DNA LOOPING

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INTRODUCTION

DNA looping is generated by a protein or complex of proteins that simultaneously binds to two different sites on a DNA molecule. Consequently, the intervening tens to thousands of base pairs of DNA loop out. This seemingly simple phenomenon is central in the regulation of many biochemical transactions involving DNA. The most prominent current examples of looping are in the regulation of the expression of prokaryotic and eukaryotic genes, regulation of site-specific recombination, and in the regulation of DNA replication.

WHY NATURE USES DNA LOOPING

Multiple proteins can be required to regulate properly the activity of a complex on DNA. For example, transcription of a gene could require the simultaneous presence of four different conditions, the status of each of which must be transmitted by a protein sensor to the initiation complex. In order that the protein sensors, which we normally call regulatory proteins, confine their activities to the correct genes, they bind to specific sequences located near the genes to be regulated. There is space for only two or three proteins to bind to DNA alongside an initiation complex. DNA looping permits additional proteins to bind in the vicinity of the complex and to interact with it. Multiple proteins could also assist or interfere with the interactions necessary for looping. Thus, a rich diversity of regulation systems can be expected to utilize DNA looping.

DNA looping also generates cooperativity in the binding of a looping protein to the two DNA sites involved. When a protein has bound to one of the two DNA sites, it is held near the other DNA site because the furthest the two sites can separate is the length of the DNA between the two. This tethering of one site to another increases the concentration of the protein near the second site and generates cooperativity in the binding to the two sites—whenever one site is bound by the protein, the other tends also to be bound.

The cooperativity resulting from DNA looping permits proteins to saturate their binding sites even though the protein concentration is well below the dissociation constant of the protein from an individual site. Thus, the microscopic dissociation of the protein from a single site can still occur, but overall, the protein remains at the two sites involved. Another way to view this phenomenon is that the protein does not come free of the DNA and diffuse away from the vicinity of the binding sites unless it lets go of both DNA-binding sites simultaneously. As a result, comparatively low concentrations of proteins are required to saturate DNA-binding sites involved with looping, and the sites need not be constructed to possess particularly high affinities for the proteins.
Why would nature want to saturate DNA sites while using low concentrations of protein, and without building the DNA sites with particularly high affinities? One answer seems to be that a large number of regulatory proteins, perhaps 50,000 in a eukaryotic cell, must simultaneously be present in the nucleus. Therefore, the concentration of any single one must be low, perhaps $10^{-10}$ M. While this concentration of protein could saturate sites with dissociation constants of $10^{-11}$ M, binding many proteins to DNA with such a high affinity could well interfere with other DNA processes such as replication and recombination. DNA looping takes care of this problem through the cooperativity. The DNA sites involved can have individual dissociation constants for the protein much lower, say $10^{-9}$ M, but by virtue of looping, pairs of such sites would still be nearly fully occupied. Thus, by virtue of looping, the objectives of low concentrations of the proteins, binding sites that do not weld the proteins to the DNA, and nearly complete occupancy of the binding sites can all be simultaneously achieved.

At one extreme DNA looping is generated by one protein with two DNA-binding sites. At the other extreme, two different proteins bind the two different sites, and because they are then held near one another by the DNA, they bind to each other and form a loop. Therefore, we can expect DNA looping to be generated by a single protein, by an oligomeric protein, or by two proteins that only associate at high concentrations or in the presence of DNA containing their DNA-binding sites.

BRIEF HISTORY AND OVERVIEW

DNA looping was an unanticipated discovery. In fact, it was sufficiently unexpected that a number of clues to its existence were overlooked in the years preceding its discovery. When it was finally proposed and presented with strong supporting evidence, the idea was sufficiently alien that it was not readily accepted for publication. Once reasonably solid data did get published, however, acceptance of looping came rapidly because it aided understanding of several widely observed phenomena.

The clue that motivated the experiments that ultimately uncovered DNA looping was an observation made by Englesberg on the arabinose operon in *Escherichia coli* (1). He obtained genetic evidence for a site lying upstream of the araBAD promoter that acted to depress activity of the promoter. A long series of studies followed that were aimed at determining the mechanism by which repression in the ara system could operate from the upstream site. Ultimately, it was found that this was accomplished by protein bound to the upstream site as well as to a site beside the promoter 210 base pairs downstream (2–9). Subsequently, DNA looping has been shown to function in a wide variety of other systems, both prokaryotic and eukaryotic.
The objective of this review is to give the reader an understanding of how and why looping occurs, and what it has been found to accomplish. First, the various assays that have been used to detect and measure looping are described. These different methods give an idea of the richness and diversity of the looping phenomena, as they range from detecting changes in the conformation of the DNA involved in looping to measuring changes in the regulation of genes in growing cells. As the heart of looping is the physical chemistry, an overview of the physical basis for looping is given. Then, a number of important biological systems are described in which looping plays a prominent role. This review does not contain an exhaustive list of references relevant to DNA looping; the available electronic bibliographic databases can provide this for the interested reader.

ASSAYS OF LOOPING

Helical-Twist Experiments in vivo and in vitro

A variety of assays have been developed to detect and quantitate DNA looping. The first of these, used with the intention of demonstrating looping, was the helical-twist experiment in the arabinose operon (4). In this type of experiment the two sites involved in looping are rotated around the axis of the cylindrical DNA with respect to one another by the insertion or deletion of differing amounts of DNA between the sites (Figure 1, Top). If the two sites are positioned for easiest looping, insertion of five base pairs between them rotates one site with respect to the other half a turn around the DNA. As a result, the formation of a loop now requires not only bending the DNA to bring the sites near one another, but also twisting of the DNA to bring the sites to the correct face of the DNA. Because DNA resists torsional stress, such a positioning increases the energetic costs of looping, thereby decreasing its frequency of occurrence. Of course, the amount of interference with looping depends upon the size, shape, and flexibility of the proteins involved, the distance separating the binding sites, and the energies available for looping. The most convincing helical-twist experiments measure the responses to large numbers of spacing changes and show multiple cycles of oscillations as the sites are positioned alternatingly to favor and disfavor looping (Figure 1).

The conceptual basis for helical-twist experiments comes from DNA cyclization experiments first done by Shore, Langowski, & Baldwin (10). For DNA several hundred base pairs in length, the cyclization rate of linear molecules to covalently closed circles in the presence of DNA ligase depended not only on the total length of the DNA, but also on the relative phases of the short single-stranded ends. Misphasing the ends by half a helical turn dramatically decreased the cyclization rate.
DNA LOOPING

Figure 1  Top. Two DNA-binding sites on the same face of the DNA favor DNA looping. Insertion of five base pairs between the sites rotates one of the sites half a turn around the DNA axis so that now the proteins are on the opposite sides of the DNA and looping is disfavored. Bottom. The DNA looping abilities of multiple spacings between the protein-binding sites oscillates with a period equal to the helical repeat of the DNA.

Electrophoresis of Looped Small Circles

The binding of proteins to sites located on small supercoiled DNA molecules of about 400 base pairs, as well as electrophoresis of the complexes, has proven to be a particularly useful tool for studying DNA looping (11). The power of the assay results from the fact that weakly looping systems can be studied. This results from the fact that the supercoiling places the sites in very close proximity even in the absence of the protein. As long as the sites are located on the proper face of the DNA, little of the protein-DNA or protein-protein binding energy needs to be expended to bend the DNA or position the sites so that the protein can simultaneously contact both.

The assay built upon gel retardation experiments in which the binding of a protein to DNA can be detected by performing electrophoresis under conditions where the protein remains bound during the electrophoretic run. Normally in these experiments the protein-DNA complex migrates more
slowly than the DNA molecules free of protein. On small supercoiled circles such retardation can be seen when the DNA contains only a single binding site for a protein. When looping between two sites on the circle occurs, however, the complex may migrate faster than either the free DNA or the DNA with protein bound to a single site (11). The reason for the mobility increase when two operators are present must be that looping between the two sites holds the DNA in a highly compact structure.

Although the dependence on both protein-binding sites for the existence of the altered mobility species suggests DNA looping, additional properties strengthen this conclusion. First, the complex is much more stable when two binding sites are present than when only a single site is present (11, 12). That is, its dissociation rate is much reduced when the two sites are present. Second, the increased stability and faster mobility properties show helical-twist effects when one site is rotated to the opposite face of the DNA.

**Generation of Sandwich (Crossjoined) Structures**

In DNA looping, a protein or complex of proteins binds to two DNA sites. There is no fundamental reason that the same two sites could not be on two different DNA molecules. Then, the DNA molecules would be held together by the protein(s), and a sandwich structure would be formed. The detection of such sandwiches is easy in the gel retardation assay in which proteins remain bound to DNA during the electrophoresis. Unfortunately, such sandwiches are hard to form. As described above, one reason looping occurs is because the two DNA sites are held in the vicinity of one another by the intervening DNA. This greatly reduces the entropic costs of loop formation. When the two sites are on different molecules, the entropic costs of forming the sandwich are high. Therefore many looping systems lack sufficient binding energy to form sandwiches, although their presence has occasionally been used to support conclusions that a system loops.

**Altered Structure of DNA in Loops**

Looped DNA has to be bent. When the loop is small enough, the bending will be great enough to affect substantially the widths of the major and minor grooves of the DNA. These width changes can be revealed by DNaseI digestion since the activity of this enzyme is sensitive to the width of the minor groove (13, 14). The regions facing inward will be so compressed that the enzyme will have little activity there, but portions of the regions facing outward will be suitably spaced for rapid attack by the enzyme, and will be readily cleaved. In some cases then, the pattern of sites that have gained and lost DNaseI sensitivity reflects the helical repeat of the DNA and shows periodicities of 10 or 11 base pairs.

A dramatic example of this phenomenon is an artificial looping situation
involving lambda phage repressor (15). Normally the repressor dimers bind to DNA alongside one another and also interact with one another via protein-protein interactions. These protein-protein interactions are sufficiently strong that if the binding sites are moved 55 base pairs apart, the proteins will still interact with one another and in doing so, will form a DNA loop. The region between the binding sites shows areas of strongly protected and enhanced DNase sensitivity.

Looping can alter not only the conformation of DNA lying between the two protein-binding sites, but it can also alter DNA beyond the looped region. The holding of the two protein-binding sites near one another directs the segments of DNA coming into the loop and leaving the loop to lie in similar directions. This constraint tends to hold the ends of the DNA near one another. Such a proximity increase can increase the rate at which the ends will ligate together when DNA ligase is added (16). Such ligation rate effects have been observed in a looping system comprising a DNA replication protein from plasmid RP6 (16).

**Electron Microscopic Observation of Looping**

DNA looping formed by protein binding to two sites separated by more than about 50 base pairs should be readily observable by electron microscopy. Such looping has been observed in a number of systems (17–21). Although seeing is close to believing, the labor of obtaining statistically valid data has greatly limited the use of microscopy in looping studies. Careful control experiments are also necessary in this type of experiment because random aggregation of protein that has bound to the correct sites can generate a loop, but one without biological significance.

**Cooperativity from a Distance**

As described earlier, the binding of a bivalent protein to one site on the DNA increases the concentration of the protein's second DNA-binding site to nearby DNA sequences. Simultaneous binding to a second site generates looping. The affinity of the second site for the protein can be so weak and the concentration of the protein so low that the site is occupied only by virtue of looping. Another way to describe the phenomenon is that binding to the two DNA sites shows cooperativity. The presence of one binding site increases the binding to a second site. Chemists call this phenomenon the chelate effect, and its origins can equally well be discussed in terms of the decreased entropy. The existence of cooperativity has been used as an argument for DNA looping in a number of systems. The cooperativity can be observed physiologically, as for example, in the lac operon where a mutant lac operator binds repressor so weakly that only minimal repression is observed (22). The
introduction of a second operator in the vicinity increases the repressor occupancy of the first operator.

**Tethering**

DNA looping is an attractive means by which a protein bound at one site can interact with a DNA site or protein bound to a DNA site located within a few hundred or few thousand base pairs. Instead of looping, some have suggested that two nearby sites might interact by direct communication along the DNA separating the sites through crawling, threading, or conformational telegraphic mechanisms. The two basic options of looping vs DNA-mediated communication can be distinguished by tethering two sites near one another without providing a direct DNA connection between the two. In this case, communication between the sites should be blocked if normally they communicate by mechanisms directly utilizing the intervening DNA, but communication between the sites will not be blocked if they normally utilize DNA-looping mechanisms.

Two ways of fastening sites near one another have been used. These are (a) use of interlocked DNA circles, called catenanes, and (b) linking DNA by a protein bridge. Interlocked circles have been used in vitro with the gln system to demonstrate that stimulation of transcription by NtrC was via looping. The NtrC-binding site was placed on one circle and the promoter on the other (23). The promoter was activated by NtrC despite being bound to a different DNA molecule than the promoter. Analogous experiments were performed with the enhancer and promoter for ribosomal RNA synthesis in *Xenopus*, although there the interlocked circles were injected into oocytes for assay of enhancement (24). In these experiments the topological interlocking was removed in vivo within 30 minutes, but the enhancement persisted for more than 18 hours. This implies that a stable initiation stimulating complex was set up early on. DNA molecules can also be linked by a protein bridge of avidin or streptavidin binding to biotinylated ends of DNA molecules (25). In this case too, the enhancer worked fine.

**SOME PHYSICAL CHEMISTRY OF DNA LOOPING**

**General Considerations**

Too many effects complicate in vivo DNA-looping situations for us to be able to perform meaningful calculations on looping probabilities in growing cells. For some cases, however, we can estimate whether looping will be more or less favored by certain changes in the system. In looping, the protein-binding sites must approach within a distance equal to the size of the looping protein and the orientation of the DNA at the sites must be constrained as well. Therefore DNA looping is closely related to ring closure of DNA molecules,
and a number of results from physical chemistry on ring closure are applicable to or at least provide insight into this aspect of looping, particularly looping in vitro. Below are listed a few of the factors that clearly affect looping. I also discuss a few of the calculations related to looping with the objectives of seeing the consequences of changes in DNA length, lateral stiffness, and torsional stiffness.

LENGTH AND LATERAL STIFFNESS OF THE DNA  Two sites can only interact if the DNA is sufficiently flexible and the sites are sufficiently far apart that the DNA can bend to position the two sites the proper distance apart in three dimensions. The stiffer the DNA, the more difficult it is to form small loops.

A measure of the stiffness of polymers is their persistence length. Roughly this can be thought of, within a factor of two, as the lengths of inflexible rods connected by flexible joints that approximate the statistical behavior of the polymer. Therefore, bending the DNA into a circle of size near the persistence length would be very difficult because of the stiffness of the DNA.

The formal definition of persistence length comes from a consideration of the statistics of polymers. Consider a collection of polymers with one end at the origin. The average of the locations of the other end will also lie at the origin. Now consider orienting each of the polymers so that the initial direction of the polymer is along the x axis. Then, on average, the other ends of the polymers will be displaced some distance in the x direction. The amount of this displacement as the polymer length approaches infinity is defined as the persistence length.

TORSIONAL STIFFNESS OF THE DNA  If two sites are misoriented around the DNA, twisting of the DNA between the sites may be required to orient the sites properly for looping. On the other hand, if the sites are already properly oriented, torsional stiffness of the DNA holds the sites in the correct orientation and increases the ease of loop formation.

FLEXIBILITY OF THE LOOPING PROTEINS  If the looping protein is inflexible, then the DNA must assume exactly the correct conformation for looping to occur. A highly flexible protein would tolerate a large collection of DNA conformations and would loop more easily. Such a protein would not require the DNA to bend in the limit of small loops, ones in which the protein can span the distance between the two DNA sites.

THE GLOBAL STRUCTURE OF SUPERCOILED DNA  Points well separated from one another on a circle of 5000 base pairs are brought close to one
another by supercoiling of the circle. In general the effective concentration of one point near another is increased by supercoiling. This concentration increase facilitates loop formation, but the magnitude of the increase decreases as the separation between sites becomes very large.

ADDITIONAL SECOND-ORDER EFFECTS Proteins not involved with a particular looping reaction could affect the reaction. For example, a competing looped structure could hold the DNA such that the sites required for the first looping reaction could not occur. Alternatively, a protein could bind within a region that normally would be included in a loop and prevent loop formation if the length of the DNA is insufficient to reach around the interloper.

DNA kinks or bends specified by sequence could help or hinder formation of small loops by altering the amount of bend required to loop. They could also affect formation of larger loops by altering the conformations available to the DNA and therefore alter the looping probability.

A Zeroth Order Approximation, A Perfectly Flexible and Twistable String

How might the possibility of looping vary with DNA length if we consider the role of the intervening DNA as merely to hold the two sites in the vicinity of one another and not to let them wander independently of one another anywhere in the cell? Consider first the problem of estimating the average distance separating closest molecules in a solution of molarity M. Let us approximate the situation by taking the molecules to be located on the lattice points of a cubic lattice. By taking a one molar solution to contain Avogadro’s number of molecules in 1000 cm$^3$, the spacing between adjacent lattice points turns out to be approximately $12/M^{1/3}$ Å. For a protein present at $10^{-8}$ M in cells, which is about 10 molecules per bacterial cell and about 10,000 per eukaryotic cell, the spacing is then about $6 \times 10^4$ Å. That is, the average spacing between such molecules is about $6 \times 10^4$ Å. If a pair of the protein molecules is forced closer together, the effective concentration of one of the proteins in the presence of the other is raised above $10^{-8}$ M. Consider that the protein is bound to a site on DNA. What is its effective concentration for binding to a second site $d$ Å away on the DNA? Since the other DNA site is confined to the vicinity of the first site by the length $d$ Å of the “string,” we can invert the above formula to obtain an effective concentration of $M = (12/d)^3$. If $d$ were 300 Å, which might correspond to about 100 base pairs of spacing, the effective concentration is about $3 \times 10^{-5}$ M, which is a substantial increase from $10^{-8}$ M. This means that holding a bivalent protein at one site increases its effective concentration at the other DNA-binding site 300 base pairs away.
Including Lateral Stiffness

In the limit of small circles where the size of the circle approaches the persistence length of the DNA, circle formation becomes energetically more and more expensive as the circle size decreases. The persistence length of double-stranded DNA in physiological buffers is about 450 Å. In the other extreme, as the DNA becomes longer and longer, the concentration of one end in the presence of the other falls off, but rather slowly, and in this limit, can be calculated (26). Due to the random-walk nature of the path of long DNA, this concentration falls as $(3/2\pi n l^2)^{-3/2}$, where $l$ is the step length in the random walk, or interbase distance, and $n$ is the number of steps, or bases.

In the above calculation, only the presence of the two ends in the same volume element is calculated, independent of the directions from which the two ends approach one another. For some ring-closure problems and some looping problems the DNA segments should be oriented properly. A more complicated calculation by Yamakawa & Stockmayer (27) has considered this case. In such a situation we expect that the concentration of one end in the presence of the other will be low for short DNA, for which the necessary bending is difficult, will rise to a maximum for some intermediate length of DNA, and will then fall as $(\text{length})^{3/2}$ as in the random-walk situation, due to the reducing probability that the two ends will occupy the same volume. The quantitative calculations bear this out (27).

Including Torsional Stiffness

In the example just considered, the torsional stiffness in the connecting string can increase or decrease the effective concentration of one site in the vicinity of the other. If the sites were properly oriented, then each collision would be a productive collision and functionally the effective concentration would be increased. The converse is true if the sites are misoriented. The torsional stiffness of DNA is such that it costs about 2 kcal/mole to twist a stretch of 200 base pairs half a turn (28). Of course, the precise values depend upon the nucleotide sequence and the buffer conditions. This energetic cost is inversely proportional to the length of the DNA separating the sites. Since the typical energies involved in protein-DNA binding are 10 to 15 kcal/mole, torsional stiffness can be expected to play an important role for looping involving sites closer together than about 500 base pairs. As mentioned earlier, if the binding energies are substantially greater or if the protein is unusually flexible or extended, such helical-twist effects can be greatly reduced.

Including Both Lateral and Torsional Stiffness

The consequences to ring closure of both lateral and torsional stiffness in the DNA have been considered by Shimada & Yamakawa (29). Their calculation shows that the probability of ring closure for DNA of less than about 500 base
pairs long oscillates with period of 10.5 base pairs while following roughly the same shape of ring-closure curve as is followed when twisting is ignored. This is the behavior expected. When the ends are in phase with one another, the rate of ring closure is high, and when they are out of phase with each other, twisting of the DNA is required in addition to bringing the ends together, and the rate is reduced.

Monte Carlo Calculations

The ring-closure problem for a laterally and torsionally stiff polymer is just about as complex a problem as can be solved analytically. For some of the biological problems, however, we will need to consider the effects of natural bends in the DNA, the consequences of other proteins bound to the DNA, and of supercoiling of the DNA. In these cases, Monte Carlo calculations are the best computational approach. In such approaches one computes over and over, millions of times, the behavior of a system when particular randomly chosen values are applied to each of the variables that describe the system.

For example, in a Monte Carlo calculation of DNA conformation, the variables would be the various angles describing the path of the phosphodiester backbone, the angles describing the sugar conformation, and the angle of the base-sugar bond. The values assigned to the random variables are weighted in accordance with the distribution that occurs in nature. For example, if there is complete freedom of rotation about a particular bond, then any value between 0 and 360 degrees will be randomly chosen for the angle. On the other hand, if the bond is constrained to lie near 10 degrees, most of the time the angles will be chosen to lie near 10 degrees. The probability of deviating an amount δ from 10 degrees would be appropriately weighted.

In performing a Monte Carlo calculation of the conformation of DNA, the path of a DNA molecule is then computed for a set of the randomly chosen angles. This is done over and over, and the fraction of conformations that forms loops is obtained. This can be converted to an effective concentration of one end in the presence of the other. Levene & Crothers (30) did this ignoring the torsion angle, and later Hagerman included computation of the torsion angle (31). When applied to situations similar to those for which the analytical solutions of Shimada & Yamakawa apply, the results closely agree, suggesting that both the analytical and numerical approaches are correct (or most unlikely, both are incorrect).

LOOPING IN GENE REGULATION

This section describes and surveys a number of the most carefully studied DNA looping systems. Both an historical and a mechanistic perspective is taken in these descriptions. The systems illustrate the diversity of the uses to which nature has put DNA looping and show the generality of looping.
Prokaryotic Systems

ARA Englesberg found a deletion entering the \textit{araCBAD} operon from upstream of the \textit{araC} gene and ending before the \textit{araB} gene had unusual properties (1) (Figure 2). When AraC protein was provided in \textit{trans}, the deletion did not impair normal inducibility of the \textit{arapBAD} promoter, but it did alter the uninduced, or basal level of the promoter in the presence of AraC protein. In the absence of arabinose, the uninduced level of the \textit{araBAD} genes was about 10 times normal. That is, the deletion appeared to remove a site through which AraC protein acted to repress its own activation of the \textit{arapBAD} promoter. Presumably, in the absence of arabinose, a small fraction of AraC would be in the inducing state, and would weakly activate transcription of the \textit{arapBAD} promoter if the site required for repression by AraC were absent. Englesberg's deletion would remove this site.

At about the same time the \textit{ara} operon was being probed genetically, biochemical studies were unraveling the \textit{lac} operon. There repression occurred through steric hindrance in the binding of repressor or RNA polymerase. Therefore the possibility that a repressor could function from a position upstream of all the sites that are required for induction, as the genetic data in the \textit{ara} system indicated, was not easily understandable. This led to an extensive study of the phenomenon of repression from upstream. Standard genetics reproduced the upstream repression phenomenon (2, 4). Subsequently, locating the AraC protein–binding sites on the DNA led to the mistaken conclusion that repression was mediated by the \textit{araO1} site (32). This is located immediately adjacent to the initiation complex (Figure 2). A protein bound in such a position can be imagined to be capable of generating repression by a mechanism involving direct side-by-side touching of proteins all in a row along the DNA; from AraC protein bound at \textit{araO1} contacting the cyclic AMP receptor protein contacting AraC protein bound at \textit{araI} finally contacting RNA polymerase bound at the promoter.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{ara_operon.png}
\caption{The regulatory region of the L-arabinose operon of Escherichia coli. The promoter \textit{pC} serves the \textit{araC} gene, which begins just to the left of \textit{araO2}. The \textit{araO1} site helps regulate \textit{pC}. The \textit{araI} site activates transcription from \textit{pBAD}, and the \textit{araBAD} genes lie just to the right of \textit{pBAD}.}
\end{figure}
Genetic engineering techniques eventually permitted more accurate mapping of the upstream site required for repression of the arabinose operon. It was found not to be $araO_1$, but another site that lies more than 200 base pairs upstream from the induction complex at the promoter (4, 6, 7), called $araO_2$. That is, a deletion ending as far as 200 base pairs upstream from $p_{BAD}$ eliminated the self-repression by AraC protein. Clearly some signal was being sent from $araO_2$ to the initiation complex at $p_{BAD}$, and the domino mechanism of direct protein-to-protein interaction for transmitting a signal from one site to another by a continuous row of proteins on the DNA was impossible. Amongst the possibilities for transmission of the signal was DNA looping whereby AraC protein bound at the $araO_2$ site would contact AraC protein bound at the $aral$ site at $p_{BAD}$.

Helical-twist experiments were used to demonstrate that indeed, looping was the mechanism of interaction between $araO_2$ and $aral$ (4). Inserting five base pairs at an irrelevant site between $araO_2$ and $aral$ effectively rotates these sites one half a turn of the DNA helix with respect to one another. If previously the sites faced one another and looping required bringing proteins bound to these sites into contact, the insertion of five bases required not only bringing the proteins together, but also twisting the DNA half a helical turn. The energetic costs of such a twist are about 2 kcal/mole, which in the arabinose operon were sufficient to interfere substantially with looping. Inserting 10 base pairs at the same site restored looping. The only reasonable mechanism compatible with these observations is DNA looping.

One virtue of the arabinose system was the relative simplicity of utilizing genetics to study looping. One might expect that mutations in both the DNA and in the AraC protein would interfere with looping. Both types have been found and studied. Repression-negative mutations in the DNA were confined to the two AraC protein–binding sites, $araO_2$ and $aral$ (7). Those in $araO_2$ reduced the binding affinity to this site about 30-fold. Those in $aral$ were more complicated, and originally appeared to decrease the ability of AraC bound at $aral$ to repress without affecting the binding ability of AraC. More accurate binding measurements have shown they slightly increase the binding affinity of AraC protein and they may be constitutive in nature (R. Lobell and R. Schleif, unpublished). Mutations in AraC have also been found that either increase or decrease repression; their molecular mechanism is not yet understood.

The $araO_2$ site involved in DNA looping binds AraC protein about 50-fold less tightly than the $aral$ site. This is sufficiently weak binding that in the absence of looping to $aral$, the $araO_2$ site is not significantly occupied by AraC protein. The cooperativity in binding between $araO_2$ and $aral$ as measured with an in vivo footprinting system developed for the purpose provided good supporting data for the existence of looping in the $ara$ system (7, 8).
Extensive helical-twist experiments have been done on the ara system (9). About 75 different spacings have been generated and the degree of repression in each measured. Sufficient data is available that by eye or by Fourier transform there is no question that the periodicity in the ability to repress expression oscillates with a period of a little over 11 base pairs, and is clearly not 10.5 base pairs per turn. The dramatic difference in helical repeat from the oft-measured value of 10.5 base pairs per turn of linear DNA in vitro must result from the linking-number deficit of DNA in vivo. DNA extracted from bacteria has a linking-number deficit that is commonly measured as supercoiling. In reality, the linking-number deficit can be partitioned between unwinding the DNA, which increases the number of base pairs per turn, and supercoiling. Clever measurements show that in vivo the partitioning between the two is roughly half and half (33). At the physiological linking-number deficit of -0.06 and such a 50-50 partitioning, the helical repeat of the DNA would be about 10.8 base pairs per turn. Therefore there remains 0.3 or so of base pairs per turn unaccounted for. It will be interesting when sufficiently accurate measurements have been made on other systems to see their helical repeat values.

AraC protein is a dimer in solution and binds to linear DNA as a dimer (34, 35). Therefore it was expected that looping in the ara system would involve the binding of a dimer at aral to a dimer bound to araO2, and it was a shock to discover that, in fact, just a single dimer of AraC protein generates the loops (36). When arabinose is added, the loop opens, contacts to araO2 are lost, and DNA contacts are made only at aral. This behavior raises the question of what is it that makes the loop open and AraC protein now contact a larger stretch of DNA at aral? One attractive mechanism that could generate this behavior is subunit orientation or position. The subunits could be located in the absence of arabinose such that it is easier for them to contact two well-separated sites on the DNA via looping than two adjacent sites on the DNA. That is, the subunits could normally be misoriented. The addition of arabinose could alter the orientation such that the subunits become well positioned for binding to two adjacent sites on the DNA. This mechanism then could easily control looping, and could be extended to other systems in which looping is regulated.

LAC Several early hints for the existence of looping in the lac system were not pursued. When, however, the powerful tools of the lac operon were then turned to investigating looping, a variety of wonderful experiments were done.

Amongst the hints for looping in the lac system were experiments with chimeric lac repressor-β-galactosidase molecules, the existence of multiple pseudo-operators, and the behavior of operator mutants. In 1977 Kania & Müller-Hill reported the DNA-binding abilities of tetrameric hybrids of β-
galactosidase consisting of the normal β-galactosidase subunit and β-
galactosidase subunits containing the N-terminal 60–80 amino acids of lac
repressor (37). The various types of hybrids were partially separated from one
another and their binding to linear DNA was investigated with the filter-
binding assay in which uncomplexed DNA freely passes through a filter, but
the protein bound on the DNA retards the DNA on the filter. Although
significant cross-contamination existed in samples with differing numbers
of DNA-binding domains, and there could be questions about proper positioning
of DNA-binding domains or conformational effects on tetramers containing
two or four of the repressor domains, a tighter DNA binding was seen in the
fractions thought to contain molecules with four repressor domains than in the
fractions enriched in hybrids containing two repressor domains. The authors
suggested that these results could result from the binding of repressor to two
operators on the DNA simultaneously. The authors did not propose that the
effect occurs in vivo, and this work was apparently not pursued between 1977
and 1986. In retrospect, it is difficult to understand any higher binding affinity
seen by the tetrameric molecule because subsequent studies have shown that
supercoiling is required for the natural operators to be able to engage in
looping (38, 39).

Cooperativity in repressor binding to lac operators was also an overlooked
cue to the existence of DNA looping. For some years, the behavior of
operator mutants in the lac operon was most puzzling. Point mutations in the
operator seemed to raise the basal level of expression of the operon at most
only 20-fold, yet study of the binding affinities to the operators in vitro and
consideration of the in vivo concentrations of lac repressor suggested that the
mutations should have had a much greater effect on repression (40).
Apparently DNA looping ameliorated the effects of the operator-constitutive
mutations. When wild-type lac operator was placed various distances from a
mutant operator, it was apparent that the presence of the wild-type operator
aided the binding of repressor to the mutant operator. By this time the concept
of looping as a mechanism in gene regulation was accepted and the data was
interpreted as possibly resulting from looping (41). Similarly, placing the lac
operators upstream of the lac promoter gave no repression and placing an
operator downstream gave moderate repression, but both operators together
generated strong repression (42). Whether the repression results from loop-
ing, helping the repressor bind to the downstream site and thereby serve as a
blockade to an active RNA polymerase, or whether the loop poses a steric
hindrance to the binding of the polymerase is not yet known.

An elegant experiment showed that looping in the lac system is a natural
part of the operon's regulation. This made use of the two pseudo-operators
found near the lac regulatory region. Since 1974 it had been known that in
addition to the primary lac operator located just downstream from the
transcription initiation site, two additional weaker binding operators existed in the lac operon (43, 44). For more than 10 years these were thought merely to be remnants of evolution. To investigate a role of the downstream operator O₂, which is located 481 base pairs downstream from the primary operator, eight bases in lac O₂ were altered in such a way as not to change the amino acids encoded by this region of the DNA (45). Thus the β-galactosidase remained unaltered, but O₂ was so greatly altered that lac repressor no longer could bind. These changes were found to decrease the repression of the operon about fivefold. That is, they raised the uninduced or basal level fivefold, but had almost no effect on the induced activity of the promoter. This result indicates that the second lac operator does play a role in repressing the operon (46). Apparently DNA looping frequently occurs in vivo between the main lac operator and the downstream pseudo-operator.

The second pseudo-operator is located 83 base pairs upstream from O₁, at the end of lacI. It also apparently plays a role in normal regulation of the operon, for its destruction raises the basal level of lacZ about threefold (46).

Nearly the full set of experiments that can demonstrate looping have now been applied to the lac operon, and with dramatic success (17). In vitro and in vivo footprinting has been used to show cooperativity in the binding of repressor to the main lac operator and the two pseudo-operators O₂ and O₃ located 83 and 481 base pairs from the operator (47, 48).

DNA-binding studies of lac repressor-operator interactions have been performed with the filter-binding assay. The dissociation rate of repressor from such DNA is much reduced if the DNA is supercoiled and if it contains one of the pseudo-operators in addition to the operator at the promoter (38, 39).

In addition to using supercoiled DNA and the natural lac operators, looping in lac has also been studied by increasing the energies available for looping by using perfectly symmetric lac operators. Repressor binds to such operators about 100 times as tightly as it binds to the wild-type operator. With such operators, linear DNA could be forced to loop and at high repressor concentrations, "crossjoined" or sandwich structures could also be formed (17). The structures could be detected by gel retardation or observed by electron microscopy. They were identified as looped structures by their enhanced stability and their absence when the two operators were rotated half a turn with respect to one another. Additionally, the presence of these loops on the DNA generated the characteristic alternating pattern of DNase-I-hypersensitive and hyposensitive sites on the DNA between the two operators.

In an effort to apply principles of statistical mechanics to looping in vivo in the lac operon, Mossing & Record examined operator spacings of 118, 185, and 283 base pairs and concluded that supercoiled DNA in vivo possessed the same stiffness and therefore experienced the same difficulty in bending
sharply to form small loops as would be predicted from the hydrodynamically determined stiffness of linear DNA (49). Later it was learned that the inability of the closest spaced operators, 118 base pairs, to loop in their experiment apparently resulted not from stiffness of the DNA, but from the fact that they were on opposite faces of the DNA as the helical repeat of DNA in vivo is not 10.5 base pairs per turn as it is on linear DNA in vitro. This they found in a subsequent helical-twist experiment in which the repression abilities of 12 different spacing mutations were clearly inconsistent with an helical repeat in vivo of 10.5 base pairs per turn (50). These authors have also considered the decrease in looping that occurs as loops become particularly large. This should occur when entropic effects might begin to become more important than the gains from not having to bend the DNA so sharply (50). This problem is particularly difficult, however, for in supercoiled DNA distal sites are forced into close contact with one another due to the wrapping, and simple polymer statistics should not be applied to the problem. The deo system is an example where operator separations of up to 4000 base pairs showed only a small distance effect (51). An additional difficulty in attempting to calculate looping is the unknown flexibility of the protein. Undoubtedly some proteins possess significant flexibility and their bending will reduce the amount of bending required by the DNA to form a loop.

DEO The protein products of the deo operon in Escherichia coli permit the cells to catabolize nucleosides. At least four enzymes are involved in the catabolism and three proteins are involved in the regulation. The synthesis of deoC, deoA, deoB, and deoD message initiates from two promoters separated by 599 base pairs and lying upstream of the four deo genes (52). Messenger synthesis is regulated by three proteins, the cyclic AMP receptor protein, which activates transcription, and the CytR and DeoR repressor proteins. In the absence of CytR protein, DeoR can repress expression of the Deo proteins about 60-fold (53). This repression requires three operator sites, one overlapping each of the -10 regions of the two promoters, and a third site lying an additional 270 base pairs upstream of the first two (54). The integrity of all three operators is required for maximal repression, suggesting that a double DNA loop might form. Indeed, such is possible, for the molecular weight of DeoR protein based on gel exclusion chromatography indicates that the protein is an octamer, and therefore it could bind three, or possibly four of the 16-base-pair palindromic binding sites simultaneously (55). Double looped structures can be formed in vitro and observed by electron microscopy (18).

The potential energies available for looping of the deo system must be large, for loops will form on linear DNA, and particularly large loops can form and repress in vivo. When only two operators are present, one of which overlaps the -10 region of a promoter, repression falls from 23-fold to
3.6-fold to 3.0-fold as the spacing between the two operators increases from 567 base pairs to 3700 base pairs to 4685 base pairs (51).

**OTHER LOOPING SYSTEMS** The gal operon of *Escherichia coli* is subject to a complex control as galactose can be catabolized when it is available, but also must always be present to fulfill needs for cell wall synthesis. Two promoters separated by about 10 base pairs serve the *gal*ETK genes. The activity of one is stimulated by cyclic AMP receptor protein, whereas the other is repressed by cyclic AMP receptor protein. The activity of both is repressed by GalR repressor, which binds both at positions -60 and at +55 with respect to the start of transcription (56). Instead of repressing via steric hindrance in which repressor bound to either of the operators blocks the binding of RNA polymerase or cyclic AMP receptor protein, a DNA loop formed by repressor bound at both sites generates a loop that represses the promoter. Most likely the binding rate of RNA polymerase to such a sterically constrained promoter is much reduced from the nonlooped state. An elegant experiment showing that the repression was generated by looping was the finding that substitution of either binding site for the *lac* repressor binding site left the system poorly repressed, but when both of the *gal* operators were changed to *lac* operators, repression was strong when *lac* repressor was present (57). As expected, if a mutant *lac* repressor unable to form tetramers and therefore unable to loop was used, repression was poor (20).

The genes involved in the uptake and metabolism of *N*-acetylglucosamine are controlled by the NagC protein, a repressor that loops. This may be a particularly good system for physical studies as short linear DNA fragments can be looped by the NagC protein (58).

**Activator Systems**

A complex system utilizing a two-component signal-transducing system as well as protein phosphorylation regulates synthesis from the genes involved in nitrogen metabolism in *Escherichia coli* (59). Transcription of the *gln* genes does not use the major form of the RNA polymerase that contains the $\sigma^{70}$ subunit. Instead the promoters require RNA polymerase containing $\sigma^{54}$, a subunit encoded by the *rpoN* gene whose other aliases are *glnF* and *ntrA* (60). The major difference between transcription from genes using the $\sigma^{54}$ polymerase and those using $\sigma^{70}$ is that transcription from the former requires an additional activating protein. Transcription of the *gln* genes requires either the NtrC protein or the NifA protein, which bind to DNA one or two hundred base pairs away from the promoter (61) and form a loop in the DNA as they interact with RNA polymerase.

Although no plausible mechanism except DNA looping could explain the action-at-a-distance effects shown by the activating protein NtrC in the
transcription of \( \text{glnALG} \) genes, a nice looping confirmation was performed using linked or concatenated DNA circles as described earlier (23). The electron microscopic evidence for looping in the \( \text{nif} \) system is rather inconclusive as only 5% of the DNA molecules with bound protein were observed to be looped (19). Undoubtedly NtrC and bound RNA polymerase can interact in the samples prepared for microscopy, but random aggregation of protein would produce the same result.

The regulatory proteins NtrC and NifA regulate nitrogen fixation genes called \( \text{nif} \) in \( \text{Klebsella pneumoniae} \), a close relative of \( \text{Escherichia coli} \). Helical-twist experiments in which which 5, 11, 15, and 21 base pairs were added to the natural spacing of about 150 base pairs between the NtrC-binding site and the promoter for \( \text{nifLA} \) showed a cyclic dependence (62). Additional evidence for DNA looping in \( \text{nif} \) genes is that even if the natural spacing of one hundred or so base pairs from the promoter to the NifA-binding site were increased to as many as several thousand, activation by NifA could still be detected (63).

**Eukaryotic Enhancers in General**

Eukaryotic enhancers activate and regulate transcription initiating at promoters located hundreds, thousands, and sometimes tens of thousands of base pairs away. Enhancers can be moved about and turned end for end, usually with only a small effect on their stimulatory powers (63a, 64a). Enhancers were first recognized in animal viruses, and initially were hypothesized as being entry points for RNA polymerase molecules that would then drift to the promoter to start transcription (65).

One test of the drifting model is to place a blockade between the enhancer and promoter. Ptashne & Brent did this with the \( \text{gal4} \) system in yeast and found strong interference with promoter activity (65). This they interpreted as evidence in favor of the bind and drift model. After finding evidence for looping in vitro with the lambda repressor system, looping seemed more plausible and the blockade was interpreted as interfering with looping instead (66).

Relatively few definitive experiments have been done to prove that proteins bound to enhancers generate loops as they interact with other parts of the transcription apparatus at the promoter. Nonetheless, the prevailing opinion seems to be that most enhancers use a looping mechanism. This certainly seems the most plausible for enhancers with large separations between them and their promoters. Further, DNA looping is almost the only way that transvection (67) in \( \text{Drosophila} \) could work (68). There an enhancer on one chromosome activates a promoter on a different but homologous chromosome.
A few helical-twist experiments have been done with eukaryotic enhancers. Those that showed some helical effects were the late promoter of SV40 (69), and the heat-shock promoter of *Drosophila* (70). Other promoters fail to show helical-twist effects (71–74). These negative findings can be the result of several factors. The energies available for loop formation may greatly exceed the energetic costs of torsionally twisting the DNA. Another reason may be that the proteins involved are sufficiently flexible that they can accommodate substantial misorientations of their binding sites. This is highly likely, for many of the eukaryotic enhancer binding proteins seem to consist of loosely connected domains that can freely be interchanged without loss of activity.

As discussed earlier, tethering experiments support DNA looping. They have been done with the ribosomal promoter of *Xenopus* and the viral SV40 promoter (24, 25). Electron microscopy of enhancer binding proteins binding together and to two sites on the DNA suggest, but hardly prove, the existence of DNA looping.

**Looping in Genetic Recombination**

Because the DNA sites participating in site-specific genetic recombination must be close to one another at the time of strand exchange, proteins bound to these sites bind to each other. Consequently DNA looping, or its equivalent if the sites are on two different DNA molecules, occurs. These types of reactions are found in the *hin* inversion system of *Salmonella*, the *gin* and *pin* inversion systems of phages Mu and P1, resolution reactions of transposons, the transposition system of Mu, and the integration-excision system of lambdoid phages (75–77).

The resolvase protein encoded by transposons such as Tn3 and Mu phage catalyzes an exchange reaction between two *res* sites in the same orientation on the same DNA molecule (78). When the sites are oppositely oriented there is no reaction or a greatly reduced reaction. Initially this fact was taken as evidence for a DNA-threading reaction by resolvase from one *res* site to the other (78). By this means the recombinase could determine the relative orientation of the two sites. It did not seem possible that the two sites could communicate their relative orientations by any other means. Closely reasoned topological experiments (79) followed by an elegant linked-circle experiment with the Mu phage transposition system however, changed thinking on this issue (80). DNA molecules with two properly oriented Mu *att* sites that were capable of recombining were used in this experiment. The DNA substrates for this experiment also contained lambda phage *attB* and *attP* sites oriented such (Figure 3) that the integration reaction between them would either invert one of the Mu *att* sites or separate the two *att* sites onto two separate DNA molecules. Once the DNA was supercoiled and capable of supporting a reaction between the *att* sites, the lambda phage integrase reaction between
the sites, which either inverted an \textit{att} site or placed the two sites on two different molecules, had no effect on the reaction. The explanation for these results is that the two \textit{att} sites are placed in close proximity and in the proper spatial orientation via the supercoiling of the DNA. If this supercoiling persists while the DNA molecule is cut and rejoined at sites elsewhere, the reaction between the \textit{att} sites is unaltered. The cutting and rejoining by the lambda \textit{int} system can separate the sites or even invert them topologically. In summary, what is important for interactions between two \textit{att} sites is their local spatial orientation not their global topological structure.

The lambda phage integration-excision structure itself can also be thought of as a loop. The complex of Int, IHF, and Xis proteins at the phage attachment region is sharply bent by the proteins and appears to be nearly looped itself. Of course the Int protein participates in the strand scission and rejoining reactions, but it also helps hold the DNA looped by binding both at the crossover region as well as 125 and 150 base pairs away. Although six dimers of proteins, including three dimers of IHF, are present in the structure, they appear not to require direct protein-protein interaction. The cyclic AMP...
receptor protein bends DNA about as much as IHF protein (81–83), and it can replace IHF protein in the intasome. Also, a sequence of DNA possessing oriented runs of As and Ts so that it naturally bends about 100 degrees can replace one of the IHF proteins (83).

Looping in Regulation of DNA Replication

DNA replication is a highly important process, and it is likely that as discussed earlier, proteins that bind to DNA and that interact are able to form loops in the DNA. As a collection of proteins binds to the origin of replication of phage lambda and E. coli, it seems reasonable that the activity of most origins will require binding by multiple proteins. Such a situation opens the possibility of DNA looping, and indeed looping between two lambda replication origins that had been placed on the same DNA molecule mediated by the lambda O protein has been observed (84). It remains to be seen whether this type of looping is of biological significance. It is clear that the replication-initiator protein for plasmid R6K binds weakly to an active origin, but by virtue of DNA looping from a collection of much tighter binding sites, the weaker site is occupied by the initiator (85). This is a case where the cooperativity provided by looping helps transfer proteins to a DNA site.

SUMMARY

DNA looping is widely used in nature. It is well documented in the regulation of prokaryotic and eukaryotic gene expression, DNA replication, and site-specific DNA recombination. Undoubtedly looping also functions in other protein-DNA transactions such as repair and chromosome segregation. While the underlying physical chemistry of DNA looping is common to all systems, the precise biochemical details of looping and the utilization of looping by different systems varies widely. Looping appears to have been chosen by nature in such a wide variety of contexts because it solves problems both of binding and of geometry. The cooperativity inherent in binding a protein to multiple sites on DNA facilitates high occupancy of DNA sites by low concentrations of proteins. DNA looping permits a sizeable number of DNA-binding proteins to interact with one of their number, for example RNA polymerase. Finally, DNA looping may simplify evolution by not requiring a precise spacing between a protein's binding site and a second site on the DNA.

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