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Biochemistry

Echinomycin-induced hypersensitivity to osmium tetroxide of DNA fragments incapable of forming Hoogsteen base pairs

(antibiotic binding/DNA structure/nucleotide analogs)

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ABSTRACT **To show conclusively that the critical structural deformation of double-helical DNA that is induced by the binding of quinoxaline antibiotics does not involve the formation of Hoogsteen base pairs, we have prepared a DNA fragment containing the nucleoside analog 7-deaza-2′-deoxyadenosine in one of the two strands. This DNA fragment was subjected to treatment with the thymidine-specific reagent osmium tetroxide and to DNase I "footprinting" in the presence or absence of micromolar concentrations of echinomycin. We report that this anti-tumor antibiotic binds to DNA containing the nucleoside analog as well as to natural DNA and that the previously reported hypersensitivity to osmium tetroxide of certain thymidine residues adjacent to echinomycin binding sites is maintained in analog-containing DNA. Since these thymidines are rendered incapable of participating in Hoogsteen base pairs by the incorporation of 7-deaza-2′-deoxyadenosine, we conclude that this unusual base-pairing scheme is not the cause of the observed hypersensitivity to osmium tetroxide and that it therefore results from a large local unwinding of the DNA in the presence of the antibiotic. Moreover, preventing the possibility of Hoogsteen base pairing does not preclude echinomycin binding.**

The physical consequences of the binding of quinoxaline antibiotics to DNA have been the subject of some controversy in recent years. Thus, while crystallographic studies (1, 2) have strongly suggested that base pairs adjacent to drug-binding sites adopt the unusual Hoogsteen (3) base-pairing scheme, NMR evidence indicates (4, 19) that this is not always the case and that A·T base pairs at the ends of some sequences adopt this configuration while others remain paired via the normal Watson–Crick scheme.

There also exist conflicting reports regarding the implications of the hypersensitivity of certain regions of DNA fragments to attack by chemical reagents in the presence of micromolar concentrations of these antibiotics (5–8). Thus, Mendel and Dervan (5) observed that some purines (mainly adenine residues) adjacent to echinomycin binding sites become hypersensitive to reaction with diethyl pyrocarbonate (DEPC) when the antibiotic is bound and concluded that this reaction was consistent with the formation of Hoogsteen base pairs in which the adenine base is in the *syn* conformation about the glycosidic bond. They reasoned that since N-7 of a Hoogsteen-paired adenine moiety is no longer available for reaction with DEPC (since it is involved in hydrogen bonding), the reaction site must be at either N-1 or N-3. We have recently shown (8) that in tubercidin (7-deazaadenosine), DEPC reaction may indeed occur at N-1 or N-3, but this reaction does not lead to scission of the glycosidic bond, and thus reaction at these positions is unlikely to give rise to the results observed by Mendel and Dervan (5) as well as others (6–8).

By using a range of chemical and enzymatic probes that should be sensitive to the presence of Hoogsteen base pairs, we showed that the complex patterns of reactivities observed in the presence of echinomycin (Fig. 1) do not tally with those expected if this unusual DNA structure were in fact present. In particular, we found (8) that certain thymidine residues are rendered hypersensitive to modification by OsO_4 in the presence of echinomycin. While it is conceivable that this is a consequence of the rotation about the glycosidic bond of their complementary adenines (a necessity for Hoogsteen base pairing), we felt that the results were better explained as a consequence of the known widening of the major groove (9) on binding of the antibiotic.

To resolve this outstanding question, we have prepared large quantities of single-stranded *tyrT* DNA by cloning the double-stranded DNA fragment from pKMΔ-98 (10) into M13mp19 and isolating single-stranded phage DNA. The complementary strand was then synthesized in the presence of 7-deaza-2′-deoxyadenosine 5′-triphosphate or 2′-deoxyadenosine 5′-triphosphate and the double-stranded DNA fragments were then released by cleavage with restriction endonucleases. The overhanging ends were filled in with radioactive nucleotides so that the original strand (i.e., non-analog-containing) was radiolabeled.

These fragments were then digested with DNase I in the presence or absence of echinomycin to determine whether the antibiotic still binds to DNA containing the analog. There appears to be no significant difference in the strength of binding to such DNA fragments, as judged by the intensity of DNase I footprints. OsO_4 reactions were performed on the fragments, and we observed an almost identical pattern of reactivity in the presence of echinomycin, whether or not the DNA samples were substituted with the unnatural nucleotide. Since the 7-deaza-2′-deoxyadenosine residues opposite the thymidines that exhibit this reaction are incapable of forming Hoogsteen pairs, we conclude that the hyperreactivity to OsO_4 previously observed (8) is due to unwinding of the DNA upon intercalation and does not reflect the presence of Hoogsteen pairing.

MATERIALS AND METHODS

Preparation of Single-Stranded DNA Substrates. A 600-base-pair (bp) DNA fragment containing the *tyrT* promoter and the λt_{RI} terminator region was liberated from pKMΔ-98 (10) by digestion with *Eco*RI and *Eco*RV endonucleases. The sticky end generated by cleavage with *Eco*RI was filled in by DNA polymerase (Klenow fragment) in the presence of dATP and dTTP, the fragments were separated by PAGE, and the 600-bp fragment was excised, eluted, and recovered by precipitation from ethanol. This fragment was then ligated

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Abbreviation: DEPC, diethyl pyrocarbonate.

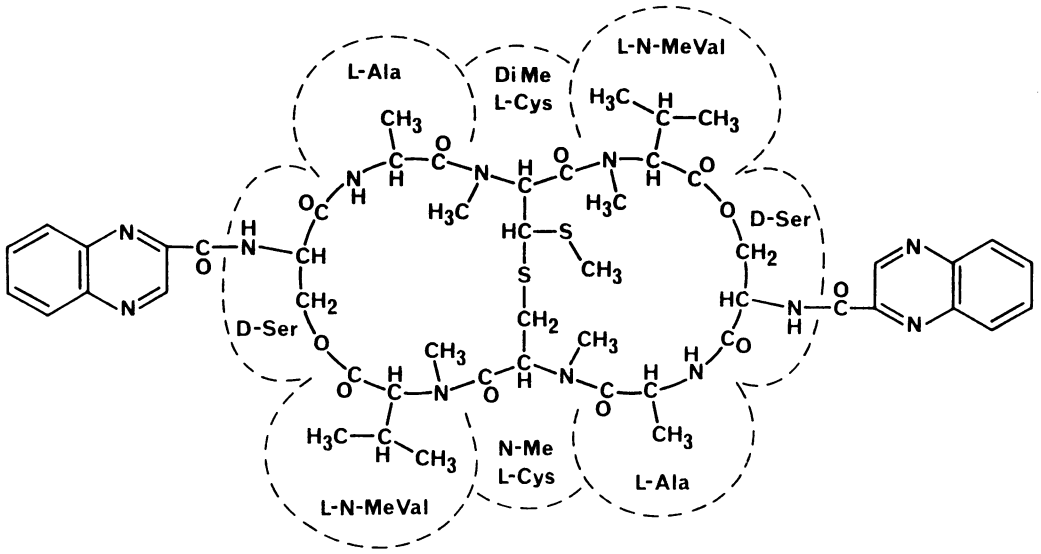

FIG. 1. Chemical structure of echinomycin.

into the *Sma* I site of M13mp19 (gift of Mike Way, Medical Research Council Laboratory of Molecular Biology, Cambridge), and the ligation mixture was used to transform *Escherichia coli* TG-1 cells. Heat shocked cells were plated according to published protocols (11) and incubated at 37°C overnight. Colorless plaques were transferred by toothpick into tubes containing a 1:100 dilution of an overnight growth of TG-1 cells in 1.5 ml of 2× TY medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per liter adjusted to pH 7.4) and incubated with vigorous shaking at 37°C for 2 hr. The cultures were then transferred to 1.5-ml Eppendorf tubes and spun in an Eppendorf centrifuge for 10 min. The supernatants were added to 150 ml of a 1:100 dilution of an overnight growth of TG-1 cells in 2× TY medium, and these flasks were incubated with vigorous shaking at 37°C for 5 hr. To the supernatants obtained after centrifugation was added 20 ml of 20% polyethylene glycol, and the tubes were Vortex mixed thoroughly and left to stand at room temperature for 20 min. Single-stranded recombinant phage DNA was then isolated from the phage pellet by scaling up of the published procedure (11). The growth and isolation conditions outlined above gave an average of 400 μg of single-stranded DNA from each original plaque.

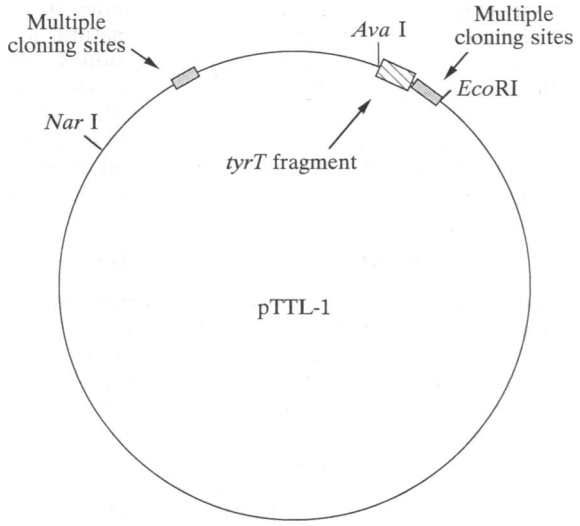

FIG. 2. Construction of pTTL-1, showing the relative positions of the *tyrT* fragment and the M13mp19 multiple cloning sites.

The presence of inserts and their orientations relative to the M13 polylinker were then determined by dideoxynucleotide sequencing strictly according to published protocols (11, 12). By this means a clone containing the entire *Eco*RI/*Eco*RV fragment was identified and designated pTTL-1. Its constitution is shown in Fig. 2. In pTTL-1, the fragment has inserted such that the lower strand of the original *tyrT* fragment is present in the single-stranded virion DNA. Synthesis of the complementary strand of this molecule was achieved by using the Klenow fragment of DNA polymerase to extend a short complementary primer as described below.

Preparation of Double-Stranded DNA Substrates. A synthetic DNA oligomer 17 nucleotides long was kindly prepared by M. J. Gait (Medical Research Council Laboratory of Molecular Biology, Cambridge, U.K.) (using an Applied Biosystems 380A DNA synthesizer) that corresponded to the complementary strand of the M13mp19 polylinker extending from 7 bp to the right of the *Eco*RI site to the *Sac* I site. It was annealed to the single-stranded template. This mixture was then treated with DNA polymerase (Klenow fragment) in the presence of 100 μM deoxynucleoside triphosphates. Alternatively, 2'-deoxyadenosine 5'-triphosphate was replaced in the reaction mixture by its 7-deaza- analog, thus generating molecules in which one of the DNA strands contains the 7-deazaadenine nucleotide in place of the naturally occurring adenine nucleotide. The reactions were allowed to proceed at

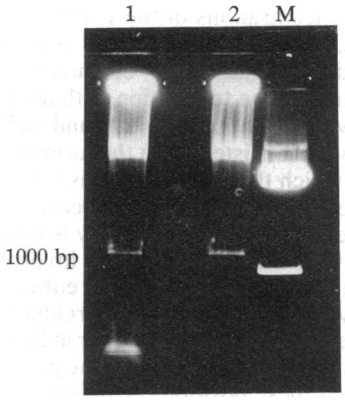

FIG. 3. Gel electrophoresis of double-stranded *Eco*RI/*Nar* I fragments obtained after synthesis of the complementary strand of pTTL-1 in the presence of dATP (lane 1) or of 7-deaza-dATP (lane 2). Lane M, marker from an *Eco*RI/*Nar* I digest of pKMΔ-98.

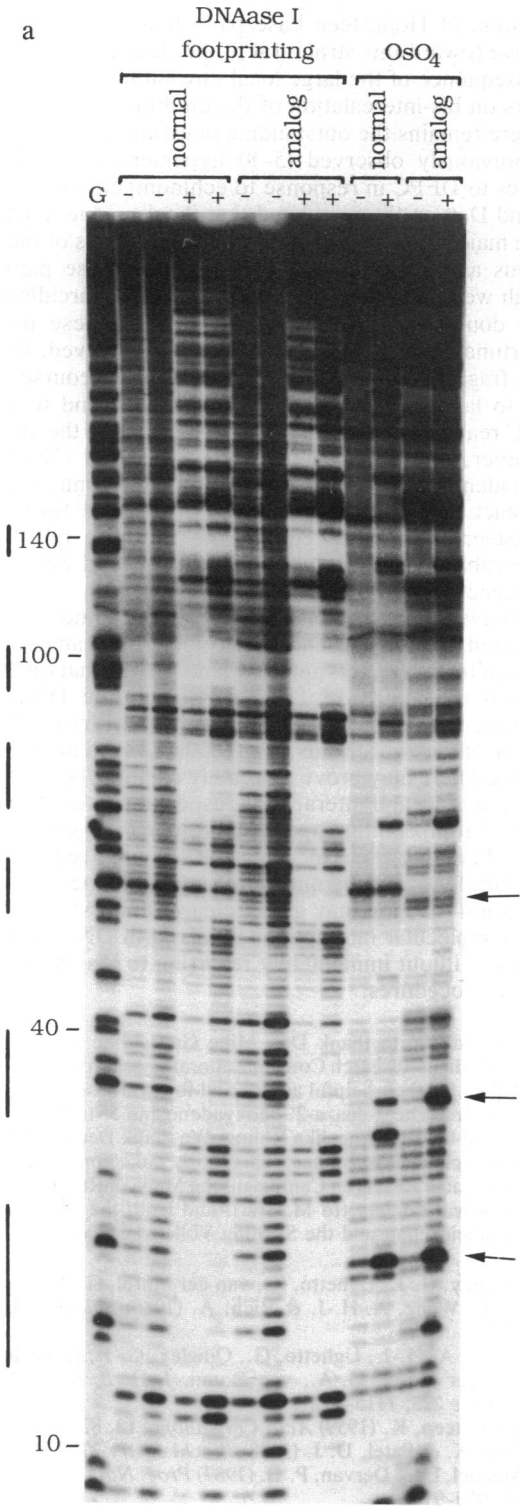

FIG. 4. (*a*) Autoradiograph showing the results of DNase I cleavage of DNA fragments containing deoxyadenosine in both strands (lanes marked "normal") or containing 7-deazadeoxyadenosine in one strand (lanes marked "analog") in the absence (lanes −) or presence (lanes +) of 4 μM echinomycin. For each experiment, two samples were run in adjacent lanes, corresponding to 1-min and 5-min digestions with the enzyme. Numbers on the left refer to nucleotide coordinates in the original *tyrT* fragment. Bars highlight footprints or antibiotic-binding sites—i.e., regions of diminished cleavage by DNase I in the presence of echinomycin. Also shown are the results of OsO_4 reactions with these two fragments in the absence (lanes −) or presence (lanes +) of 4 μM echinomycin. The three thymidine residues immediately 3′ to echinomycin-binding sites in this strand are marked with arrows. Lane G, dimethyl sulfate/piperidine marker specific for guanine. (*b*) Sequence of the lower (Crick) strand of the *tyrT* DNA fragment from positions 10–65 for comparison with the autoradiograph in *a*. Echinomycin-binding sites, centered around CpG steps, are underlined and the same three thymidine nucleotides are marked with arrows.

37°C for 2 hr, and the enzyme was removed by extraction into buffer-saturated phenol. Residual traces of phenol were removed by three extractions into diethyl ether, and the DNA was recovered by precipitation from ethanol. The DNA was redissolved and digested with *Eco*RI and *Nar* I to liberate an ≈1000-bp fragment containing the *tyrT* promoter region close to the *Eco*RI end. The reaction mixtures were loaded onto a 1% agarose gel, which was run in the presence of ethidium bromide (0.5 μg/ml), and the desired fragments were located under UV light, run into a trough cut in the gel, and recovered. Small particles of agarose were removed by filtration, the filtrates were extracted with phenol and diethyl ether, and the DNA fragments were recovered by precipitation from ethanol. The lower strands were then radiolabeled at the *Eco*RI sites at their 3′ ends by the action of DNA polymerase (Klenow fragment) in the presence of [α-^{32}P]-dATP.

Fig. 3 shows the results of agarose gel electrophoresis of these reaction mixtures. In the sample for lane 2, the polymerase reaction was performed in the presence of 7-deaza-2′-deoxyadenosine 5′-triphosphate. It is known (13) that the presence of this analog in a restriction enzyme recognition sequence drastically reduces the ability of the enzyme to cleave at its cognate site. Since the recognition sequence for *Nar* I does not contain any A·T base pairs, there should not be any difference in the relative amounts of fragment generated when surrounding sequences contain the nucleotide analog. That this is in fact the case can be clearly seen by comparing lanes 1 and 2.

Other Methods. DNase I footprinting, OsO_4 modification, and gel electrophoresis were performed as described (8). Because the extent of the OsO_4 reaction with DNA depends on numerous variables, including reagent concentration, temperature, and so on, rather aggressive conditions were deliberately chosen to produce a limited amount of "background" reaction with unmodified DNA. We reasoned that such conditions would be most conducive to detecting changes (positive or negative) induced by the binding of antibiotic molecules.

RESULTS

The two radiolabeled DNA fragments were subjected to cleavage by DNase I or to treatment with OsO_4, in the presence or absence of echinomycin, with the results shown in Fig. 4*a*. Lanes marked "normal" result from DNase I cleavage of DNA containing all four natural nucleotides, in the absence (lanes −) or presence (lanes +) of 4 μM echinomycin. Lanes marked "analog" result from identical treatment of DNA containing 7-deaza-2′-deoxyadenosine. It can be seen that there are no significant differences in either the sequence selectivity or in the extent of antibiotic binding to the analog-containing DNA compared to the natural DNA. The regions protected from endonucleolytic cleavage in the presence of echinomycin occur at the same places in either DNA fragment (highlighted on the left of Fig. 4*a*) and appear to be equally well protected. Thus, the incorporation of the analog into one strand of the DNA does not affect echinomycin binding *per se*. The resolution of the method does not allow us to exclude the possibility of a modest change in the

binding constant, although previous experience (6, 8) suggests that a change of as much as an order of magnitude would be detected, and we have noted no dependence of the footprinting pattern on DNA concentration.

Also shown in Fig. 4 are the results of OsO_4 reaction on these two DNA fragments in the absence or presence of echinomycin. We previously observed (8) that of the three thymidines immediately 3′ to echinomycin binding sites on this strand of the *tyrT* DNA fragment (arrows on right of Fig. 4*a*), only two become hypersensitive to modification by OsO_4 in the presence of echinomycin. That this phenomenon is independent of fragment length is clearly shown in Fig. 4*b*. Thus, the only thymidines adjacent to antibiotic binding sites that exhibit OsO_4 hypersensitivity are those at positions 21 and 34. It is also perfectly clear that the presence of a 7-deazadeoxyadenosine residue opposite these thymidines does not render them refractory to attack by this reagent. If anything, the reactivity at thymidines adjacent to antibiotic binding sites is actually enhanced when they are paired with the nucleoside analog.

There are other OsO_4-reactive sites in these DNA fragments which again show no diminution in reactivity when the complementary strand contains the analog (for example, at positions 69, 87, 89, and 137), although there are some regions that exhibit different behavior in the presence of this unusual deoxynucleotide. Of these, the most notable is the thymidine at position 31 (marked with an asterisk in Fig. 4*b*), which reacts very strongly in natural DNA and yet remains only feebly reactive when it is paired with 7-deazadeoxyadenosine. Why should this be so? We previously observed (8) that those thymidines that exhibit the most acute sensitivity to OsO_4 in the presence of the antibiotic are ones that are embedded in A+T-rich regions of the helix, such as at position 31. It is perhaps no coincidence that these regions would be expected to be the most easily unwound upon bis-intercalation of the antibiotic. We believe that it is this large unwinding of the DNA that leads to the observed chemical reactivity, and this effect can clearly be transmitted to quite distant regions of the duplex.

It should be noted that the thymidine at position 31 is actually at the end of a 4-bp homopolymeric tract of A·T, and such runs are known to have an unusual DNA structure (14–17). We feel that the absence of the nitrogen at position 7 of the imidazole ring might well strengthen the hydrophobic interactions between adjacent deazadeoxyadenosine residues so that they will stack better than their "normal" counterparts. Thus, it may be that such runs of consecutive analog·thymidine nucleotide pairs will tend to resist the unwinding forced upon them by antibiotic intercalation, and, if so, it might be expected that their reactivity toward OsO_4 would be reduced.

A similar situation is observed for the OsO_4-hypersensitive thymidines at positions 134 and 137. That is, in the presence of the analog, only one of these residues reacts with the probe, whereas both exhibit hypersensitivity in "natural" DNA. It should again be noted that neither of these thymidines is adjacent to an echinomycin-binding site.

DISCUSSION

We have shown that in a DNA fragment containing 7-deaza-A·T base pairs, which are incapable of adopting the Hoogsteen configuration, thymidines adjacent to echinomycin-binding sites remain hypersensitive to reaction with OsO_4 in the presence of the antibiotic. Since it appears that the binding of echinomycin is unaffected by the incorporation of 7-deazaadenine residues into a DNA fragment, we conclude that the same structural deformation of the helix gives rise to the observed reactivity as that pertaining in DNA containing only the natural nucleotides. That is to say, it is not the formation of Hoogsteen base pairs that makes thymidines reactive toward this structural probe. Rather, it seems to be a consequence of the large local unwinding of the helix that occurs on bis-intercalation of the antibiotic.

There remains the outstanding question as to what causes the previously observed (5–8) hypersensitivity of certain purines to DEPC in response to echinomycin binding. Mendel and Dervan (5) postulated that it arises due to exposure in the major groove of the N-1 or N-3 positions of the purine nucleus as a consequence of Hoogsteen base pairing, although we feel that our experiments with tubercidin (8) cast some doubt on the DEPC sensitivity of these positions. Unfortunately, this question is not easily resolved. Using the DNA fragments described here, it would of course be possible to label the analog-containing strand and to perform DEPC reactions in the presence or absence of the antibiotic. However, it has previously been shown (18) that 7-deazaadenine residues in single-stranded oligonucleotides do not react with this probe. Furthermore, the formation of Hoogsteen base pairs requires the presence of N-7, thus inextricably linking the postulated cause and effect of this hyperreactivity.

We have also shown that the binding of echinomycin is not prevented by the presence of a nucleoside analog in one strand of the DNA. It is apparent, moreover, that (in this case at least) the ability of DNase I to cut the DNA is not diminished by the incorporation of 7-deazadeoxyadenosine into one of the two strands. We feel that a system such as that described here may prove extremely useful in better defining nucleic acid–ligand interactions, especially in cases in which DNase I footprinting experiments appear to give overestimates of binding-site size. Thus, the judicious replacement of the naturally occurring nucleotides with analogs in the polymerization reaction may give a clearer understanding of the precise molecular interactions involved in DNA–ligand recognition without immediately resorting to lengthy crystallographic procedures.

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1. Quigley, G. J., Ughetto, G., van der Marel, G. A., van Boom, J. H., Wang, A. H.-J. & Rich, A. (1986) *Science* **232,** 1255–1258.
2. Wang, A. H.-J., Ughetto, G., Quigley, G. J., Hakoshima, T., van der Marel, G. A., van Boom, J. H. & Rich, A. (1984) *Science* **225,** 1115–1121.
3. Hoogsteen, K. (1959) *Acta Crystallogr.* **12,** 822–823.
4. Gao, X. & Patel, D. J. (1988) *Biochemistry* **27,** 1744–1751.
5. Mendel, D. & Dervan, P. B. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 910–914.
6. Portugal, J., Fox, K. R., McLean, M. J., Richenberg, J. L. & Waring, M. J. (1988) *Nucleic Acids Res.* **16,** 3655–3670.
7. Jeppesen, C. & Nielsen, P. E. (1988) *FEBS Lett.* **231,** 172–176.
8. McLean, M. J. & Waring, M. J. (1988) *J. Mol. Recognition* **1,** 138–151.
9. Waring, M. J. & Wakelin, L. P. G. (1974) *Nature (London)* **252,** 653–657.
10. Drew, H. R. & Travers, A. A. (1984) *Cell* **37,** 491–502.
11. Bankier, A. T., Weston, K. M. & Barrell, B. G. (1987) *Methods Enzymol.* **155,** 51–93.
12. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
13. Seela, F. & Kehne, A. (1987) *Biochemistry* **26,** 2232–2238.
14. Nelson, H. C. M., Finch, J. T., Bonaventura, F. L. & Klug, A. (1987) *Nature (London)* **330,** 221–226.

15. Ulanovsky, L. E. & Trifonov, E. N. (1987) *Nature (London)* **326,** 720–722.
16. Koo, H.-S., Wu, H.-M. & Crothers, D. M. (1986) *Nature (London)* **320,** 501–506.
17. Wells, R. D. & Harvey, S. C., eds. (1988) *Unusual DNA Structures* (Springer, New York).
18. Rosenthal, A., Billwitz, H., Kehne, A. & Seela, F. (1988) *Nucleic Acids Res.* **16,** 1631–1632.
19. Gilbert, D. E., van der Marel, G. A., van Boom, J. H. & Feigon, J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3006–3010.