

DNA Footprinting: Hitherto Unaddressed Problems

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Abstract

Footprinting is the process of identifying the specific sites on DNA to which a molecule, usually a protein or group of proteins, is bound. The technique was described at least as long ago as 1974¹ but it remains central to a very wide range of study today.

Recent research reports making use of footprinting describe the passage of RNA polymerase in real time along DNA in nucleosomes², the binding sites of autoantibodies to double-stranded DNA³, the binding of a component protein from the RNA polymerase complex⁴, the binding of a specific thyroid hormone receptor⁵, specific genetic interference by double-stranded RNA⁶, a study of archaeal chromatin⁷, the binding of sequence-specific transcription factors^{8,9}, the destabilisation of a nucleosome position¹⁰, transitions in the heterochromatin of plant subtelomeres¹¹, methylation-dependent chromatin fiber compaction¹², the role of histone H3 in the transcription of chromatin¹³, the interaction of a platinum anticancer drug with nucleosomal DNA¹⁴, and the attenuation of charge transport by nucleosome formation¹⁵.

From the research protocols, the local winding periodicities of nucleosomal DNA can best be determined using *deoxyribonuclease 1* (DNase 1) but not by *micrococcal nuclease* (MN), while the translational position of nucleosomes along a specimen of DNA can best be determined by MN but not by DNase 1.

The theory behind these particular uses and modes of operation of MN and DNase 1 has never been explained.

Papers report the cleavage patterns of naked DNA in solution which all show a 10bp periodicity corresponding to the pitch of the double helix. It has never been explained why such a periodicity should be observed at all when the cylindrically symmetrical, random sequence, double helix suspended in solution can be approached by a general endonuclease from any side. A continuous series of cuts at each base pair might be expected from a solution.

Until there is a clear understanding of how both the general endonucleases cleave naked DNA in solution only every 10bp, why MN is preferred to DNase 1 for the identification of nucleosome position, and why DNase 1 is preferred to MN for identifying rotational twist on a nucleosome core, there is a risk that the understanding of the *modus operandi* of more complex systems on DNA will be poorly understood.

This paper attempts to address these issues.

Key Words: DNA, DNA Models, Paranemic DNA, DNA Footprinting, DNA in Medical Research

Introduction: Outstanding Problems In DNA Footprinting

In order to address some outstanding problems in DNA footprinting, it is necessary to review problems evident in the simplest DNA:protein systems, that is, DNA interacting with a single protein, before considering the more complex interactions of DNA with two or more proteins simultaneously.

(A) Naked DNA

While placing some emphasis upon, and making reference to, the work of Klug & Rhodes, and of Drew & Travers, the reader may wish to keep in mind that their work is selected for comment precisely because it can be considered to rank among the most carefully researched, reliable, detailed and scrupulously developed of any work in the field of nuclease recognition of DNA.

Rhodes and Klug ¹⁸, using notionally random sequence DNA digested with DNase 1 while adsorbed on "smooth" mineral surfaces, remark:

"The intensities of the fine bands vary sinusoidally, showing broad maxima at regular intervals, about 10 bases apart... The two strands must be cut in a symmetrical fashion to give rise to such a clean pattern... **it seems at first sight unlikely** that all the DNA molecules would lie down in the same orientation... " **[My bold italics – CSD]**

Rhodes and Klug ¹⁹, working now with defined sequence DNA digested with MN and DNase 1, remark:

"The helical repeat of poly (dA.dT) bound to calcium phosphate, obtained directly from the densitometer tracings, is 10.5 bp with both nucleases... Poly (dA.dT) bound to mica showed a slightly lower helical periodicity... of 10.4 bases...

The densitometer tracing in Figure 3 shows that the helical periodicity of poly dA. poly dT is distinctly different from that of DNA or poly (dA.dT). The repeat length of the fragments produced is 10.0 bases. The periodicity observed using MN is the same as that found with DNase 1..."

Thus, even where defined sequence DNA having a regular pattern of preferred binding or variation in groove width is employed, the nucleases still cleave their substrate with a periodicity apparently conforming to the pitch of the helix, and not, for example, uniformly every 2 bp in poly (dA.dT), even though this molecule should have a regular cylindrical symmetry if it is a double helix.

Another report of the preferred cleavage sites of DNase 1 on naked DNA, for example, is shown in their Fig.1B Lane 1 ⁴. It is evident that cleavage occurs every 10 base pairs, with cleavages often around the mid-point between the 10bp periodicity. Lane 2 shows enhanced cutting sites every 10bp for DNA wrapped around histone cores compared with Lane 1 because the DNA in Lane 2 is locked into one orientation on the histones whereas naked DNA can oscillate between several helical states of closely similar energy dependent upon the aggregate of the stacking stabilisation energies of closely similar rotational isomers.

As a cylindrical double helix, duplex DNA of high sequence specificity, such as (dA.dT)_n, might be thought able to adopt many rotational positions on a substrate. After endonuclease cutting, applied to many molecules *en masse*, fragments of many random lengths should result, but this is not the case.

Therefore duplex DNA, even of high sequence specificity, cannot have a high, cylindrical symmetry, since only fragments which are multiples of the helical repeat are found. Moreover, these fragments being multiples of the helical repeat are from starting molecules which must have been cleaved at a very limited range of common positions along the parent molecules.

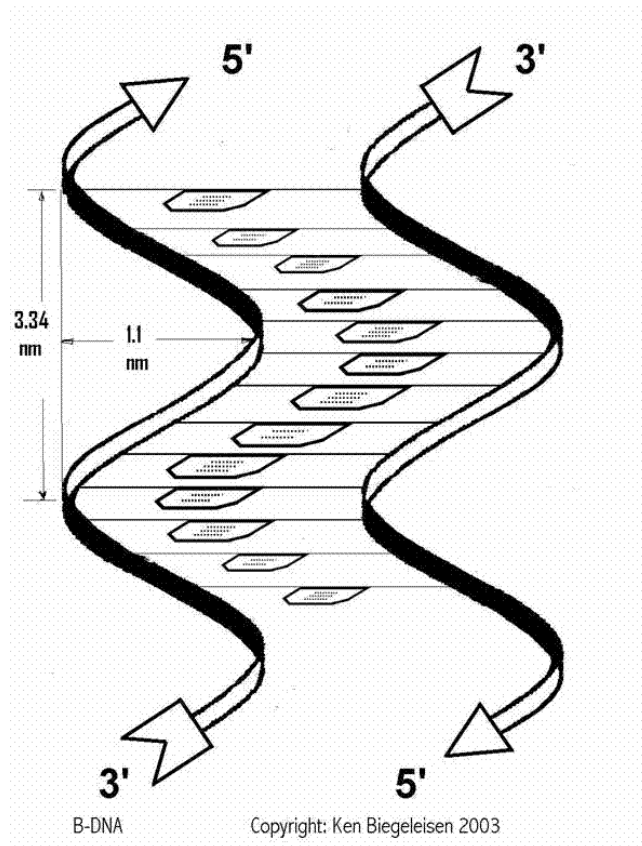
Several other models for duplex DNA have attracted sustained attention. Sasisekharan and his team²⁰⁻²³, and Rodley and his coworkers²⁴⁻²⁷ have refined duplex models which incorporate alternating left- and right-handed double-helical half turns and have a more restricted cylindrical symmetry. These models would offer restricted access to an endonuclease were such molecules to be laid down upon a substrate, or even suspended in solution, and this might account for the limited range of fragment lengths as multiples of the helical pitch.

A further model for duplex DNA²⁸⁻³² does not have any cylindrical symmetry at all. The paranemic model, shown diagrammatically below in its B form as FIG 1, has a “rear” face at which phosphate ionic charges predominate, and a “front” face from which the stack of base pairs project outwards. This offers a new paradigm for understanding the results of DNA footprinting.

The Watson-Crick base pair width of 1.1 nm imposes an individual sugar-phosphate helical diameter of 1.1 nm upon the geometry of the paranemic model, while the helical pitch remains unchanged at 3.34 nm.

FIG 1 A Diagram Of B Form Duplex DNA According To The Paranemic Model

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The helical diameter of the paranemic duplex, with dimensions dictated and determined by the geometry of the standard Watson-Crick base pair width of 1.1 nm adopted in the model, agree exactly with the experimental

results reported by Lee et al.³³, Figures 3(b) & 3(d) and by Beebe et al.³⁴ as well as by the results of James & Mazia³⁵, reviewed recently^{28, Appendix 1; 31}, using monomolecular DNA films in a Langmuir-Blodgett trough.

More recently, Wu & Wu¹¹¹ demonstrated in their Figure 7 that the sense and antisense strands of covalently closed circular plasmid HTB4 could be separated by electrophoresis in a denaturing gel after the sense strand had been given a greater relative molecular mass by allowing a length of mRNA to remain bound to it. Their results lend support to the suggestion of the existence *in vivo* of topologically non-linked duplex DNA, i.e., paranemic DNA.

Hopkins³⁶ noted that even for a Watson-Crick double helix there are two possible right-handed isomers. So it is with the paranemic duplex also. The reasons why this particular paranemic isomer of the two possibilities has been chosen have been set out^(28, Chapter 11).

(B) A Covalently Closed Circular 169 Bp Duplex

A random sequence 169 bp covalently closed circular (ccc) duplex, just about 16 full turns of the B form in solution, was hydrolysed with the general endonuclease deoxyribonuclease 1 (DNase 1). Drew & Travers³⁷ found that sugar-phosphate bonds around positions 15, 25, 35, 45 and 55 were cut frequently, while those around 20, 30, 40 and 50 were cut infrequently, if at all:

"This suggests that the circular DNA adopts a highly preferred configuration, with one side facing in and the other facing out, and that this bending is restricted to one plane of a topological circle... It is perhaps unexpected that a small protein such as DNase 1 should experience hindered access, ***but the result speaks for itself.***" [My bold italics - CSD]

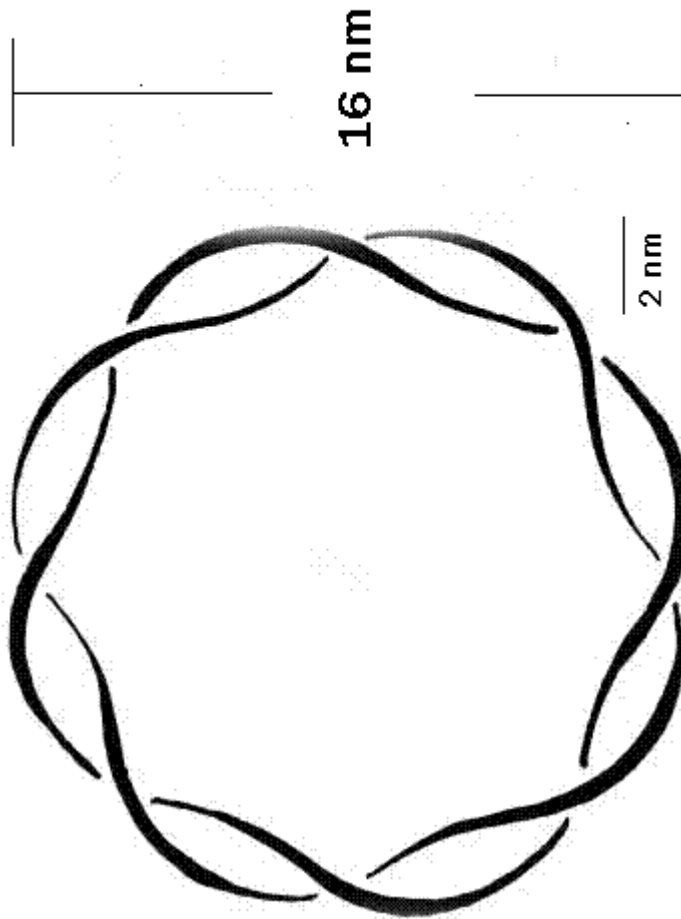
There are many difficulties with this passage.

First, experimental results never speak for themselves. They have no meaning outside a theoretical framework or paradigm within which the results can be ordered, organised, interpreted and tested. What Drew & Travers have discovered is that their results do not conform with their expectations using a double-helical paradigm.

Next, DNase 1, with RMM of 31,000³⁸ and relative density 1.3, say, would have an estimated diameter of about 4 nm if spherical and Suck et al.³⁹ give the dimensions of DNase 1 as 3.5 nm x 4.0 nm x 4.5 nm.

A closed circle of 169 bp in B-form with pitch 3.34 nm per 10 bp would have an innermost diameter of some 16 nm, portrayed diagrammatically in Fig. 2.

**Fig 2 Representation
of Drew & Travers
ccc Duplex DNA**



Consequently a plane circle with that diameter could hardly obstruct the activity on the inner face of a 4 nm nuclease. In addition, if the circular duplex showed the flexibility normally visible in electron micrographs of DNA, access to the "inner" face would be even less hindered.

Moreover, the double helix is cylindrically symmetrical and can lie at any rotational angle on the substrate. The fragments from each strand, lying at any rotational angle on the surface show a high regularity. It is not clear how the nuclease would evidently be able only to cleave the sugar-phosphate bonds from a position on one side of the topological plane of the ccc duplex.

If the double helix can lie at any rotational angle on its substrate, it is not clear why cleavages at any base pair are absent, or to express it another way, it is not clear why there would be such high regularity of cleavage at 10 base pair intervals.

Drew and Travers compared the cutting of the ccc DNA with same sequence linear DNA.

In their Figures 5 a) and b) are recorded some 50 cleavages of circularised 169 bp duplex, adding the cleavages in both strands together. Only four or five of these are not also present in the cleavage pattern of the linear duplex. Put another way, some 45 cleavages in the circular duplex are also seen in the linear duplex where a total of over 80 cleavage points can be distinguished. Therefore the cleavages in the circular DNA are a subset of the cleavages of the linear DNA.

Now the linear DNA cleavage pattern is not a continuous smear, since only about one quarter of the sugar-phosphate bonds are cleaved, and therefore DNase 1 is not cleaving the linear duplex at all azimuthal angles but is showing a pattern of preferred cutting sites. It has been customary to describe DNase 1 cutting sites in terms of base-pair sequence^{40, 41} or, latterly, base-pair sequence preferences and the local width of the DNA minor groove^{40, 41, 42, 43}.

The difficulty with this approach is the high regularity of the cutting of naked DNA (for example, 18, 44) and nucleosomal DNA (for example, 45 - 52). In the present study both DNA strands are cut with a 10 bp frequency over long stretches in both linear and circular forms of the random sequence 169 bp molecule. For example, the non-coding strand in the linear duplex shows cutting at 20, 40, 60, 70, 80, 90, 100, 110, 120 and 140 as well as the cuts at 15, 25, 35, 45, 55 present in the same strand of the circular duplex also.

The coding strand in the linear duplex shows cutting at 40, 60, 90, 100, 110, 130, 140 as well as at 65, 75, 85, 105, 125, 135, and 145. Many cuts, drawn from both series, are present also in the same strand in its ccc duplex form.

Since the 169 bp sequence used by Drew and Travers is random, for all practical purposes, but nevertheless shows a high periodicity based on 10 bp in both linear and circular duplexes, and the circular duplex cleavage positions are a subset of the linear duplex cleavage set on both strands, it hardly seems likely that base sequence, *per se*, or preferred directions of bending within different sequences can be exerting much influence on the cleavage pattern because any preferred cutting sites and groove widths would then have to be spaced exactly and fortuitously one pitch apart (or possibly one half pitch⁹²) from each other across the whole length of these random sequence specimens.

The crucial question can now be framed:

How is it that a cylindrically symmetrical, linear double helix of random sequence, which can be approached from any azimuthal angle by a deoxyribonuclease, should display a high periodicity of cleavage in solution, apparently based on 10 bp (the helical repeat), which cleavage pattern for both coding and non-coding strands contains the virtually exact subset of cleaved bonds observed in the respective strands of the circularly closed form of the molecule, rather than a continuous, or even an irregular smear of cleavages resulting from cutting sugar-phosphate bonds at random azimuthal angles, especially when taken over a large number of DNA molecules ?

Or, put another way, how is it that the direction of approach of the deoxyribonuclease to the cylindrically symmetrical random sequence linear double helix is always such, even in solution, that the high periodicity of cleavage, taken over a large number of DNA molecules, always tends to include the same limited set of cleaved sugar-phosphate bonds, which are found to be spaced at 5 or 10 bp intervals?

The new, paranemic model for the structure of DNA offers a different paradigm which permits a very different explanation of the results of Klug and Rhodes, and of Drew and Travers.

In Drew and Travers' experiments, the circularly closed 169 bp duplex offers the same access as the linear form. Both molecules are organised according to the diagram in Fig 1 and have a rear and a front face.

The nuclease DNase 1 therefore has a choice of a limited number of possible modes of interacting with DNA. It is necessary to recall that DNase 1 may bind in a manner different from, and possibly in a different nucleotide position from that in which it actually cleaves its chosen, primary sugar-phosphate bond before cleaving the other, secondary bond on the complementary strand. Nevertheless, and keeping the distinction between binding position and cutting position in mind, DNase 1 can interact with DNA, in principle, from the rear face, front face, from the side or from a circular or helical embrace of the whole equatorial section of the DNA molecule.

In this paper is offered an account of the interaction of DNase 1 with paranemic DNA and with chromatin by postulating that this nuclease binds to the front face of duplex DNA and that it makes primary cuts to one side.

The DNase 1 would pass along the DNA duplex in a longitudinal manner, or more probably would register a large number of separate "single hits", does not proceed in any sort of helical manner, and remains on the same face ^(see, for example, 53).

It recognises a constant feature of the DNA molecule, probably the crest of each helical turn (as would be seen in a side view of Fig 1) and cleaves a sugar-phosphate bond in a fixed relation to the structural feature of DNA at which it binds.

The primary cut must be to one side of the paranemic duplex because that site is shared with the primary cutting site of micrococcal nuclease (MN) which, it is postulated, operates itself from the rear face of the DNA duplex, as will be examined shortly.

In moving longitudinally along one face of the duplex, or, more probably, in cleaving duplex DNA with a large number of "single hits" spread over many independent DNA molecules, and always recognising a constant feature of DNA structure, such as the outermost sugar-phosphate bonds which are closest to itself, DNase 1 necessarily cleaves these bonds with a periodicity founded upon that of the DNA substrate at the repeated structural feature of the equivalent sites upon which it alights, that is, say, crests.

A possible explanation can be seen now of the observation that the linear duplex cleavage pattern includes exactly the cleavage pattern seen in the circular duplex but contains additional cleavages also.

DNA does indeed have preferred rotamer conformations which are the result of the interplay of minimising the free energy arising from base pair stacking and from the interplay of hydrogen bonds of one base pair with those of base pairs immediately above and below it in the sequence, and this results in certain sequences being preferentially variously located at troughs, at crests, or in between.

This may be the explanation of the supposed sequence preferences of the general endonucleases DNase 1 and MN ^{54 - 59, 60 - 60 (c)}, which, predominantly at least, actually recognise a specific geometric feature of the DNA substrate ^(for example, 59).

Thus the rotamer adopted by nucleosomal DNA can be transformed into another rotamer, for example, by antibiotics ⁶¹, and, in principle, will adopt a different rotamer conformation from that of the same DNA in its naked state. The same geometric feature in nucleosomal DNA is recognised, and cleaved without the regularity being much disturbed by any easily perceptible sequence specificity.

Some support for the proposal that DNase 1 severs DNA by operating from the front face (in contact with the bases) while MN operates from the rear face (in contact with the sugar-phosphate chain) can be found in Drew and Travers ⁴² :

"It is now clear that different sequences in a DNA molecule can be distinguished in either of two ways: by contacts with the base pairs, or by contacts with the sugar-phosphate backbones..."

The equatorial view down the length of the duplex in Fig 1 implies that base pairs to the front (crest) or rear (trough) of a helical turn, and 5 bp away from each other, enjoy greater overlap with the aromatic rings of their immediate neighbours than do those 2 bp away on either side, and so they may enjoy greater stacking energy stabilisation.

Thus DNA duplexes may be able to choose between competing rotational isomers such that certain base pairs in a sequence tend to be found at the rear or front if they and their immediate neighbours offer such energy stacking stabilisations due to the overlap of aromatic rings in adjacent purines. (The double bonds in pyrimidines are only weakly delocalised.)

Such rotational isomers will often have energies close to each other, so that in a population of DNA duplexes of identical random sequence there will be a proportion of each rotational isomer at any instant, and individual duplexes will be converting at any instant into another rotational isomer, in true chemical equilibrium.

When such isomers are in equilibrium with each other the crest of one will be 5 bp out of phase with the trough of the other, as "perceived" by an endonuclease recognising a constant geometric feature such as a crest, and this phenomenon can explain the cut lengths reported by Rhodes and Klug^{18, 19}.

In the case of a circularly closed duplex, such as that composed of 169 bp constructed by Drew and Travers³⁷, transitions between rotational isomers are likely to be constrained since, in a circular molecule, these transitions must become cooperative around the whole circle as they will otherwise be extinguished by the damping of adjacent base pairs.

In a linear duplex a rotational transition might start at either end and will work its way along the duplex in a progressive fashion having a lower activation energy than would the ccc duplex. Thus circular duplexes will tend to adopt and retain the rotational conformation of only the lowest energy rotamer, and show a subset of DNase 1 cleavages relative to the linear molecule's cleavage pattern.

(C) Nucleosomal DNA

Taking duplex DNA to have the paranemic structure, at least as a hypothesis to test against the reported patterns of endonuclease cleavage, it is evident that the duplex can wrap itself around the nucleosomal core histones in one of two orientations; namely with either its rear face or its front face in contact with the core histone proteins.

It must be the rear face in contact with the histones because transcription factors, replication complexes and restriction endonucleases must be able to rapidly identify their operator base sequences and it is only likely to be easy to do this when the base pairs are facing outwards. That is, the rear face of the duplex, predominantly negative with phosphate ions, must be in contact with the core histones, with their predominantly positive protonated amino groups.

Typical of outstanding current problems in DNA footprinting are:

- 1 Footprinting of the core nucleosomal histones in chromatin stripped of histone H1 can be carried out with micrococcal nuclease (MN)⁶¹ which, in light digests at least, results in DNA fragments of approximate length 145 bp but such footprinting is generally not carried out with deoxyribonuclease 1 (DNase 1) because the products of that digestion tend to be a wide range of fragments mostly smaller than 145 bp and which are all multiples of 10 bp

- 2 In complete contrast, proteins which recognise specific base pair sequences, which histones in nucleosomes do not, form complexes with DNA which can be footprinted with DNase 1^{62, 63} but cannot generally be footprinted well with MN because it tends to give rise to a large number of mostly small DNA fragments which are all multiples of 10 bp in length.

Why should the footprinting of proteins which recognise specific base pair sequences, in their complexes with DNA, respond to the general nucleases DNase 1 and MN in a manner which is normally entirely complementary to the action of these nucleases on complexes of DNA with non-sequence-specific proteins ?

3 It is known ^{64,65} that the action of DNase 1 on both linker DNA and on nucleosomal DNA in chromatin stripped of H1 produces a large range of fragments which have lengths which are multiples of 10 bp of each other, for both the linker region and the nucleosomal region, but why should the spectrum of lengths of fragments of linker DNA be maintained in 10 bp phase with the fragments of nucleosomal DNA when the linker DNA can be attacked by DNase 1 from any side and the nucleosomal DNA can be attacked only from the side away from the histones?

4 In some transcription systems ⁶⁶ some or all of the operator sequences binding promoter proteins with masses of many kilodaltons reside within the transcribed DNA sequence yet massive transcribing proteins such as the RNA pol III complex can transcribe the whole sequence without being obstructed by the promoter and without the promoter being displaced from its operator sequence.

Numerous studies of nuclease cleavage of nucleosomal DNA have demonstrated a very high periodicity of cutting despite presumed bending of DNA or variations of pitch or groove width with base sequence.

Simpson and Whitlock ⁶⁷, working with random sequence DNA on nucleosomes digested with DNase 1, report that:

"The results show that the nucleosome contains a potential cleavage site every 10 nucleotides, with the exception of the site 80 nucleotides from the 5' end."

(In ref. 28, Chapter 11, is offered an explanation as to why site 80 might be different from the others in respect of cleavage by DNase 1, though it is cut relatively strongly by MN and DNase 2 ⁴⁹.)

Lutter ⁴⁵ reached a very similar conclusion. His Figure 4 shows cutting at 10, 20, 30, 40, 50, 60, 70, 90 and 100 nucleotides from the 5' end, with minimal cutting at 80, using DNase 1.

In summary then, whether DNA is on or off the histone proteins and whether it is random sequence or of specified sequence, several general nucleases cleave it with high regularity at or about 10 bp intervals. The primary cutting sites of DNase 1 and MN coincide on the nucleosome, and, according to the results of Jessee et al. ⁶⁸, these cutting sites would seem to coincide off the nucleosome also, that is, in the linker DNA.

For naked DNA it is emphatically not the case that MN and DNase 1 approach their duplex DNA substrate from random directions, either compared each enzyme with the other, or comparing one enzyme alone cutting individual duplex molecules.

This situation is poorly explained on the basis of the high cylindrical symmetry of the double helix, but is easily explained by the proposition that the DNA duplex has a rear and a front face, each worked by MN and DNase 1 respectively, and adopts a highly preferred base sequence - specific rotational conformation to maximise base stacking stabilisation and hydrogen bonding between adjacent base pairs. It is probably this preferred conformation which gives rise in some studies to what has hitherto been assumed to represent base and sequence-dependent cutting preferences on the part of the nucleases themselves.

There is, in the report of Mirzabekov et al. ⁶⁹ on their study of the sequence in which histones are brought into contact with DNA wrapped around the nucleosomal core proteins, the fascinating observation that the first 20 bp of both 5' ends of the DNA are not in close association with any core histones even though their partner strands, the 3' ends, are in close association with parts of histones H2A and H3. What has remained unexplained, in a double helix where two full turns of DNA would exist in a length of 20 bp, is that only the 3' ends of strands are in close association with core histones but not the partner, 5' ends.

The paranemic model allows an explanation of their result. At both ends of the nucleosomal DNA, the 5' ends are not in contact with the histone core because only one sugar-phosphate helix of the pair is in such contact, namely the 3' end due to the postulated axial twisting of the DNA duplex through 90° as it leaves the nucleosome.

The double helix model cannot allow this possibility because the two strands are wound round each other through 2 full helical turns at each end.

With the paranemic duplex DNA structure it is possible to offer a straightforward explanation of the rapid and potent specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*⁶. The sequence specific *unc-22* gene presents its base sequence outwards into its surrounding environment because it is the rear face which is wound onto the nucleosomal core. To this sequence the double-stranded RNA can immediately bind as a stable series of Löwdin Tetrads, that is, the pairing of like base pairs with each other²⁸.

Appendix 3.

(D) The Action of Endonucleases on Chromatin

It has long been known that in eucaryotic DNA the duplex is wound onto a succession of nucleosome cores composed of eight histones (2 sets of H2A, H2B, H3 and H4) and kept steady in its place around these core proteins by a further histone, H1.

The DNA and its core are further found to resemble a wedge-shaped, squat cylinder, or, alternatively a prolate spheroid, of approximate major axis diameter 11 nm and minor axis diameter 5.7 nm. The individual nucleosome, that is, the DNA and 8 histones around which it is wrapped, can be severed on both its ends from the remaining chromatin by digestion with MN, when it is found that some 146 bp are wound round the histones in a so-called superhelix, which is left-handed⁴⁵ and constrained in 1.75 turns around the histones (see, for example, 46, 70 - 73). Between the nucleosomes, which are spaced like "beads on a string" along the chromatin⁷⁴, is found "linker DNA".

Now, when chromatin stripped of H1 is digested with general endonucleases of low individual base specificity, such as MN and DNase 1, some quite different behaviours of the nucleases can be distinguished, both between the nucleases and between linker and nucleosomal DNA.

MN attacks linker DNA in marked preference to nucleosomal DNA, which is cut at rather few places by this nuclease^{46, 75, 76} in light digests anyway.

On the other hand, digestion of chromatin with DNase 1 proceeds with very different results⁷⁷:

"Whereas primary cleavage by micrococcal nuclease occurs only in the linker region, pancreatic DNAase [DNase 1] attacks the DNA of both classes even during brief digestions."

To emphasise the contrasting location of cuts seen with MN compared to DNase 1, Cockell et al.⁴⁶ remind us that:

"MN not only cleaves between nucleosomes but, as the digestion [of chromatin] is allowed to proceed to yield nucleosome core particles, a small number of cuts ... are also introduced within the nucleosome core DNA..."

So, only a few cuts are introduced by MN within the DNA wound onto the histone proteins, with the great majority of cuts falling in the linker DNA region.

Now, the cleavage of DNA by a general endonuclease of low base specificity could conceivably take place, in terms of the paranemic model, by encirclement of the thread of DNA, or by reaction from the front face, rear face or side. If it was known in which orientation, that is, front or rear face pointing outwards, DNA wound itself

onto the basic, histone proteins constituting the core of the nucleosomal particle, it would be possible to review the modes of action of MN and DNase 1.

Now it is known that nucleohistones bear positive charges ^{75, 76} and so a natural working hypothesis is that DNA winds onto the histone proteins with its rear face in contact with the proteins, because the DNA phosphate anions would be nearer to the histone positive charges. In addition, as mentioned already, the base sequence is readily accessible when the front face points outwards.

This view accords exactly with the finding of Littau et al. ⁷⁸ that nucleohistones associate closely with the phosphate ions in DNA. Thus, as a working hypothesis, the front of the DNA duplex, bearing the exposed base pair sequence, can be considered to face outward from the nucleosome.

This particular disposition of the DNA in the nucleosome carries with it

1 a vitally important implication not offered by the double helical paradigm, because it suggests how the 30 nm chromatin fibre is assembled from the 10 nm nucleosome, utilising repetitive DNA, and offers insights into the forces which lead to the assembly and maintenance of the 30 nm fibre and higher order aggregates which the double helix model can only attempt with difficulty,

2 a ready explanation of the easy recognition of operator sequences in nucleosomal DNA by transcription factors ^{37, 66, 79}, glucocorticoid receptors ⁸⁰, restriction endonucleases ^{81, 82} and repressor-operator interactions ⁸³, for example, without the need to unwind great lengths of duplex DNA, and

3 a ready explanation of the great reduction in the activity of some antibiotics, such as the anthracyclines ¹¹², on nucleosomal DNA where the cationic amino sugar of the drug can no longer easily approach the phosphate ions now held against the nucleohistones.

Preliminary support for this new approach to the winding of duplex DNA onto histones so that only the rear face makes contact with them is offered by McGhee and Felsenfeld ^{84, 85}, quoted in Ramsay et al. ⁸⁶:

"We have shown previously that, in nucleosome core particles, histones do not make extensive contact with the DNA bases, either in the major groove or, a less certain claim, in the DNA minor groove."

Very strong support for the proposal that DNA winds onto nucleosomal core histones so that only its rear face is in contact with the proteins comes from the experimental results of McGhee & Felsenfeld ⁸⁴ who reacted nucleosomal DNA with dimethyl sulphate. They recorded their surprise at their results:

"We are unable to detect any significant difference between the reactivity of N⁷ of guanines in nucleosome DNA and of that in naked DNA... Contrary to our expectation, there is no detectable periodic modulation of reactivity corresponding to the twist of DNA on the nucleosome surface... judging from the relative concentration of intact unreacted DNA remaining, the guanines in the nucleosome reacted about 20 - 30% **faster** than in the isolated DNA... *[Their bold italics]*

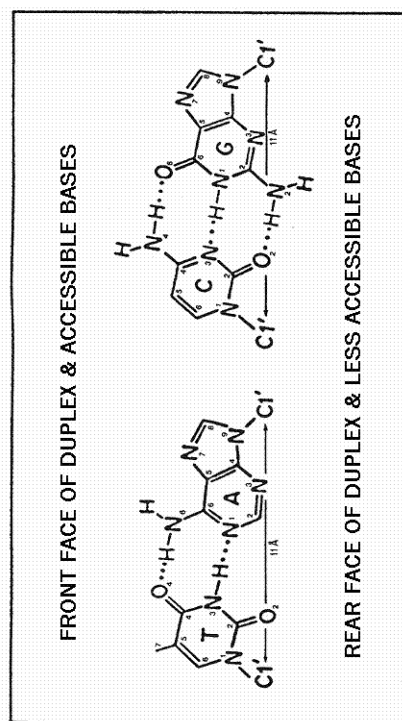
...In preliminary experiments, no significant degree of protection was detected by using the larger probe, diethyl sulfate."

According to the double helix model for the structure of DNA, about half the DNA wrapped around a nucleosome core should lie on the inside in contact with the histone proteins, or up against adjacent turns of the DNA and therefore offer reduced accessibility to methylation reagents.

Crucially, the double helix offers no clue as to how the methylation of nucleosomal DNA could be faster than it is for naked DNA.

With the paranemic model, nucleosomal DNA offers no periodic modulation of reactivity to dimethyl sulphate because only the rear face of the duplex is in contact with the histone proteins. The front face of the duplex points outwards all around the nucleosome, and N⁷ on guanine is on the leading, outward pointing edge of the Watson-Crick base pair (Fig 3). This is why there is no periodic modulation of the reactivity of N⁷ on guanine on and off the nucleosome. It presents itself to the reagent in a similar manner in both situations as the dimethyl sulphate approaches the front face.

**Fig 3 Watson-Crick Base Pairs
Illustrating The Important
Implications of the Front &
Rear Faces of the Duplex**



Indeed, on the nucleosome, the base pairs, pointing outwards towards a greater radius of curvature, are allowed more space for their leading edges, and this could be why the dimethyl sulphate reacts 20 - 30% faster with nucleosomal DNA than with naked DNA and why the larger probe, diethyl sulphate, is able to react with N⁷ on nucleosomal guanines without any evident protection of N⁷.

McGhee & Felsenfeld (496) offer further support to the proposition that contact of the DNA with nucleosomal histones is from the rear face:

"...The combined results suggest that contacts between histones and DNA are **localised to the phosphodiester backbone**... Our experiments support a model for the nucleosome core particle in which the DNA is extremely accessible to the solvent and available for interaction with other molecules..." **[My bold italics - CSD]**

Using the paranemic model, an explanation of the behaviour of MN and DNase 1 can be discerned. MN would bind on the rear face of duplex DNA and cut to one side, for reasons explored soon. MN does cut nucleosomal DNA rather infrequently, where, although the rear face is largely held close to the histones, at a limited number of sites, the rear face is accessible, perhaps because the DNA is traversing a region where it is unsupported, maybe while it is crossing from one histone to its neighbour. MN is a small protein (RMM 16,800⁸⁷) of likely diameter about 3 nm (if spherical) in proportion to an equivalent diameter of about 4 nm for DNase 1 (RMM 31,000^{38, 39}).

Then DNase 1 has its binding site on the front face of the duplex and is able to cut linker DNA and nucleosomal DNA at every turn of the helix⁷⁷ because the front face of the duplex is continuously accessible to DNase 1.

Evidently, DNase 1 cuts to one side of the duplex because⁴⁶:

"The MN primary cleavage sites lie close (on average, within 0.5 nucleotide to those previously determined by Lutter (Nucl. Acids Res. 1981, **9**, 4251-4265)) for the nuclease DNase 1 and DNase 2..."

Now, if MN and DNase 1 proceed on opposite faces of the duplex and share a common primary cleavage site, it is likely that this site lies to one side of the duplex because DNase 1 is unlikely to cut on the face on the other side of the DNA from its binding site since the nucleosomal histones would obstruct this. Likewise, MN is unlikely to cut on the face of the DNA opposite its binding site for two reasons:

1. It is a small protein with RMM of 16,800, about half that of DNase 1 at 31,000 daltons, and as it has a similar size to the cross-section of DNA it is hard to imagine that the cutting region would reach around the duplex to cut the front face.

2. In an important paper⁶⁸, in this context at least, Jessee et al:

"...find 1,10-phenanthroline cuprous complex to recognise the same sites and cleave with the same relative preferences as micrococcal nuclease..."

This must be taken with the observation of Elgin et al.⁸⁸:

"It can readily be shown that this (presumed) intercalating agent (1,10-phenanthroline cuprous ion) recognises structural differences along the chromatin fibre cutting in the linker regions and generating an oligonucleosome ladder indistinguishable from that produced with micrococcal nuclease...it essentially duplicates the cleavage pattern of micrococcal nuclease."

Two features of this passage are of interest.

Firstly, that two utterly different reagents, MN and the 1,10-phenanthroline-cuprous ion show the same pattern and frequency of cleavage in linker and nucleosomal DNA, and second, that the small cuprous ion must cleave at or near its binding site since it has no means of acting at any significant distance from its binding site because it acts by generating OH⁻ from H₂O₂.

Therefore, MN must cleave DNA at or near its binding site, and does not reach round the DNA duplex to make cuts away from its binding location. This deduction accords with the finding of Cotton et al.⁸⁹, whose crystallographic study of MN shows the binding and cutting sites to be relatively close to each other.

The paper of Jessee et al.⁶⁸ contains another observation regarding naked DNA:

"Major cleavage sites of MN as seen in Figures 1 and 4 map to approximately the same locations as DNase 1 cleavages on the same DNA substrate, although some differences are visible."

Jessee et al. worked with native DNA, unfortunately using only low-resolution gels, and it would be of great interest to repeat this experiment with the same random sequence DNA using a high resolution gel, because Cockell et al. ⁴⁶, already quoted, showed that the same locations were used by MN and DNASE 1 (where they had a common site) in cleavages on nucleosomal DNA.

If it could be established beyond reproach that the cutting sites of MN and DNASE 1 were the same in random sequence DNA on or off the nucleosomal core proteins then it would be evident that duplex DNA lays itself down on the histones in the same orientation as it enjoys naked in solution, and this would be very hard to explain on the basis of a double helical paradigm of high cylindrical symmetry.

(E) The Phasing Problem

It has been reported ^{45, 65, 90} that adjacent nucleosomes along a stretch of DNA seemed to be spaced apart from each other by multiples of 10 bp, a phenomenon which was termed the "phasing" of nucleosomes.

Tatchell & van Holde developed their work on this ⁴⁹:

"...if the protein cores are to remain stacked in a parallel array, this extra DNA (between nucleosomes) can be inserted only in 10 bp increments. This is required because the ends of the DNA strands in the inserted segment must match. Any other length (say 7 bp, for example) will lead to either a twisting of one core with respect to the next or a deformation of the DNA if the histone cores remain parallel..."

Drew & Calladine ⁹¹, in a detailed study of the spacing of histones on an 860 bp length of DNA, remarked:

"...it seemed remarkable that the centres of regions 1, 2, 3A, 3B & 4 should all be at multiples of approximately 10 bonds from one another, at positions 71.5, 212.5, 332.5, 421.5 and 592.5; and we wondered whether the folding of the DNA about the protein might be influenced by some long-range folding of the core particles themselves."

The difficulty presented by these extracts is that the double helix configuration on histones allows no explanation of why, or using what forces, adjacent nucleosomes would align themselves in parallel at separations which are multiples of 10 bp.

Using the paranemic model, if DNA wound itself onto histones in the same manner on each occasion, for example to maximise the electrostatic attraction of phosphate charges to the positive charges on histones, then adjacent nucleosomes would automatically be parallel to each other and could only be spaced at one, or higher, multiples 10 bp apart.

A result was reported in 1974 by Noll ¹, and, in greater detail, in 1977 by Lohr et al. ⁶⁵ which poses a very severe test for the double helical model for the structure of DNA. These latter workers equilibrated histone cores onto DNA, cleaved the complexes with DNase 1 and succeeded in resolving the sizes of the resulting fragments of DNA up to a maximum length of some 300 bp.

They reported that:

"Since the average size of the nucleosomal repeat in yeast is 160 bp... the presence of discrete, regularly spaced bands between 160 and 300 bases shows that... there is structural regularity extending over regions much larger than one nucleosomal repeat unit. Even when digestion produces fragments so large that they must have arisen from cleavages within two different nucleosomes, the fragments are still of discrete sizes, spaced at intervals of 10 bases.

"Figure 2A shows the results of DNase 1 digestion of small dimers and trimers... of... chicken erythrocyte chromatin. The extended ladder is extremely clear and background free.

"Yeast nuclei possess endogenous endonuclease... which also produces an extended ladder of fragments to 300 or more bases with fragments spaced at 10 bp intervals."

Not only does DNase 1 cleave nucleosomal DNA at 10 bp intervals, and the spacer DNA between nucleosomes at 10 bp intervals also, but the two sets of fragments are also 10 bp apart from each other, producing an "... extended ladder... extremely clear and background free."

How can DNase 1 score single-hit cleavages on physically separate chromatin sequences both in the spacer and in the nucleosomal DNA, at random, in such a way that electrophoresis of the aggregate of fragments, some of a length extending across several spacers and nucleosomes, always produced length differences of 10 bp, "extremely clear and background free", when the nuclease can approach the double helix from any side in the linker DNA?

Cylindrically symmetrical double helical spacer DNA must allow DNase 1 to bind and cut having approached the DNA from any side, yet intermediate fragment lengths between multiples of 10 bp are not observed.

Though these experimental results are inexplicable in terms of the double helix, the result is comprehensible using the paranemic model.

Winding duplex DNA around the histones locks the DNA into one rotational isomer with its rear face in contact with the histones. DNase 1 can cleave the DNA along the exposed front face of the spacer or nucleosomal DNA because it binds to the front face and always cuts the duplex DNA at the same geometric contour (a crest, a trough, or most probably half way between those two positions since it has a cutting site identical to MN, which binds to the rear face.)

With this scenario, only fragments with lengths a multiple of 10 bp can be produced.

(F) The Classification Of Endonucleases And Chemical Cleavage Agents

In terms of the paranemic model for duplex DNA, endonucleases and chemical cleavage agents can be classified according to whether they bind from the front or rear faces of the duplex.

By comparison of these nucleases and cleavage agents with MN and DNase 1 this approach can be extended and its implications for the structure of nucleosomes and for "footprinting" the interaction of proteins with their operator sequences in DNA can be explored.

Experimental support for this general approach to the classification of nucleases is offered by the work of Jessee et al.⁶⁸ and Elgin et al.⁸⁸ who have shown that MN and the 1,10-phenanthroline-cuprous complex cleave both DNA and chromatin with a virtually identical pattern of products which is different in some respects from that arising from similar digestions with DNase 1, and, for example, from the work of Drew and Travers⁴³ who have shown that the patterns of digestion products arising from comparison of DNase 1 with DNase 2 are such as to be generally opposite and complementary in many respects:

"..in the sequence stretching from position 110 to position 135 on the upper strand...there are 8 DNase 2 peaks, six of which correspond to DNase 1 minima. In the same region there are 5 major DNase 1 peaks, four of which are DNase 2 minima... At only 3 bonds in the entire sequence do strong DNase 1 and DNase 2 peaks coincide... Local rates of attack for DNase 1 and DNase 2 turn up strongly out of phase with one another at any phosphate..."

It is tempting to conclude from this passage that DNase 1 and DNase 2 approach duplex DNA from opposite faces but recognise the same structural contour, for example, the crest of the duplex nearest to each nuclease.

Then, should cleavage occur near the binding site, the respective cleavage patterns of DNase 1 and DNase 2 would always be exactly 1/2 turn out of phase with each other. That is, the position of maximum cleavage for one nuclease would always be that of minimal cleavage for the other, assuming that the DNA sequence being cleaved had a strong preference for a particular rotational conformer.

While DNase 1 and DNase 2 may approach free DNA from the front and rear faces respectively, such a scenario would not seem to constitute a complete statement of their approach to DNA prior to cleavage because Lutter⁹³ found that DNA wound onto nucleosomal histones was cleaved at essentially the same locations by DNase 1 and by DNase 2, though with rather different cutting frequencies.

The site numbers of the nucleosomal DNA represent the regular crests of the duplex as it is wound onto the nucleosomal histone core.

It is noticeable that the cutting frequencies of DNase 1 and DNase 2 are largely complementary except at nucleosome positions S7 and S11 where the enzymes cut with about the same frequency, or perhaps with equal difficulty of access to their substrate. Furthermore, the cutting rate of DNase 2 is anyway sufficiently high at S7 that cleavage by DNase 2 at that site is known to sever the nucleosomal DNA in half before other cuts by DNase 2 become prominent in the digests⁹⁴.

Now, the results of Cockell et al.⁴⁶ on the cleavage sites of DNase 1 and MN on nucleosomal DNA showed that cleavage positions for MN at sites 4, 5 and 12 had centres of cleavage some 5 bases shorter than the centres for DNase 1, corresponding to attack from opposite faces of the DNA, and that, in a number of other respects the cleavage patterns for MN and DNase 1 were complementary (their Figure 1):

- a) weak cleavage by DNase 1 at sites 7 and 9 was matched by zero cleavage at sites 7 and 9 by MN,
- b) strong cleavage at sites 8 and 10 by MN were matched by zero and weak cleavage respectively DNase 1, though it is noteworthy that
- c) sites 6 and 11 were virtually uncleaved by either nuclease.

Thus MN is more obviously engaged on the opposite face to that used by DNase 1.

The spread of cutting at sites 4, 5 and 12 to a distance up to five bases away from the DNase 1 sites would seem to arise from secondary sites of cutting by MN, which Drew and Calladine⁹¹ have calculated are 5 bases away from the primary sites of MN on nucleosomal DNA.

Now, Cockell, Rhodes and Klug⁴⁶ found that the primary cutting sites of DNase 1, DNase 2 and MN were coincident and, in large measure, the cutting frequencies of MN and DNase 1 were complementary. The cutting sites of MN are rather fewer, and, as was established early in studies on chromatin digestion with MN, the nucleosome is an arrest point for MN in light digests. MN is by far the smallest of the three nucleases (RMM 16,800) where DNase 1 has an RMM of 31,000 and DNase 2 has an RMM of 38,000-40,000 daltons.

MN shows evidence of secondary cleavage at sites 4, 5 and 12 to the 5' side of its primary cutting sites. This evidence seems to be consonant with a small nuclease being partly underneath, or perhaps to the side of the duplex DNA at sites 8 and 10 where no secondary cutting is evident, completely behind the DNA at sites 4, 5 and 12 so that secondary cutting can take place, perhaps by successive single hits which leave primary and secondary cutting on both strands when viewed over a large number of nucleosomes, and with sites 6 and 11 rather obscured by histones because neither DNase 1 nor MN could cut at those locations.

Table 1

Cutting Of Nucleosomal DNA
By DNase 1, DNase 2 & MN

SITE No.	DNASE 1 (45, 50)	DNASE 2 (93)	MN (46)
1 (5')	many cuts	less cuts	zero cuts
2	few cuts	less cuts	zero cuts
3	v.few cuts	v.few cuts	v.few cuts
4	many cuts	less cuts	v.few cuts
5	many cuts	zero cuts	less cuts
6	zero cuts	v.few cuts	v.few cuts
7	few cuts	few cuts	few cuts
8	zero cuts	few cuts	less cuts
9	few cuts	less cuts	less cuts
10	few cuts	many cuts	many cuts
11	zero cuts	zero cuts	zero cuts
12	few cuts	many cuts	many cuts
13 (3')	less cuts	many cuts	many cuts

NOTE: Many cuts > less cuts > few cuts > v.few cuts > zero cuts

At site 8 the approach to the front face of the duplex may be obstructed by histones so that there is no cleavage by DNase 1, but evidently the rear face is accessible to MN cleavage. Perhaps the DNA is traversing the boundary between two core histones at that point such that the front face is obstructed by overhanging core histones, though normally it would be the rear face which makes contact with the histones, while, at site 8, the nucleosomal histones may not be in contact with the rear of the duplex. Certainly, at sites 3, 6, 8 and 11, symmetrically disposed about site 7, it is known that the DNA is sharply bent ¹⁰⁹ around the "tails" of the core histones ¹¹⁰ so it is possible that the deformation of the normal DNA structure, assisted perhaps by the passage of DNA from contact with one histone to that of its neighbour, is enough to obstruct the action of DNase 1, DNase 2 and MN in some degree and to account for the observed cleavage pattern at these sites.

The work of Lutter⁹³ and Cockell et al.⁴⁶ invites further consideration of the behaviour of DNase 2. On free DNA, Drew and Travers⁴³ found its local cutting frequencies to be generally out of phase with those of DNase 1, though on nucleosomes DNase 1, DNase 2 and MN all shared the same sites of primary cleavage. At the same time, DNase 2 and MN show marked differences in cutting frequencies from each other. In particular, MN exhibited zero cutting at site 9 where DNase 2 showed moderate cutting. Again, DNase 2 showed zero cutting at site 5 where MN showed moderate cutting.

If MN and DNase 1 operate on opposite faces in free and nucleosomal DNA, and DNase 1 and DNase 2 show complementary cutting frequencies with DNase 2 different from both DNase 1 and MN in nucleosomal DNA, then it may be that DNase 2, being the largest of the three nucleases binds to the side of the duplex in nucleosomes, where the large size of DNase 2 (about 3.5 nm) would seem to preclude it from operating on the rear face and render access even to the sides difficult in most positions. Only at position 9 would DNase 2 seem to have a higher cutting frequency than both DNase 1 and MN where one side of duplex DNA may be accessible, with histone on the other side to obstruct front and rear.

Some support for the idea that DNase 2 attacks to the side of duplex DNA is offered by Drew³⁸:

"DNase 2 does not seem to require a double-stranded track as does DNase 1... Perhaps it has a very narrow binding domain, and can reach down into the groove of the double helix so as to bind one strand with only minimal interference from the opposing strand."

The observations by Drew accord with these of Lutter⁹³:

"A comparison of the DNase 2 exposure pattern...with that of DNase 1 shows both similarities and differences...the DNase 2 pattern in general tends to be more uniform than that of DNase 1, **especially toward the middle of the DNA [My bold italics - CSD]**...Put another way, DNase 2 does not seem to differentiate among sites' accessibilities as effectively as does DNase 1..."

Now DNase 2 at RMM 38,000 is larger than DNase 1 at RMM 31,000 so its ability to cleave relatively inaccessible sites seems to imply that it is able to bind and cut an exposed length of one strand of the duplex, and would therefore be able to attack duplex DNA from the side.

TABLE 1 helps to bring out not only insights into the comparative modes of operation of the nucleases but also structural insights into the nucleosome itself. Labelling the 5' and 3' ends of the nucleosomal DNA as sites 0 to 14 respectively, corresponding to the typical nucleosomal duplex length of 145 bp, with site 7 therefore as the centre site, we see indications of apparent asymmetry about site 7, for example⁴⁵, (Figure 5(c)),⁴⁶ (Figure 5 (a)). Sites 5 and 9, at ± 2 sites from 7, shows DNase 1 to be very active at site 5 but not at 9.

A similar remark can be made about a high cleavage rate for DNase 1 at site 4, though not at site 10, ± 3 sites away from site 7. Indeed, at site 10 it is DNase 2 and MN which are highly active, though these are much less so at site 4. Sites 4 and 10 seem to show converse activities from each other for all three general endonucleases.

On the other hand, sites 3 and 11, ± 4 sites from site 7, show a similar pattern of endonuclease activity. It is interesting that the converse patterns of nuclease activity at sites 4 & 10, and the rather low levels of activity at sites 6 & 8, are those identified as sites of sharp bends and strong binding to H4¹⁰⁹.

**Table 2 A Summary
Of The Mode Of Interaction
Of Endonucleases And
Chemical Cleavage Agents
With Paranemic DNA**

Endonuclease Or Cleavage Agent	Mode Of Interaction
DNase 1	front face
DNase 2	side
MN	rear face
Restriction Endonucleases	front face
o-phenanthroline- cuprous-complex	intercalation from rear face
methidium-propyl- EDTA-Fe(II) (refs in ref 11)	intercalation from rear face
Bleomycin-ferrous Complex (95,96)	front face
Metalloporphyrins (60,97,98)	intercalation from rear face

This classification of hydrolytic cleavage agents allows an explanation of the protocols presently used to footprint DNA, such as those reported by Fitzgerald & Anderson ¹⁶:

“... Translational positioning was assessed by ... micrococcal nuclease digestion (MNase) studies. ... DNase1 digestion studies were used to determine the rotational orientation of sequences along the nucleosomal DNAs.”

MN can be used to determine the translational position of the nucleosomal histone core because the MN acts on the rear face of the paranemic duplex where the non-sequence-specific histone proteins are bound, whereas DNase 1 is used to define the rotational orientation of the DNA wound onto a nucleosome because that endonuclease acts on the other, sequence-specific front face by cleaving at a regular contour of the paranemic helix, possibly the crest of each turn.

Similarly, Wong et al. ⁵ have used MN cleavage of the linker DNA in nucleosomal DNA to determine the translational position of the thyroid hormone response element (TRE) in *Xenopus*. This is possible because the nucleosomal histones bind to the rear face in paranemic DNA and act as stop signals to MN also acting on the

rear face. Thus DNase 1 cannot be used to determine the translational position of TRE because this nuclease acts on the front face and will cleave the DNA every 10bp along the entire length.

Again, Prunell ¹⁷ reports that in topological studies of the twist of DNA around nucleosomal histones carried out in order to resolve the Linking Number Paradox, MN was not used but that DNase 1 was so used. MN could not be used because it acts on the rear face and is stopped with a periodicity determined by proteins bound to the rear face, in this case the nucleosomal octamer.

(G) Enhanced Rates Of Cleavage Of Duplex DNA By Nucleases

Sakonju & Brown ⁶⁶ reported on the contacts made by a 40,000 dalton transcription factor, tf III A, with its substrate, an intragenic control region in the 5S RNA gene of *Xenopus*.

They examined these regions of contact by determining which parts of the DNA sequence were protected from cleavage by the general endonuclease DNase 1, which has an RMM of 31,000 daltons. Thus tf III A is composed of approximately 2500 C,N,O atoms and DNase 1 contains of the order of 2000 C,N,O atoms.

Their Figure 5 shows a total region of protection extending from bases 44 to 90, and, within that one protected region, at bases 60 & 61, there is strong enhancement of cutting of the DNA : tf III A complex by DNase 1, itself a substantial protein having some 2000 C,N,O atoms.

Now Sakonju & Brown demonstrated that tf III A is in intimate association with nearly every base between 81 and 91, and assumed that this must involve the major groove since methylation in this region at 7-Guanine decreases binding, and they assumed further that unwinding of the duplex must occur during transcription.

So tf III A protects the greater part of 4.5 turns from 44 to about 90. This cannot all be single stranded since DNase 1 cleaves bases 60 & 61, and 74 & 76, but DNase 1 is known to bind only to double stranded DNA.

Since bases 60 & 61 enjoy enhanced cleavage they must be more accessible than they were in naked DNA. This increased accessibility takes place in a region of length 4.5 turns x 3.4 nm (some 15 nm) where the regions around 60 & 61 and 74 - 76 must still be double stranded, where a sizeable transcription factor with some 2500 C,N,O atoms is bound and where a sizeable nuclease of some 2000 C,N,O atoms experiences increased accessibility to its substrate at bases 60 & 61, in the middle of the protected region.

Drew & Travers ³⁷ record their frustration with this situation:

"Many eukaryotic transcription factors, notably tf III A from *Xenopus*, protect up to 50 bp of DNA from enzymatic cleavage, yet **somehow** manage to induce regions of enhanced cleavage within their binding sites..."
[My bold italics - CSD]

If tf III A were operating in the major groove of its binding site it must contrive to have vacated that groove around regions 60 - 61 and 74 - 76, though not in the region 77 - 90, but, being a bulky protein, it is difficult to see how this would lead to enhanced cleavage at 60 & 61 by a bulky nuclease, DNase 1.

One possibility here is that the double helical duplex is wound around tf III A so that bases 60 & 61 are left exposed and readily accessible on an outer surface to easy approach by DNase 1. The main problem with this is that bases 74 - 76 are still cleaved by DNase 1 with slight enhancement and are 1.5 double helical turns away from 60 & 61. If bases 60 & 61 are on an outside surface, bases 74, 75 & 76 must lie on an inside surface, yet they are still cleaved efficiently.

Daniela Rhodes ⁷⁹ has studied the cleavage of the internal control region in DNA, bound and unbound to nucleosomal histones, in the presence and absence of tf III A, with DNase 1 as a structural probe. She identified protection between bases 45 - 97. Her Figure 1 shows enhanced cutting on the non-coding strand at bases 39, 62, and 92, with continuing cleavage also at 40 - 44, 60 - 64, 74 - 76 and 91 - 94, that is, at

multiples of 10 bp, for DNase 1 cutting of DNA complexed with tf III A. This periodicity would seem to make it difficult for tf III A to bind to its DNA substrate within the major groove (and 7-Guanine positions do not project into the minor groove at all so that minor groove binding is already excluded.)

In her study, Rhodes recorded these observations:

"The results presented in Figure 3 show that tf III A forms a stable complex (or aggregate) both with nucleosome cores containing the 5S RNA gene and naked DNA containing the same gene. In the two experiments, saturation of binding is reached at the same concentration of tf III A, ***although binding of tf III A to the nucleosome appears more gradual than to the naked DNA.*** [My bold italics - CSD]

"Comparison of the DNase 1 cutting patterns in Figure 5a shows that, upon tf III A binding, the pattern of digestion of the nucleosome core in the region of the internal control sequence is lost, and instead becomes characteristic of the tf III A - DNA complex. Because the positions and relative intensities of other DNase 1 cutting sites within the nucleosome core do not change, it is clear that the histone octamer does not "slide" in order to accommodate tf III A binding."

Sakonju & Brown showed in naked DNA that methylation of 7-Guanine at 70, 71, 81, 82, 85, 86, 87 and 89 on the non-coding strand, and 91 on the coding strand, significantly reduces the binding of tf III A, while phosphate ions at 69, 70, 79, 80, 82, 84, 85 and 86 are also in contact with tf III A. Now, using the paranemic model, the phosphates are variously at the side, or to the rear, while 7-Guanine positions are on the front face. Tf III A can hardly recognise the phosphates as such, so that it must recognise the front face between 80 and 90 (and 70 - 72), while the bulk of the protein moves round to the rear face in intimate contact with the phosphates.

The whole protected region might be wound onto the tf III A in a manner very similar to that observed in nucleosomes, with the rear face in contact with the tf III A, reminiscent of the rear face of DNA being in contact with nucleosomal histones. This could be why Rhodes found that the binding of tf III A to a nucleosome containing the DNA control region was more gradual, because both the histones and the tf III A were competing to occupy the rear face.

This might explain also why the DNase 1 cleavage pattern of the ternary histone-DNA-tf III A complex was that of the DNA-tf III A binary complex in the control region, where, in both complexes, the tf III A was bound to the rear face in much of the control region and DNase 1 operated on the front face. It might seem unlikely at first sight that some or all of the bulky tf III A molecule could interpose itself between the nucleosomal histones and the DNA but many NMR studies^{99, 100, 101} have shown that there are mobile, and, indeed, highly mobile regions of the histone core.

Enhanced cutting with DNase 1 is now possible within the protected region because, as deduced earlier, DNase 1 operates on the front face while tf III A would seem to bind to the rear face over much of the protected region. Rhodes identified continuing cleavage of DNA by DNase 1 at about 10 bp intervals, again reminiscent of nucleosomal cleavage by DNase 1, but some cleavages, for example, 50 - 54 and 80 - 84 are weak or missing, perhaps because of steric obstruction at those sites by tf III A, also reminiscent of nucleosomal cleavage patterns. As it is likely that tf III A bends its DNA substrate¹⁰², the DNA-tf III A complex may be a nucleosome-like particle with the rear face of the DNA kept in contact with the transcription factor.

At no stage is it necessary to postulate the unwinding of the DNA internal control region, simultaneously supercoiled around the nucleosome, since unwinding is unnecessary, and, of the highest importance, RNA polymerase III (pol III), in its transcribing complex, RMM = 650,000 daltons¹⁰³, can reach and pass by tf III A which is bound to the rear face of the DNA, because the polymerase needs to read the genetic code which is accessible to it from the unobstructed front face.

Thus it is possible to reconcile all the known facts, some hitherto intractable, by use of a paranemic model employing front and rear faces rather than one giving rise to helical major and minor grooves.

A extensive review of thinking regarding the DNA - tf III A interaction is that of Kingsman & Kingsman ¹⁰⁴. Thus a fuller discussion would include the precise rôles, and binding sites ¹⁰⁵, of tf III C and tf III B which complex in that order with tf III A and DNA after tf III A has attached itself to its part of the internal control region. It has been suggested that pol III can pass by the complex of transcription factors, which are attached to the DNA at a number of places, by a mechanism in which, successively, these places of attachment are detached prior to, and re-attached after its passage.

A difficulty here is that for many, or perhaps all of the occasions when such detachment would occur, the pol III, of RMM upwards of 250,000 daltons, would have to squeeze through the loop formed by the two adjacent attachment sites of the rather smaller transcription factors constituting one part of the circumference of the loop, and a short length of DNA constituting the other part of the circumference between the transcription factor attachment sites still in place.

Until physical dimensions can be given to the loop circumference and to pol III, this means of passage might seem speculative. In any case it hardly explains convincingly how the pol III transcription system would operate on chromatin more effectively than on bare DNA for certain genes in the presence of histone cores ¹⁰⁴, or the enhanced cleavage with DNase 1 observed for the DNA - tf III A complex at bases 60 & 61.

It has been possible to offer an account of the approach and binding of tf III A to naked and to nucleosomal *Xenopus* DNA so as to explain the enhancement of certain cleavage positions and the protection of others, using the paranemic model.

Basically, this explanation turns on the transcription factors in *Xenopus* becoming attached to the rear face of the duplex after tf III A has found its recognition site using the front face. This accounts for the fact that the assembly of the transcription factors on vertebrate DNA generates little or no signature when footprinted with DNase 1 (which acts on the front face).

However, the equivalent *Saccharomyces cerevisiae* recognition sequence, which has a very high homology with that of *Xenopus*, does allow its transcription factors to be footprinted with DNase 1 ¹⁰⁵, but it is not established that the yeast transcription factors are still on the front face when transcription is actually in progress.

(H) An Experiment To Distinguish Between The Paranemic & Plectonemic Models

An interesting experiment suggests itself at this point which may make it possible to distinguish decisively between a model implying the existence of double-helical major and minor grooves on the one hand and front and rear faces on the other.

Ramsay et al. ⁸⁶ identified a 145 bp DNA sequence which adopts a single preferred position on the nucleosome core, having a sequence they define on page 2610. According to the double helical model some significant length of the major groove will be in contact with, or very close to the histone proteins, perhaps one third to one half.

Consequently in any methylation processes which alkylate N⁶-Adenine or ⁵Cytidine, say, especially after only a brief exposure to the methylases so that these proteins would have less opportunity to interpose themselves between the DNA and the histone core, we should expect to find alkylated bases at multiples of about 10.4 bp apart from each other in each of the N⁶-Adenine or ⁵Cytidine series if the distribution of methylation sites allowed this.

Relative to methylation at a particular site, we would not expect to find alkylation at removes of odd multiples of 5.2 bp from this site because all, or a great majority, of these multiples would lie in contact with the histones and be relatively inaccessible to the methylases.

On the other hand, the paranemic model suggests that methylation will lead to alkylation of all allowed N⁶-Adenine and ⁵Cytidine sites, though very likely with some variation in methylation rate since some of the A and C bases will lie at or near crests and others at or near the slightly less accessible troughs.

It is fortunate that the sequence used by Ramsay et al. contains a large number of A and C bases at separations close to or at multiples of 5.2 and 10.4 from other A and C bases respectively, so making this sequence especially inviting for use in the proposed experiment.

Some of the experimental difficulties to be encountered, for example, the sequence influence on methylation rates at ⁵C, and the influence of prior methylation at nearby sites on the complementary strand are discussed by Bolden et al. ¹⁰⁶. It may be necessary to study the methylation of the naked DNA sequence as a control. However, the 145 bp sequence contains methylatable EcoR II and Hind II sites, unfortunately an inconvenient 8 bp apart, but also Mbo II and Sph I sites which may be methylatable. There would seem to be an important site 14 bp downstream of the EcoR II site in the sequence TCGmA cleaved by Taq I and TthHB8 I ¹⁰⁷.

It may also be the case that the 145 bp sequence may include appropriate sites of N⁶A methylatable with the systems described by Smith et al. ¹⁰⁸, so that a map of accessibilities of ⁵C and N⁶A sites could be deduced. Any separated by odd multiples of 5.2 bp should not show a pattern of protection by histones using the paranemic model.

(I) Summary

Experimental results drawn entirely from the literature have been identified and compared in order to offer an explanation of hitherto unaddressed problems:

1. a possible explanation is offered as to why DNase 1 might approach paranemic DNA from only one side and not the whole range of directions of approach allowed to a cylindrically symmetrical double helix by considering the similarities in the points of cleavage in a 169 bp length of naked linear DNA compared with the pattern for the same sequence when circularly closed,
2. a possible explanation is offered as to why a number of studies of transcription might indicate that the DNA-protein complex is in contact along substantially one face only of a paranemic duplex,
3. an account is offered of the cutting frequencies and the pattern of cleavage of nucleosomal DNA by DNase 1, DNase 2 and MN in terms of cleavage from the front, side and rear of the paranemic DNA duplex, respectively,
4. an account is offered of the close similarity in cleavage patterns of MN and the 1,10-phenanthroline-Cu⁺ ion, even though these cleavage agents are very different chemically, so deducing that they both act from the same face of the paranemic DNA duplex,
5. an account is offered of the action of DNase 1 on duplex paranemic DNA adsorbed onto mineral surfaces in which it is noted that the linear DNA disposes itself onto the mineral surface in a mixture of only two orientations, variously front face onto the mineral or rear face onto the mineral, but that DNase 1 will cut only those molecules lying rear face down on the mineral,
6. an account is offered of the fact that both nucleosomal, and its contiguous internucleosomal DNA is cleaved by DNase 1 into multiples of 10 bp across the whole length of the DNA demonstrating that 10bp phasing of the cleavage by DNase 1 extends without dislocation both on and off the nucleosome as the DNase 1 operates from only one face of the paranemic duplex,
7. a new classification of some nucleases and chemical cleavage agents according to which face of paranemic duplex DNA they recognise is offered.

It is possible to offer an account of Protacio & Widom's observation ² of real time transcription rates on reconstituted nucleosomes compared with naked DNA:

"... Remarkably, the sites of this increased pausing on the nucleosomal templates are also pause sites on the naked DNA. ..."

For both nucleosomal DNA and naked DNA the transcription proceeds on the front face, which is fully and permanently exposed in both situations since the nucleosomal histones are almost totally bound to the rear face,

8. how it might be possible for DNase 1 to cleave DNA with enhanced cutting rates in the presence of a large, complexed protein, tf III A, bound to the DNA,

9. how it might be possible for the RNA pol III transcribing complex to operate along a paranemic DNA sequence, within which tf III A is bound, without displacing the transcription factor and without being obstructed in its operation,

10. how it might be possible that RNA pol III can transcribe certain genes rather better when the DNA is itself wound onto histones than when it is not, and

11. a paranemic paradigm of DNA is developed to show that the experimental results reported by Lee et al. ³³, Figures 3(b) & 3(d), Beebe et al. ³⁴, James & Mazia ³⁵, and Wu & Wu ¹¹¹, accord with the physical dimensions and topology of a paranemic structure for B form duplex DNA having an oval cross-section with minor and major axes of some 1.2 and 2.1 nm respectively, while retaining a pitch of 3.34 nm.

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