to an end. The Institute of Molecular Biology continued to grow, however, and within a decade, James Shen, one of the five who went there with me, returned to serve as its Director for nine years.

HARVARD: THREE-DIMENSIONAL STRUCTURAL STUDIES

My wish to see the three-dimensional structures of DNA topoisomerases had an early history. In the mid-1970s when Helen Berman came to spend a sabbatical leave in my laboratory, we started a collaboration to crystallize *E. coli* DNA topoisomerase I. Almost everyone in my group joined an effort to make a large preparation of the enzyme from the "side fractions" that Paul Modrich generously gave us from his preparation of EcoRI restriction endonuclease from several thousand liters of *E. coli* cells. We obtained about 90 mg of the topoisomerase, but the preparation refused to crystallize.

In the late 1980s, the tide shifted. The dawn of the recombinant DNA era, and our success in cloning the genes encoding the DNA topoisomerases, made things much easier. Alfonso Mondragon, then a postdoctoral fellow in my colleague Steve Harrison's laboratory, started a collaboration with me to solve the crystal structure of *E. coli* DNA topoisomerase I, and

after he joined the faculty of Northwestern University, he and his graduate student, Chris Lima, finally succeeded in 1993. The intricately folded structure of a large fragment of the enzyme is a beauty to any beholder, and it provided an invaluable structural basis for the mechanistic insight we gained over the two decades before (76).

I was also delighted in 1990 when a newly arrived graduate student, James Berger, came to see me and told me that he had always wanted to solve the three-dimensional structure of DNA topoisomerase II. Several years and 26 preparations of the yeast enzyme later, he managed to solve the crystal structure of an 800-amino acid–long fragment of the enzyme in collaboration with Steve Gamblin of Steve Harrison's laboratory (77). This beautiful structure showed us one of nature's most elegant designs. From it, plus a 1991 structure of the *E. coli* gyrase ATPase domain obtained by a York University group in England (78) and the electron microscopy work done by my collaborator Piero Benedetti (79) and Ronald Hancock and his collaborators (80), one can literally see the operation of the two-gate model we painted earlier: How an enzyme dimer might bind a DNA double helix, how the pair of ATPase domains of the dimer would come together to close the entrance gate and capture a second DNA double helix that had entered the entrance gate, how the captured DNA was moved through the enzyme-mediated DNA gate into a large central hole enclosed by the enzyme, and how the moored DNA segment could be expelled through the exit gate (81). The crystal structure also showed the three-dimensional details of the putative exit gate, and how we might lock this exit gate to trap the second DNA double helix that had entered the enzyme and passed through the DNA gate. On the basis of the crystal structure, James mutagenized two amino acid residues of the yeast enzyme to cysteines and showed, together with Joaquim Roca, that disulfide bond formation between each of the two cysteines and its symmetry-related partner would indeed lock the exit gate (82). Their experiments completed our work over a period of 16 years with the two-gate model. James later became a faculty member at UC Berkeley and continued his beautiful structural studies of DNA enzymes.

HARVARD: FROM YEAST TO MICE

Earlier functional studies of the DNA topoisomerases in my laboratory were largely limited to *E. coli* topoisomerase I, but by the 1990s, we had also ventured into functional studies of the yeast DNA topoisomerases through the efforts of Tad Goto, Ray Kim, John Nitiss, Mary-Ann Bjornsti, and several others. Whereas the baker's yeast provided a superb system for combining biochemical and genetic studies, I felt that studies of the mammalian enzymes, including genetic analysis of their cellular roles, would be helpful in achieving a better understanding of the actions of the topoisomerase-targeting drugs and in laying a firmer foundation for the future development of drugs of this class. These goals prompted the identification, cloning, and sequencing of the human *TOP2α* gene encoding DNA topoisomerase IIα by Monika Tsai-Pflugfelder (69), the cloning and expression of the human *TOP1* gene encoding DNA topoisomerase I in collaboration with Leroy Liu's group (83), the cloning and expression of the human *TOP2β* gene encoding DNA topoisomerase IIβ in collaboration with Caroline Austin at the Newcastle University Medical School (84), and the identification and cloning of the human *TOP3α* gene encoding DNA topoisomerase IIIα by Ryo Hanai and Paul Caron (85).

With the cloned human genes and their nucleotide sequences in hand, my laboratory entered an adventure into gene knockout studies of their counterparts in mice in the late 1990s. Around that time, Oliver Smithies' laboratory reported a knockout study of the mouse *TOP1* gene, and their results showed that inactivation of this gene led to a very early death of the mutant embryos, in contrast to the dispensability of the *TOP1* gene in yeasts (86). On the basis of what had been already learned about the crucial

role of mammalian DNA topoisomerase IIα in chromosome segregation, I was convinced that mouse *top2α* knockouts lacking DNA topoisomerase IIα would fare no better than the *top1* knockouts, and therefore, I persuaded Wei Li to start with knocking out the mouse *TOP3α* gene. Wei was a PhD student in chemistry, but he did not seem to have any difficulty in forging ahead into biology in an area completely new to my group. He soon found out that despite the presence of DNA topoisomerase IIIβ, which is enzymologically very similar to the IIIα isozyme, mouse embryos lacking DNA topoisomerase IIIα would expire around the time of their implantation into the uterus wall (87). By contrast, mouse embryos lacking DNA topoisomerase IIIβ, obtained by Kelvin Kwan, another graduate student, are viable and develop to maturity with no apparent defect (88). It took much patience and work on Kelvin's part to discover that the apparent normalcy of the *top3β* mice was deceptive. As they age, these mutant mice exhibit progressively worsening inflammatory responses in multiple organs, leading to a much shortened life span. Kelvin also found, in collaboration with Peter Moens at York University in Toronto, a progressive reduction in the fecundity of the *top3β* mutant mice over time and through successive generations (89). We attributed the complex physiological consequences of inactivating the enzyme to its role in the resolution of chromosomes with entangled strands, which is consistent with a high incidence of chromosome loss observed in spermatocytes, splenocytes, and bone marrow cells of the mutant mice (89).

The physiological importance of the mammalian DNA topoisomerases was further underscored by studies of the IIβ enzyme, again initiated by Wei, who found that, although embryos lacking this enzyme can develop to term, they die at birth (90). He observed that these newborns showed no sign of any muscular movements before they expired, which suggested to us that they probably suffered a muscular and/or neural defect. I therefore contacted Steve Burden at New York University Medical School about a plausible collaboration, and our joint experiments soon established that the mutant mice indeed showed neuromuscular defects. Innervation of the diaphragm muscles by motor axons, for example, is defective, leading to a breathing impairment and death at birth (90).

The earlier studies of the *top2β* mice led Y. Lisa Lyu, who did her PhD research with Leroy and joined my group as a postdoctoral fellow, to study the role of the IIβ enzyme in brain development. Lisa found that cerebral stratification is abnormal in the mutant brain; neurons born at later stages of corticogenesis, for example, fail to migrate to the superficial layers (91). We attributed such abnormality to the effects of DNA topoisomerase IIβ in gene expression in postmitotic cells. Such a notion is consistent with the observation that expression of *Reelin*, a gene known to affect neuronal migration during the formation of the cortical layers in the neocortex, was indeed lower in fetal brains lacking the IIβ enzyme. In collaboration with Leroy's group, we also found that the expression of many other developmentally regulated genes is affected by IIβ inactivation (92). A molecular mechanism for the specific effects of the topoisomerase is still lacking, however.

Lisa also initiated the construction of a skin-specific *top2β* knockout line, in which the IIβ enzyme is absent only in the skin cells of the mutant mice (93). She soon found that these animals exhibited no defect other than a periodic hair loss over much of their bodies. Presumably, this alopecia phenotype is a manifestation of changes in gene expression in the hair follicles, and thus, we are again back to the question of how a type II DNA topoisomerase affects the expression of certain genes and not others. By then, however, the clock, which I had started ticking five years earlier for my planned retirement, was rapidly approaching its preset mark, and this time it was my turn to turn off the lights of my laboratory.

CONCLUDING REMARKS

I had my first encounter with research in the winter of 1958 while doing an undergraduate thesis on the synthesis of levulinic acid from

sucrose; there was then much discussion on how to make good use of the surplus cane sugar in Taiwan. Fifty years later, I was amused to read that levulinic acid had again popped up in discussions on biofuels and renewable raw materials. Half a century may be a long stretch, but it surely went in a flash!

Despite the havoc in my early life, by all criteria my career has been a smooth and uneventful one. I have immensely enjoyed the freedom of gazing into the unknown and experimentally testing conjectures that at times seemed farfetched. In the later years of my career, my guilty feeling for spending public funds to satisfy my own curiosity had all but evaporated when the topological properties of DNA turned out to be relevant in nearly all processes involving DNA, when the DNA topoisomerases were shown to serve critical roles in many vital processes, and when the DNA topoisomerases became well-known targets of many antibiotics and anticancer drugs.

Yet for both philosophical and personal reasons, I always had my reservations about perpetual motion, and I always believed that retirement in "due time" would be the right thing to do. I also deduced decades ago by observing others that the due time must be set way ahead of the execution point if one is to actually carry it out rather than leave it to unpredictable events.

Thanks to Harvard, access to all of its electronic subscriptions has provided an ideal medium for me to enjoy science vicariously after retirement. Writing this chapter gave me an opportunity to reflect on the past decades, and during this backtracking, I became even more appreciative of Sophia's tolerance during the long years when I spent almost all my time in the laboratory.

I would be overstretching too much the goodwill of the *Annual Review of Biochemistry* editors and the patience of the readers if I were to mention all of the unmentioned studies my coworkers and I did, but those studies, just like the ones I briefly recalled here, remain a particularly treasured part of my memory.

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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