Site-Specific Cleavage at a DNA Bulge by Neocarzinostatin Chromophore via a Novel Mechanism†

Lizzy S. Kappen and Irving H. Goldberg*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received August 5, 1993; Revised Manuscript Received September 30, 1993®

ABSTRACT: The chromophore of the anticancer drug neocarzinostatin (NCS-Chrom) oxidatively cleaves single-stranded or duplex DNA site-specifically in the absence of activating thiol provided that the DNA contains a bulged structure. Point mutations, deletions, and insertions in the DNA analogue and its complement of the 3′-terminus of yeast $tRNA^{Phe}$ show that for a single-stranded DNA to be cleaved by NCS-Chrom the DNA must generate a hairpin structure with an apical loop and at least a two-base-pair stem hinged to a region of duplex structure via a bulge containing a target nucleotide at its 3′ side. The size of the loop is not critical so long as it contains at least three nucleotides; the bulge requires a minimum of two nucleotides but must have fewer than five. With a notable exception involving base-pair changes immediately 3′ to the bulge, base changes in the bulge and base-pair changes immediately 5′ to the bulge retain substrate activity for NCS-Chrom. Maintenance of the bulged structure requires stable duplex regions on each side of the bulge. A similar bulged structure, but lacking a loop, formed by the annealing of a linear 8-mer and a 6-mer is an excellent target for cleavage in the thiol-independent reaction. Drugs such as netropsin, which sequester the DNA into nonbulge containing structures inhibit the reaction. In the absence of O_2 strand cleavage is blocked and quantitatively replaced by a presumed drug–DNA covalent adduct. The cleavage reaction has a pH optimum of about 9 and is much slower than the thiol-dependent reaction. The site-specificity of the cleavage, however, is much greater than in the thiol-dependent reaction. Thus, NCS-Chrom cleaves DNA containing a bulge via a novel mechanism not involving thiol. These studies extend the usefulness of this enediyne as a probe for unusual DNA structures.

As part of an effort to identify unusual nucleic acid structures as targets for the enediyne antibiotics and to extend the utility of the chromophore of the anticancer antiobiotic neocarzinostatin (NCS-Chrom) (**1**, Scheme I) as a probe for such structures (Williams & Goldberg, 1988; Kappen & Goldberg, 1992a,b) we found that the single-stranded DNA analogue of a 3′ terminal fragment of yeast $tRNA^{Phe}$ (Figure 1, 31-I) was cleaved by NCS-Chrom with high efficiency at a single site, T_{22} in the absence of thiol (Kappen & Goldberg, 1993). This unexpected reaction thus bypasses the two known prerequisites for DNA damage by NCS-Chrom, i.e., a thiol activator to form the diradical species of the drug (**3**, Scheme I) and a duplex DNA substrate (reviewed in Goldberg, 1991). DNA damage in the non-thiol reaction required oxygen, involved abstraction by the drug of the C-5′ hydrogen atom from the deoxyribose of the target nucleotide, and resulted in a strand break having a PO_4 at the 3′ end and a nucleoside 5′ aldehyde at the 5′ terminus (Scheme II). Most interestingly, a unique drug product resulted from this reaction, indicating a novel mechanism of drug activation to the DNA-cleaving species and implicating DNA in the activation process (Kappen & Goldberg, 1993; Hensens et al., 1993).

In this report we have explored the structural requirements for site-specific cleavage by NCS-Chrom in the non-thiol reaction using oligonucleotide substrates based on 31-I and its complement 31-II (Figure 1) and present in detail several features of this unique reaction. The data show that only oligodeoxynucleotides capable of forming certain bulged structures are substrates for this reaction.

TCCTGTGTTCGATCCACAGAAT_{22}TCGCACCAC (31-I)

GTGGTGCGAAT_{11}TCTGTGGATCGAACACAGGA (31-II)

FIGURE 1: Sequences of the DNA analogue of the 3′ terminal fragment (residues 47–76) of $tRNA^{Phe}$ (31-I) (C_{31} is an extra nucleotide) and its complement (31-II).

MATERIALS AND METHODS

Neocarzinostatin was obtained from Kayaku Antibiotics (Tokyo). Oligodeoxyribonucleotides were purchased from Chemgenes or Midland Certified Reagent Company. Radioactive materials and enzymes were from New England Nuclear and New England Biolabs, respectively. Oligomers were 5′-end-labeled with ^{32}P by standard procedures (Maniatis et al., 1982). The labeled oligomers were purified by electrophoresis on a 15% sequencing gel.

Drug Reaction. NCS-Chrom was extracted from the holoantibiotic by cold methanol in the presence of citrate (Kappen & Goldberg, 1985). A standard reaction contained 50 mM Tris-HCl, pH 8.5 (unless otherwise indicated), 1 mM EDTA, oligomer, and NCS-Chrom at concentrations indicated in the figure legends. The mixture of components was chilled in ice for 15 min prior to the addition of the drug (maximum final methanol concentration 10%). The reaction was allowed to proceed in the dark for 1 h in ice. When duplex DNA was the substrate, ^{32}P-labeled (+) strand was annealed to excess (2–3-fold) of the (–) strand in the 2× reaction buffer by heating at 90 °C for 2 min and slow cooling (Kappen et al., 1988). In the case of single-stranded substrate there was no difference in the cleavage reaction when it was used with or without the heating and cooling steps of the annealing process.

Treatment of the drug-treated DNA with piperidine or hydrazine was carried out by procedures previously described (Kappen & Goldberg, 1992a).

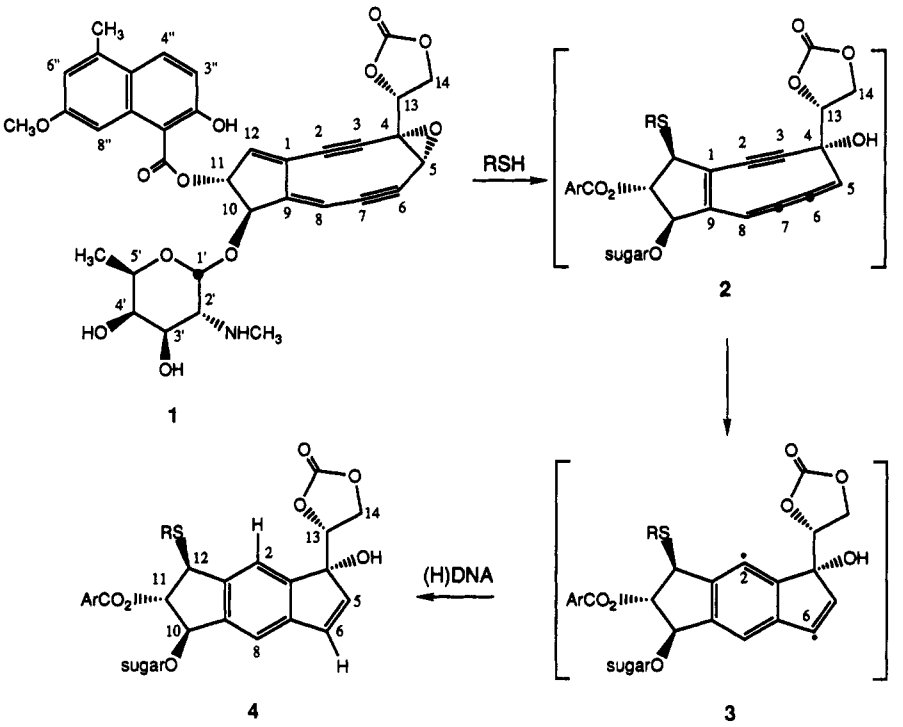
† This work was supported by U.S. Public Health Service Grant CA 44257 from the National Institutes of Health.

* To whom correspondence should be addressed.

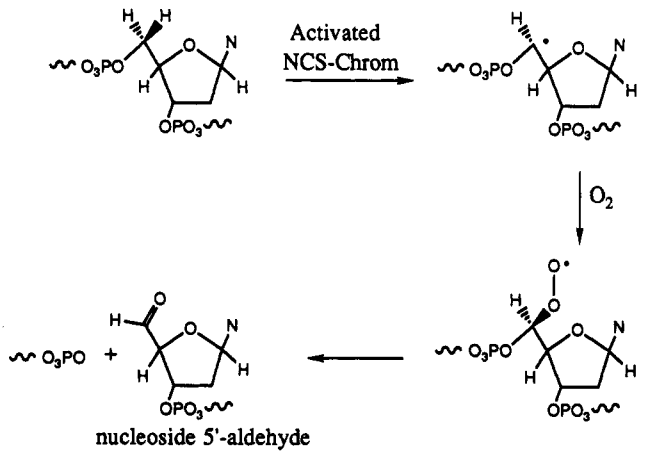
® Abstract published in *Advance ACS Abstracts,* November 15, 1993.

0006-2960/93/0432-13138$04.00/0

Scheme I: Thiol Activation of NCS-Chrom (1) to the Diradical Species



Scheme II: Proposed Mechanism of Oxidative Cleavage of DNA by NCS-Chrom Attack at C-5′



Reactions under anaerobic conditions were performed in a vessel containing a side arm as described (Kappen & Goldberg, 1985). Reactions (90 μL) contained 15 mM sodium acetate, pH 5.0, 1 mM EDTA, 300 μM ^{32}P-labeled oligomer (phosphate), 46 μM NCS-Chrom, and 75 mM Tris-HCl, pH, 8.5 (final pH of the reaction mixture was 8.4). The side arm of the reaction vessel contained the Tris buffer. The rest of the components except the drug were placed in the main chamber. After cooling the vessel in ice for 15 min, NCS-Chrom was added to the DNA in the main chamber. The contents of the flask were frozen and evacuated three times with intermittent thawing and freezing. The contents of the two chambers were then mixed and the reaction was allowed to proceed in ice for 1 h.

Product Analysis. The dried sample pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes and analyzed on a 15% sequencing gel. Quantitation of the gel bands was done on a Molecular Dynamics PhosphorImager using Image Quant software version 3.22 for data processing.

RESULTS AND DISCUSSION

Site-Specific Strand Cleavage in 31-mer DNA. Treatment of 5′-^{32}P-labeled single-stranded 31-I (Figure 1), the DNA analogue of the 3′-terminus of tRNA^{Phe} (residues 47–76), with NCS-Chrom in the absence of thiol resulted in highly efficient cleavage at a single site, T_{22} (Kappen & Goldberg, 1993). In order to test the generality of this phenomenon we tested its complementary strand, 31-II (Figure 1), as substrate for NCS-Chrom. Figure 2 shows that in a non-thiol reaction NCS-Chrom induced strand cleavage at a single site, T_{11}, only in the single-stranded DNA (lane 4). In duplex DNA there is no cleavage (lane 7). These data reaffirm the highly specific nature of the cleavage at a single site without thiol. In the presence of thiol, both in the duplex (lane 8) and in single-stranded forms (lane 6), NCS-Chrom induces strand breaks, mainly at T and A residues, in agreement with the known nucleotide preference (T > A ≫ C > G) of the drug (Hatayama et al., 1978). Cleavage of the single-stranded DNA is attributable to the generation of duplex regions intramolecularly or by the self-complementarity of regions on two separate DNA strands. The strong cleavage at C_{21} (lane 6), seen only in the thiol-dependent reaction with the single-stranded substrate, is akin to the cleavage at C_{29} in 31-I under similar experimental conditions (Kappen & Goldberg, 1993).

Structural Requirements for a Competent Substrate. In order to gain further insight into the structural and/or sequence requirements of the DNA substrate for cleavage by NCS-Chrom in the absence of thiol, several single-stranded substrates, derived from 31-I and 31-II by stepwise nucleotide deletions from the 5′ and/or the 3′ end, were first tested. Data summarized in Table I shows that the 16-mer (bases 10-25 of 31-I), designated $oligo_{10-25}$, is 73% as good as the parent substrate. Addition of three or six bases to the 3′-end of 16-mer did not make any difference (compare 19-mer and 22-mer, Table I). On the other hand, deletion of bases including C_{10} from 31-I or G_{25} from the 16-mer markedly reduces cleavage efficiency (Table I, 21-mer and 15-mer). Similarly, deletion of bases in 31-II from the 3′ end to form the 23-mer

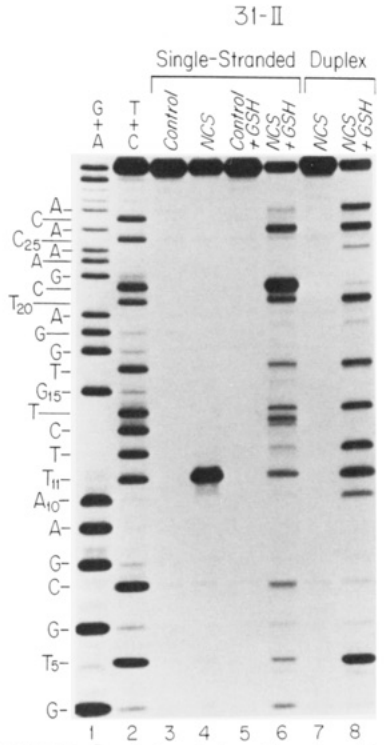


FIGURE 2: NCS-Chrom-induced strand breakage in single-stranded or duplex forms of 31-II in the absence or presence of 3 mM glutathione (GSH). Under standard reaction conditions 5′-^{32}P-labeled 31-II (225 μM) was treated with NCS-Chrom (47 μM) as described in Materials and Methods. Strand breakage at T_{11} (lane 4) in 31-II was 47% of the total substrate. Controls in lanes 3 and 5 have no drug.

Table I: Effect of End Deletions in 31-mers on Site-Specific Strand Cleavage by NCS-Chrom in the Absence of Thiol[a]

31-I			31-II		
substrate length	bases inclusive	cleavage at T_{22} (%)	substrate length	bases inclusive	cleavage at T_{11} (%)
31	1–31	100	31	1–31	100
22	10–31	71	23	1–23	41
21	11–31	39	21	1–21	18
20	12–31	ND	19	1–19	3
19	10–28	74	17	1–17	1
16	10–25	73			
15	10–24	11			
14	10–23	ND			

[a] Single-stranded substrates were treated with NCS-Chrom under standard conditions. Strand cleavage which occurred only at a single site was determined by gel analysis. 100% cleavage represents 67% and 35% of the substrate cleaved in 31-I and 31-II, respectively. ND, no detectable cleavage.

and shortened oligomers results in a progressive decrease in strand cleavage at T_{11}. These data, combined with the effect of base changes around the target T residue (see later), suggested that strand length or sequence alone is not the factor that makes an oligomer a substrate capable of mediating NCS-Chrom activation and site-specific cleavage but rather a secondary structure that provides certain conformational features is the critical determinant. Also, it was clear that a structure analogous to the T loop of $tRNA^{Phe}$, on which these DNAs were based, was not responsible for the substrate activity. Nor were duplex regions at the target site, whether generated intra- or intermolecularly, involved in the reaction.

This led us to propose the related conformations shown in Figure 3 for 31-I and 31-II, which have a hairpin with an apical loop and a two base pair stem hinged to a region of duplex structure via a two base bulge containing a target T residue at the 3′ side of the bulge. It is noteworthy that,

Table II: Effect of Mutations on NCS-Chrom-Induced Site-Specific Cleavage[a]

substrate	% cleavage at base 22
1. C_{10}GATCCACAGAAT$_{22}$TCGCACCAC	100
2. C_{10}GATCCACAGA**G**T$_{22}$TCGCACCAC	77
3. C_{10}GATCCACAG**G**AT$_{22}$TCGCACCAC	ND
4. C_{10}GATCCACAGAAT$_{22}$**C**CGCACCAC	ND
5. C_{10}GATCCACAGAA**C**$_{22}$TCGCACCAC	66
6. C_{10}GATCCACAGAAT$_{22}$TCG	100
7. C_{10}GATCCACAGAAT$_{22}$**G**CG	ND
8. C_{10}**G**GTCCACAGAAT$_{22}$**C**CG	ND
9. C_{10}G**C**TCCACAGAAT$_{22}$**G**CG	1
10. C_{10}**G**A**C**CCACAG**G**AT$_{22}$TCG	112
11. C_{10}GAT**C**CACAG**A**AC$_{22}$TCG	66
12. C_{10}GATCCACAGAA**G**$_{22}$TCG	25
13. C_{10}G**T**ACCACAG**T**AT$_{22}$ACG	ND
14. C_{10}G**TA**CCACAG**TT**A$_{22}$ACG	ND

[a] Oligomers 1 (22-mer) and 6 (16-mer) have bases 10–31 and 10–25, respectively, of 31-I (Figure 1). Strand cleavage, exclusively at position 22, was quantitated by gel analysis of standard reactions containing single-stranded oligomers in the absence of thiol. In each set of experiments cleavage in the parent oligomers (1 and 6) were taken as 100%. Mutations in 1 and 6 are indicated in bold and underlined. ND, no detectable cleavage.

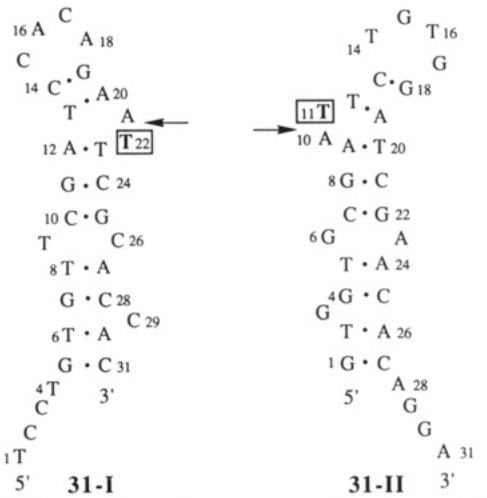


FIGURE 3: Proposed secondary structures of single-stranded DNAs 31-I and 31-II. The target residues (T_{22} and T_{11}) are in bold. Arrows indicate side of cleavage.

although the bulges of 31-I and 31-II are on opposite sides of the stem-loop structure, the duplex regions on each side of the bulge have identical sequences but with different loop sequences. The sequences immediately 5′ and 3′ to the bulge (both containing AT) and opposite the bulge are identical. Substrate 31-I is nearly twice as efficient as 31-II as a cleavage substrate (Table I), despite the immediate sequence identities, indicating that sidedness in relation to the stem-loop is important.

The proposed model was then tested by introducing several single and double point mutations and insertions in the 22-mer (oligo$_{10–31}$) and in the 16-mer (oligo$_{10–25}$), derived from 31-I, and using them as substrates for NCS-Chrom in their single-stranded form in the non-thiol reaction. As shown in Table II, while substitution of A_{21} at the bulge (Figure 3) with a G does not significantly affect cleavage at T_{22} (Table II, 2), mutation of A_{20} in the T_{13}·A_{20} base-pair at the loop stem to a G or substitution of a C for T_{23} in the A_{12}·T_{23} base-pair totally abolishes cleavage (Table II, 3 and 4). Similarly disruption of the A_{12}·T_{23} base-pair in the oligo$_{10–25}$ by substitution of T_{23} with a G (Table II, compare 6 and 7) renders the oligomer an incompetent substrate. The lack of (or trivial) activity for the G_{12}·C_{23} and C_{12}·G_{23} substitutions

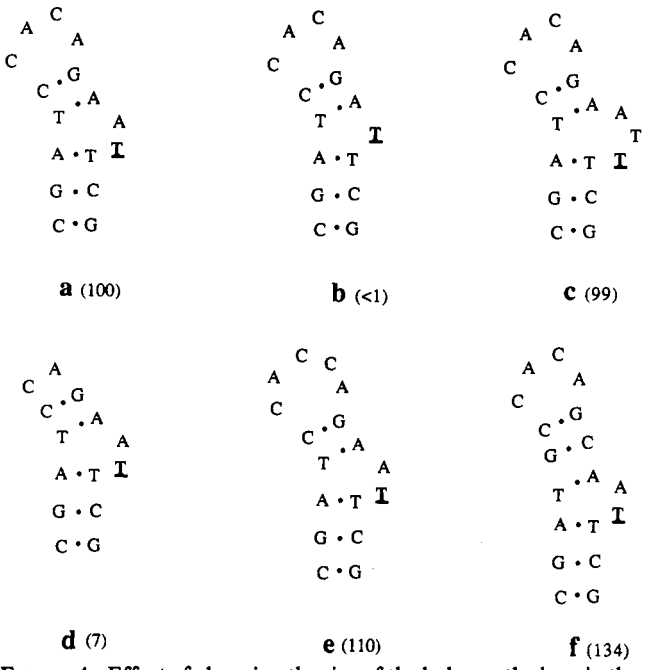


FIGURE 4: Effect of changing the size of the bulge or the loop in the 16-mer (bases$_{10-25}$ of 31-I) on site-specific cleavage by NCS-Chrom in the absence of thiol. Under standard conditions 5′-^{32}P-labeled single-stranded substrates were treated with NCS-Chrom (46 μM). All oligomers were 28 μM (in terms of molecules). Strand cleavage at the target T was quantitated (given in brackets are the percentages of strand cleavage at the T). One hundred percent cleavage in the parent 16-mer (a) represents 59% of the substrate cut at the T. (b) bulge smaller by one base; (c) bulge larger by an extra T; (d) loop smaller by two bases; (e) loop larger by one base; (f) stem longer by an extra G·C base-pair.

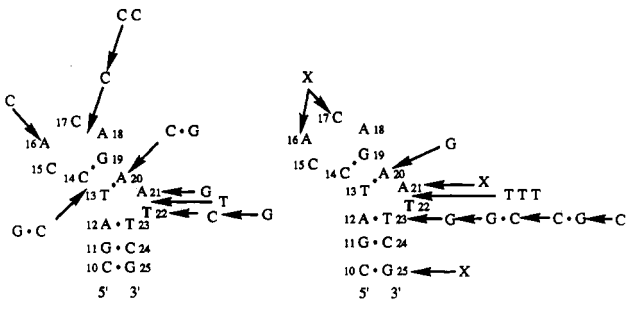


FIGURE 5: Summary of the point mutations, insertions, and deletions determining whether oligo$_{10-25}$ derived from 31-I is an active or inactive substrate. Placement position of the arrows indicates whether base-(s) is a substitution or insertion. Deletion is indicated by x.

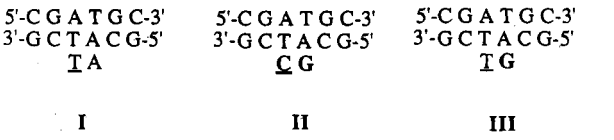


FIGURE 6: Proposed structures of the bulge-containing duplexes having base substitutions at the bulge. Base changes are shown in bold. The target nucleotide at the bulge in the duplex is underlined.

for A_{12}·T_{23} (Table II, 8 and 9) must be interpreted with caution, since spurious base-pairing elsewhere in the molecule, in particular with bases in the loop, would disrupt the bulge. On the other hand, a double mutation in the 16-mer that converts the T_{13}·A_{20} base pair to C_{13}·G_{20} slightly enhances cleavage (Table II, 10). In addition, the loss of activity observed in the deletion experiments (Table I) can also be explained on the basis that those deletions which rendered the oligomer an incompetent substrate also caused disruption of base-pairing critical for stability of the bulge structure. For instance, deletion in 31-I that resulted in the loss of C_{10}·G_{25} reduced the cleavage efficiency by 60% (Table I, 21-mer) and removal of one more base-pair, G_{11}·C_{24}, caused complete loss of activity (Table I, 20-mer).

The target T_{22} at the bulge can be replaced by a C with 66% of the cleavage efficiency as in the parent oligo$_{10-31}$ (Table II, compare 1 and 5) and in the oligo$_{10-25}$ (Table II, compare 6 and 11). Cleavage occurs also with a G at position 22 (Table II, 12), though less efficiently. It is interesting to note that this pattern of base preference is similar to that found in the duplex DNA in the thiol-dependent reaction where G is the least attacked base. While emphasis has been placed on the necessity for a two-base bulged structure in a competent substrate, not any bulge will satisfy this requirement; some type of sequence requirement may be involved. For instance, a double point mutation in the oligo$_{10-25}$, that would presumably maintain a bulged structure but would only cause the reversal of the A_{12}·T_{23} base pair to T_{12}·A_{23} and T_{13}·A_{20} to A_{13}·T_{20} rendered the oligomer an incompetent substrate (Table II, 13 and 14).

The effect of altering the size of the bulge or the loop on site-specific cleavage in single-stranded oligo$_{10-25}$ in the non-thiol reaction is shown in Figure 4. While reduction of the size of the bulge to a single nucleotide (b) virtually abolishes the cleavage at the T, increasing the size of the bulge by one T has almost no effect (compare a and c). Further, the attack site remains at the 3′ side of the bulge. Placement of three extra Ts in the bulge abolishes the activity (data not shown). Removal of two bases from the loop results in significant loss of cleavage (d); whereas increasing its size by an extra C enhances the cleavage efficiency (e). Furthermore, placement of an extra G·C base pair at the loop stem (f), which would be expected to stabilize the bulge, also renders the oligomer a better substrate. Taken together, as summarized in Figure 5, these data show that limited changes can be made at the bulge or the loop but base substitutions that potentially disrupt the base-pairing, hence the stability, of the proposed bulge conformation (Figure 3) affect the ability of the oligomer to serve as a competent substrate. Further, bulge size is critical in determining substrate effectiveness.

Competent Substrates Derived from Two Linear Oligomers. The results obtained with the single-stranded competent and incompetent substrates suggested that the critical attribute that makes an oligomer competent in mediating NCS-Chrom activation for site-specific cleavage is the presence of a bulge (minimum of two bases); the target nucleotide being the one on its 3′-side. It seemed possible, therefore, that an oligomer duplex, composed of two separate strands, but having the potential to form a bulge with a similar sequence environment as in 31-I and 31-II would be a good substrate for site-specific cleavage. Therefore, a 5′-^{32}P-labeled 8-mer annealed to a 6-mer, both having sequences that would permit the formation of a duplex region by normal base pairing with the creation of a two base pair bulge (Figure 6) was tested as a substrate for NCS-Chrom. As predicted, duplex I (Figure 6) is cleaved efficiently in the absence of thiol at a single nucleotide (T_5), which is presumably at the bulge (Figure 7, lanes 5 and 6). There is no strand breakage with NCS-Chrom when the substrate was the 8-mer alone (lane 4) or with its complement (not shown). In the presence of thiol the cleavage pattern (lanes 7–9) is the one expected for the normal duplex with breaks predominantly at T and A residues. In this case a duplex structure is formed by the self-complementary regions on the molecule (generating an *Eco*RI site) in a bimolecular interaction to form sites for NCS-Chrom-induced cleavage.

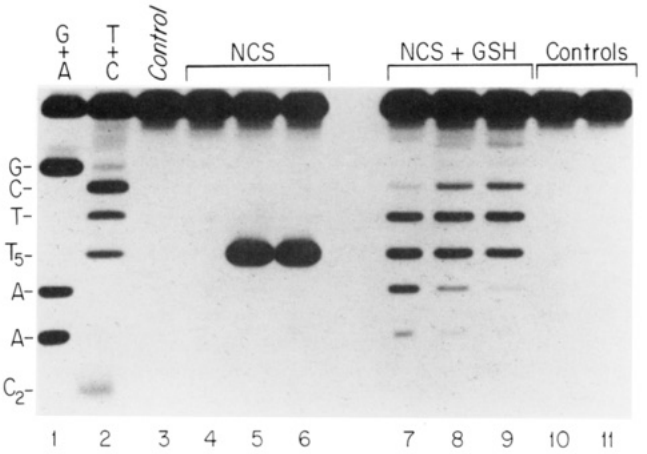


FIGURE 7: NCS-Chrom-induced site-specific cleavage in bulge-containing DNA duplex made up of two oligomers in the absence of thiol. 5′-^{32}P-labeled 8-mer (450 μM phosphate) was annealed to the 6-mer to form duplex **I** (Figure 6) and treated with NCS-Chrom (46 μM) as described in Materials and Methods. Lane 4, 8-mer alone treated with NCS-Chrom; lane 5, 8-mer, annealed with equimolar, and lane 6, with 2 × molar levels of 6-mer respectively, treated with drug. Lanes 7, 8, and 9 have substrates identical to 4, 5, and 6, respectively, but treated with drug in presence of glutathione. Controls have no drug.

Chemistry of Damage in the Non-Thiol Reaction Involves Exclusively 5′ Attack Independent of Bulge Sequence. While the predominant lesions induced in duplex DNA by NCS-Chrom in the thiol-dependent reaction are single-strand breaks at T residues due to C-5′ hydrogen atom abstraction and the generation of a strand break with a nucleoside 5′-aldehyde at its 5′ end (Kappen & Goldberg, 1985; Kappen et al., 1982; Kappen & Goldberg, 1983), it also causes sequence-specific lesions, mainly as part of double-stranded lesions involving the C of AG C (Povirk et al., 1988; Kappen et al., 1988) and the T of AGT (Dedon et al., 1992) resulting, respectively, from 1′ and 4′ attack at the target nucleotide. Attack at the C-1′ position generates, with the release of cytosine, an abasic site containing deoxyribonolactone (Kappen & Goldberg, 1989) which, upon treatment with alkali, is expressed as a break terminated with a 3′-phosphate moiety (Povirk & Goldberg, 1985; Kappen et al., 1988; Kappen et al., 1990). 4′-Chemistry at the T produces a strand break having a 3′-phosphoglycolate moiety, the presence of which causes the fragment to migrate faster than those with a 3′-phosphate terminus (Frank et al., 1991; Kappen, et al., 1991). A second product of 4′ attack is the 4′ hydroxylated abasic site, which is expressed as a break upon treatment with alkali or hydrazine to generate a DNA fragment with a phosphate or a pyridazine moiety, respectively, at its 3′ end (Saito et al., 1989; Kappen et al., 1991; Kappen & Goldberg, 1992b). In the non-thiol reaction with single-stranded 31-I strand cleavage at T_{22} involved only 5′ chemistry (Kappen & Goldberg, 1993). In order to determine if insertion of AGC and AGT sequences in the bulged region might alter the chemistry of the thiol-independent reaction from 5′ to 1′ or 4′, these sequences were substituted for AAT in duplex I (Figure 6). Data presented in Figure 8 shows that all three oligomer duplexes were good cleavage substrates in the non-thiol reaction, with strand cleavage occurring at a single nucleotide, C_5 or T_5. Cleavage at C_5 of AGC in duplex II is direct (lane 8) and is less than that at T_5 of AAT in duplex I (lane 5). On the other hand, duplex III is a better substrate than duplex I for cleavage at T_5 of AGT (lane 11). ^{32}P-Labeled 8-mer, in the single-stranded form, was not cleaved by NCS-Chrom (lanes 4, 7, and 10). With all three duplexes there is no increase in intensity of strand cleavage with piperidine (lanes 6, 9, and 12 and Table III), ruling out the generation of an abasic site from 1′ (or 4′) chemistry. Absence of any glycolate band (Figure 8) or pyridazine derivative with hydrazine (data not shown) confirms the absence of 4′ chemistry. These results show that the site-specific cleavage by NCS-Chrom in the non-thiol reaction involves only 5′-attack irrespective of the sequence at the bulge.

Effect of pH and Time Course of Strand Cleavage in the Non-Thiol Reaction. The DNA cleavage reaction involves general base catalysis, as shown by the profile in Figure 9. The reduced damage at room temperature can be attributed to the lability of the drug at the high pH at this temperature;

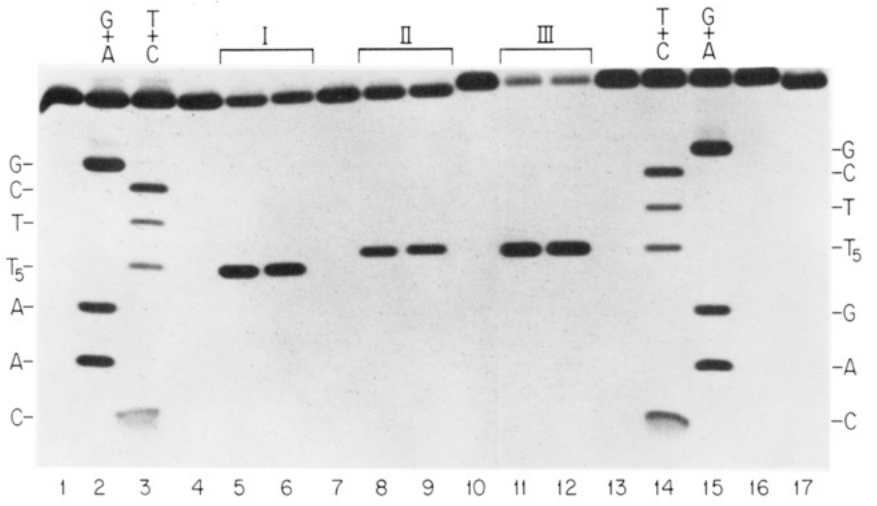


FIGURE 8: Chemistry of site-specific strand cleavage by NCS-Chrom at the C of AGC or the T of AGT sequences in bulge-containing oligomer duplexes. In standard non-thiol reactions containing each of the 5′-^{32}P-labeled 8-mer (Figure 6) (450 μM phosphate), annealed with twice its molar equivalent of the 6-mer was treated with NCS-Chrom (46 μM). Following drug treatment, portions of the reaction mixture were treated with piperidine (90 °C, 30 min) as previously described (Kappen and Goldberg, 1992a). Duplexes I, II, and III (Figure 6) having AAT (lanes 5 and 6), AGC (lanes 8 and 9), and AGT (lanes 11 and 12). Lanes 4, 7, and 10 have single-stranded ^{32}P-labeled 8-mer, respectively, of duplexes I, II, and III treated with NCS-Chrom. Lanes 6, 9, and 12 contain samples that were subjected to piperidine treatment after the drug reaction. Lanes 1 and 13 have controls with no drug, respectively, of duplexes I and III. Lanes 16 and 17 have controls with no drug, after piperidine treatment, of duplexes III and II, respectively. Lanes 2 and 3 have chemical cleavage markers for the ^{32}P strand in duplex I, and lanes 14 and 15 have those for the ^{32}P strand in duplex III.

Table III: NCS-Chrom-Induced Site-Specific Cleavage at the Bulge Having Different Sequences[a]

substrate	target sequence	% cleavage –PIP	% cleavage +PIP
duplex I	AA**T**	60	60
duplex II	AG**C**	32	33
duplex III	AG**T**	81	81

[a] Duplexes shown in Figure 6 were the substrates for NCS-Chrom in the non-thiol reaction. Cleavage at T_5 or C_5 (marked in bold and underlined) were quantitated from Figure 8. PIP, drug-treated DNA was subjected to piperidine treatment.

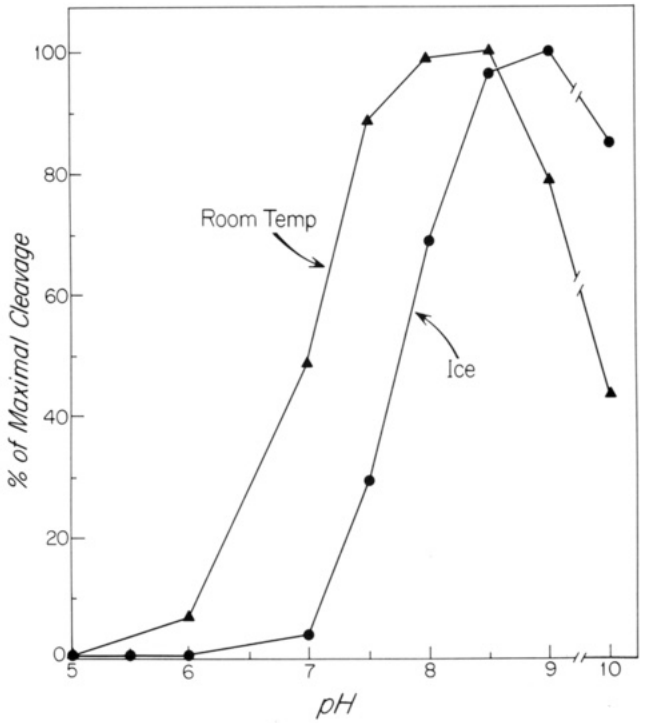


FIGURE 9: pH Dependence of site-specific cleavage by NCS-Chrom in the non-thiol reaction. 5′-^{32}P-labeled 19-mer (bases 10–28 of 31-I) in the single-stranded form (450 μM phosphate) was treated with NCS-Chrom (46 μM) under standard conditions in the following buffers: pH 5–6, sodium acetate; pH 7–9, Tris-HCl; pH 10, glycine/NaOH. Strand cleavage, exclusively at T_{22}, was quantitated. One hundred percent cleavage represents 52% and 19% of the substrate cleaved in ice and room temperature, respectively.

this is reflected in the lower pH optimum at the higher temperature. These data suggest that ionization of the phenolic hydroxyl group of the naphthoate moiety of NCS-Chrom, which has a p$K_a \sim 8.5$ (Napier & Goldberg, 1983), is involved in drug activation. These results also suggest that, independent of drug stability consideration, the true pH optimum is that at which the phenolic hydroxyl group is fully ionized. Tris buffer can be effectively substituted with buffers containing phosphate, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) or triethylammonium bicarbonate.

A time course (Figure 10) shows that strand cleavage in single-stranded DNA in the non-thiol reaction is at least 50-fold slower (rate constant $k = 1.07 \times 10^{-3}$ s^{-1}) than that in duplex DNA in the presence of thiol.

Anaerobic Reaction. Site-specific cleavage at T_{22} in single-stranded 31-I in the absence of thiol requires oxygen (Figure 11, lane 4). Under anaerobic conditions strand cleavage is almost completely inhibited (lane 5). In the anaerobic reaction a major product (presumably a DNA–drug adduct) with

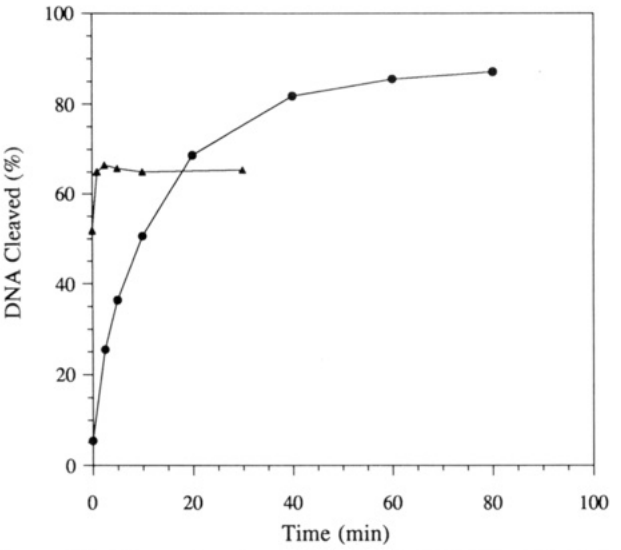


FIGURE 10: Time course of strand cleavage by NCS-Chrom in single-stranded substrate in the non-thiol reaction and in the duplex substrate in presence of glutathione (3 mM). 5′-^{32}P-labeled 31-I (450 μM) in the single-stranded or duplex form was treated with NCS-Chrom (46 μM) under standard conditions. At the indicated times aliquots were withdrawn and added to tubes containing NaOH (final pH 12.0) to stop the reaction; the samples were kept frozen until the last sample was done. The samples were then heated in a 90 °C water bath for 30 min and neutralized with HCl. After drying, the pellets were dissolved in the loading buffer for gel analysis. The amount of radioactivity in the starting material and in the band (s) was quantitated to determine the percentage of cleavage. Single-stranded DNA in the non-thiol reaction, closed circle; duplex DNA in GSH-dependent reaction, closed triangle.

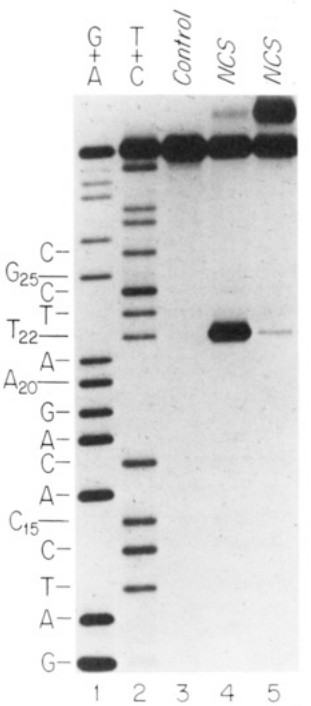


FIGURE 11: Effect of oxygen depletion on site-specific cleavage in the non-thiol reaction. 5′-^{32}P-labeled 31-I in single-stranded form was treated with NCS-Chrom under anaerobic conditions as described in Materials and Methods. Control (lane 3) received no drug. Lane 4, reaction in air; lane 5, reaction under anaerobic conditions.

mobility slower than that of the starting material is produced (lane 5). The presumed adduct is quantitatively equivalent to the strand breakage at T_{22} in the air reaction. These data show that DNA-mediated NCS-Chrom activation does not require oxygen, but oxygen is needed for expression of the

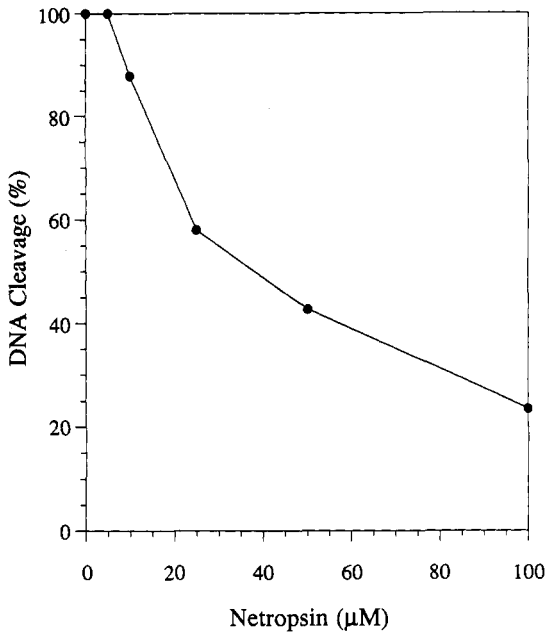


FIGURE 12: Effect of netropsin on site-specific cleavage by NCS-Chrom in the non-thiol reaction. Substrate for NCS-Chrom was 5′-^{32}P-labeled oligomer (450 μM phosphate) annealed with 2 × molar equivalent of the 6-mer (Duplex I, Figure 6). Netropsin, at the levels indicated, was added to the DNA prior to the addition of NCS-Chrom (46 μM). Strand cleavage, exclusively at T_5, was quantitated by gel analysis. One hundred percent cleavage represents 60% of the total substrate cleaved. Netropsin (100 μM) alone did not cause any strand breakage.

damage as a strand break. This finding is similar to that made in the thiol-dependent reaction with duplex DNA and NCS-Chrom where the abstraction of hydrogen atom from the C-5′ position of DNA did not require oxygen, but strand cleavage was oxygen-dependent (Kappen & Goldberg, 1985).

Effect of Netropsin on Site-Specific Cleavage. NCS-Chrom is known to bind in the minor groove of duplex DNA, and minor groove binding agents such as netropsin and distamycin have been shown to inhibit its binding (Dasgupta & Goldberg, 1985). It was hence of interest to examine the effect of netropsin on the site-specific cleavage induced by NCS in the absence of thiol. As shown in Figure 12, the strand cleavage at T_5 (in duplex I, Figure 6) decreases with increasing concentrations of netropsin. Since AATT in duplex DNA is a good binding site for netropsin (Kopka et al., 1985), the inhibition of the non-thiol reaction is, most likely, due to sequestration of the substrate by binding to the AATT duplex form rather than due to the competion with NCS-Chrom for binding at the bulge site.

CONCLUDING REMARKS

The most important determinant of competency of a DNA substrate for NCS-Chrom-induced site-specific cleavage is the presence of a bulge site containing at least two (and less than five) nucleotides and a sufficient number of base-pairs on either side of the bulge to maintain the bulged structure. The optimal bulge size is probably translated into an optimal extent of bending at the bulge (Rice & Crothers, 1989; Bhattacharyya & Lilley, 1989; Hsieh & Griffith, 1989). Since a singly-bulged pyrimidine may loop out from the double helix (Morden et al., 1983; 1990; van der Hoogen et al., 1988; Kalnik et al., 1989, 1990), in contrast to an ATA-bulge where all bulged bases are stacked within the helix (Rosen et al., 1992), the substrate with a single-bulged nucleotide (Figure 4b) may be incompetent because of its position outside the DNA helix and not necessarily because of diminished bending. It is not certain, further, whether a specific sequence is required (see, however, substrates 13 and 14 in Table II), although there appears to be some base preference (T) for the target residue at the 3′ side of the bulge.

The cleavage reaction in the non-thiol reaction is very slow compared with the thiol-dependent reaction (Figure 10). This probably is reflective of the activation step, which for glutathione in the presence of DNA is very fast (Dedon & Goldberg, 1992 and Figure 10). Nevertheless, the thiol-independent, site-specific cleavage in the bulged DNA is highly efficient and selective. A ratio of about one drug molecule per strand break was calculated under the most optimal reaction conditions (Table III, duplex III). Unlike in the thiol-dependent reaction with duplex DNA where high levels of drug cause breaks at less favored sites, no other cleavage sites are found in the bulged DNA in the non-thiol reaction. This shows that in the latter reaction DNA damage is restricted to the single site where drug activation is induced. This lesion also differs substantially from the cleavage pattern described earlier at the site of a single-base bulge in duplex DNA in the presence of thiol (Williams & Goldberg, 1988). In the latter reaction a break is generated on the strand opposite the bulged base at the residue just 3′ to the bulge; the bulged residue is not a cleavage target. This interaction between NCS-Chrom and single-base bulged DNA is consistent with studies showing that intercalating drugs bind selectively to such structures (Nelson & Tinoco, Jr., 1985; Williams & Goldberg, 1988; Williams et al., 1988; Woodson & Crothers, 1988).

Perhaps the most striking characteristic of the thiol-independent reaction is that drug activation to a DNA-cleaving species requires the presence of a substrate capable of being cleaved (Kappen and Goldberg, 1993). This result implies that the DNA bulge plays a vital role in drug activation. Recent characterization of the novel post-activation drug product, dependent on the presence of a competent DNA substrate, indicates that drug activation is due to the intramolecular attack by a nucleophile generated on the hydroxy naphthoate moiety at C12 of the enediyne (Hensens et al., 1993) to initiate the cycloaromatization reaction leading to the formation of the reactive radical species in a manner similar to that proposed for the thiol-mediated reaction (Myers, 1987) (Scheme I). Ionization of the phenolic hydroxyl group at pH 8.5 presumably initiates the reaction. The DNA bulged structure appears to mediate the intramolecular reaction and/or a subsequent one involving quenching of the radical at C2. The latter reaction would convert the drug into a monofunctional species with a radical center at C6, accounting for the formation of only single strand breaks at the target site. The role of the DNA conformation in effecting this chemistry, which leads to its own destruction, is under investigation. In particular, it is of interest to determine if the hydroxy naphthoate moiety stacks with the stacked bulged bases (Rosen et al., 1992) so as to enable its coplanarity with the enediyne portion of the molecule and thus facilitate the intramolecular reaction.

NOTE ADDED IN PROOF

Recent experiments show that reversal of the T·A base pair to A·T 3′ to the target T residue in duplex I (Figure 6) renders the substrate incompetent, further suggesting a sequence requirement.

REFERENCES

Bhattacharyya, A., & Lilley, D. M. J. (1989) *Nucleic Acids Res. 17*, 6821–6840.

Dasgupta, D., & Goldberg, I. H. (1985) *Biochemistry 24*, 6913–6920.

Dedon, P. C., Jiang, Z.-W., & Goldberg, I. H. (1992) *Biochemistry 31*, 1917–1927.

Frank, B. L., Worth, L., Jr., Christner, D. F., Kozarich, J. W., Stubbe, J., Kappen, L. S., & Goldberg, I. H. (1991) *J. Am. Chem. Soc. 113*, 2271–2275.

Goldberg, I. H. (1991) *Acc. Chem. Res. 24*, 191–198.

Hatayama, T., Goldberg, I. H., Takeshita, M., & Grollman, A. P. (1978) *Proc. Natl. Acad. U.S.A. 75*, 3603–3607.

Hensens, O. D., Helms, G. L., Zink, D. L., Chin, D.-H., Kappen, L. S., & Goldberg, I. H. (1993) *J. Am. Chem. Soc.*, in press.

Hsieh, C.-H., & Griffith, J. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4833–4837.

Kalnik, M. W., Norman, D. G., Zagorski, M. G., Swann, P. F., & Patel, D. J. (1989) *Biochemistry 28*, 294–303.

Kalnik, M. W., Norman, D. G., Li, B. F., Swann, P. F., & Patel, D. J. (1990) *J. Biol. Chem. 265*, 636–647.

Kappen, L. S., Chen, C.-Q., & Goldberg, I. H. (1988) *Biochemistry 27*, 4331–4340.

Kappen, L. S., & Goldberg, I. H. (1983) *Biochemistry 22*, 4872–4878.

Kappen, L. S., & Goldberg, I. H. (1985) *Nucleic Acids Res. 13*, 1637–1648.

Kappen, L. S., & Goldberg, I. H. (1989) *Biochemistry 28*, 1027–1032.

Kappen, L. S., & Goldberg, I. H. (1992a) *Biochemistry 31*, 9081–9089.

Kappen, L. S., & Goldberg, I. H. (1992b) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6706–6710.

Kappen, L. S., & Goldberg, I. H. (1993) *Science 261*, 1319–1321.

Kappen, L. S., Goldberg, I. H., Frank, B. L., Worth, L. J., Christner, D. F., Kozarich, J. W.; & Stubbe, J. (1991) *Biochemistry 30*, 2034–2042.

Kappen, L. S., Goldberg, I. H.; & Liesch, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 744–748.

Kappen, L. S., Goldberg, I. H., Wu, S. H., Stubbe, J., Worth, L., & Kozarich, J. W. (1990) *J. Am. Chem. Soc. 112*, 2797–2798.

Kopka, M. L., Yoon, C., Goodsell, D., Pjura, P., & Dickerson, R. E. (1985) *J. Mol. Biol. 183*, 553–563.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab.), Cold Spring Harbor, NY.

Morden, K. M., Chu, Y. G., Martin, F. H., & Tinoco, I., Jr. (1983) *Biochemistry 22*, 5557–5563.

Morden, K. M., Gunn, B. M., & Maskos, K. (1990) *Biochemistry 29*, 8835–8845.

Myers, A. G. (1987) *Tetrahedron Lett. 28*, 4493–4496.

Napier, M. A., & Goldberg, I. H. (1983) *Mol. Pharmacol. 23*, 500–510.

Nelson, J. W., & Tinoco, Jr. (1985) *Biochemistry 24*, 6416–6421.

Povirk, L. F., & Goldberg, I. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3182–3186.

Povirk, L. F., Houlgrave, C. W., & Han, Y. (1988) *J. Biol. Chem. 263*, 19263–19266.

Rice, J. A., & Crothers, D. M. (1989) *Biochemistry 28*, 4512–4516.

Rosen, M. A., Shapiro, L., & Patel, D. J. (1992) *Biochemistry 31*, 4015–4026.

Saito, I., Kawabata, H., Fujiwara, T., Sugiyama, H., & Matsuura, T. (1989) *J. Am. Chem. Soc. 111*, 8302–8303.

van den Hoogen, Y. T., van Beuzekon, A. A., van den Elst, H., van der Marel, G. A., van Boom, J. H., & Altona, C. (1988) *Nucleic Acids Res. 16*, 2971–2986.

Williams, L. D., & Goldberg, I. H. (1988) *Biochemistry 27*, 3004–3011.

Williams, L. D., Thivierge, J., & Goldberg, I. H. (1988) *Nucleic Acids Res. 16*, 11607–11615.

Woodson, S. A., & Crothers, D. M. (1988) *Biochemistry 27*, 8904–8914.