Bulge-Specific Cleavage in Transactivation Response Region RNA and Its DNA Analogue by Neocarzinostatin Chromophore†

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ABSTRACT: On the basis of the finding that in the absence of thiol the nonprotein chromophore of the antitumor drug neocarzinostatin (NCS-chrom) induces highly efficient site-specific cleavage at a single site on the 3′ side of a bulge in single-stranded DNA involving entirely 5′ chemistry [Kappen, L. S., & Goldberg, I. H. (1993) Science, *261*, 1319−1321], transactivation response region (TAR) RNA (29-mer) and its DNA analogue which presumably contain bulge structures were tested as potential substrates for NCS-chrom. In TAR RNA NCS-chrom generates a distinct but weak band due to cleavage at U_{24} in the bulge. Cleavage at U_{24} has a pH dependence and time course similar to those for previously studied DNA bulges. This band is not produced in drug reactions containing glutathione, by the protein component of native NCS, or by inactivated NCS-chrom. Cleavage at U_{24}, albeit weak, occurs in an RNA substrate made up of two linear RNA oligomers which presumably can form a bulge akin to that in TAR RNA. In the DNA analogue of TAR RNA, as well as in a DNA duplex made of two linear oligomers that can form a similar bulge, NCS-chrom causes strand cleavage at the T residues in the bulge and at the bases flanking the bulge. Cleavage at T_{25} in the bulge involves, in addition to 5′ chemistry, 4′ attack which results in a fragment with mobility characteristic of 3′-phosphoglycolate-ended fragments. Experiments using DNA substrate having deuterium selectively at the 4′ or 5′ positions of T_{25} confirm 4′ attack and show kinetic shuttling between the two positions. Sequence changes in TAR DNA show that the specificity and extent of cleavage is sequence-dependent. While TAR DNA differs from previously studied DNA bulge substrates in having multiple attack sites and 4′ chemistry at one site, it is only about 10% as good a substrate as the latter.

The principal target of the enediyne-containing anticancer drug neocarzinostatin (NCS) is DNA. NCS chromophore (NCS-chrom) binds to the minor groove of duplex DNA via an intercalative mechanism and induces, in the presence of a thiol activator, a variety of lesions of which strand cleavage is the most predominant [reviewed in Goldberg (1991); Goldberg & Kappen, 1994]. Thiol activation generates a diradical species of the drug (Kappen & Goldberg, 1985; Myers, 1987; Myers et al., 1994) which initiates the damage by sequence-specific hydrogen atom abstraction from C-5′ (Kappen & Goldberg, 1983), C-1′ (Kappen & Goldberg, 1989; Kappen et al., 1990), or C-4′ (Saito et al., 1989; Frank et al., 1991; Kappen et al., 1991) positions of deoxyribose. In the presence of molecular oxygen, the lesions on the DNA sugar ultimately result in spontaneous and/or alkali-labile breaks (Goldberg & Kappen, 1994). The precise chemistry of damage depends on the initial site of attack, which in turn, is defined by the microstructure of the drug−DNA complex. The three-dimensional structure of the glutathione postactivated chromophore (mimic of the diradical species)−DNA complex has recently been determined by NMR spectroscopy (Gao et al., 1995).

Ordinarily, in the absence of a thiol activator, there is no measurable damage by NCS-chrom in duplex or single-stranded DNA. We recently reported, however, that NCS-chrom induces highly efficient site-specific cleavage in the absence of thiol (Scheme 1) at a single nucleotide at the 3′ side of a bulge in single-stranded DNAs that are capable of folding into hairpin structures and also in related bulge-containing duplex DNAs made up of two linear oligomers (Kappen & Goldberg, 1993a,b). Cleavage at the target residue involves selective 5′-hydrogen atom abstraction by an active species of NCS-chrom and oxidative formation of a break, generating a DNA fragment with nucleoside 5′-aldehyde at its 5′ end and a phosphate at the 3′ terminus. In addition, the bulge-specific reaction generates a novel drug product (**3**; Scheme 1) containing the tritium abstracted from the C-5′ of the target nucleotide (Kappen & Goldberg, 1993a). Structure determinations of this drug product (Hensens et al., 1993) and the two main compounds produced by spontaneous decomposition of NCS-chrom under the reaction conditions but in the absence of bulged DNA led to proposals of possible mechanistic pathways for the activation of the drug in the thiol-independent reaction and the role of bulged DNA in drug product formation (Hensens et al., 1994). It appears that NCS-chrom undergoes spontaneous intramolecular activation to a diradical species in a general base-catalyzed reaction (steps 1−3, Scheme 1) followed by DNA bulge-induced quenching of one of the radical centers (step 4) to form the monofunctional drug species (**1e**) responsible for abstraction of the C-5′ hydrogen. This reaction defines a new role for DNA in effecting a conformational change in the active drug intermediate so as to enable carbon−carbon bond formation (step 4).

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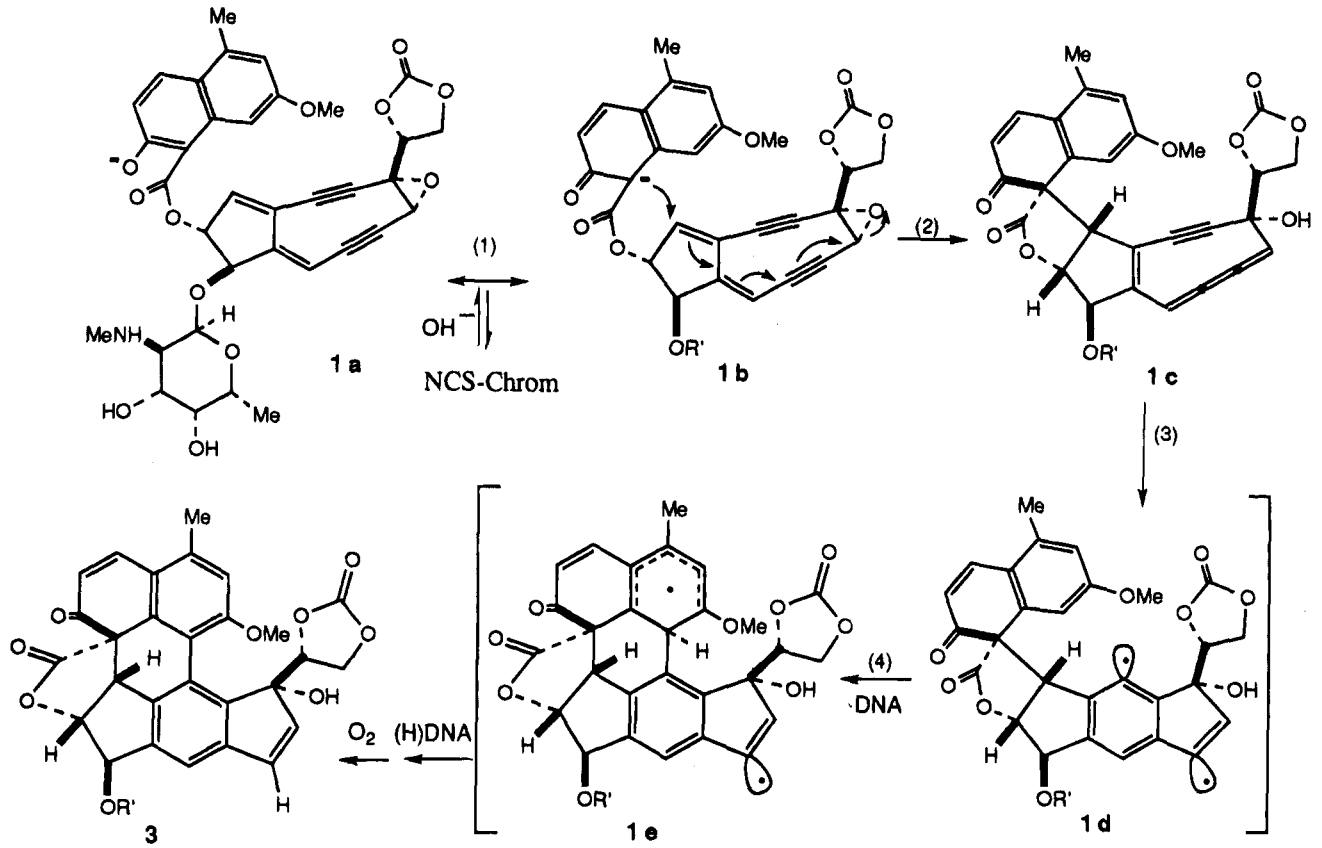
Scheme 1: Proposed Mechanism of NCS-Chrom-Induced Cleavage of Bulged DNA in the Thiol-Independent Reaction



The DNA bulge substrates used in the previous work were limited to two 31-mers (31-I, the DNA analogue of a 3′ terminal fragment—residues 47−76—of yeast $tRNA^{Phe}$, and 31-II, its complementary strand) and several oligomers derived from these by deletions and mutations (Kappen & Goldberg, 1993a,b). Despite the close similarity in their structures, 31-I was cleaved more efficiently than 31-II. This finding, taken together with the site-specific cleavage data on the other bulge substrates, suggested that the efficiency of cleavage depends upon several factors such as position of the bulge, neighboring sequences, bulge size, and, in single-stranded DNA, apical loop size. Since the thiol-independent reaction is specific for bulges, it is of interest to study other bulge-containing nucleic acid structures to ascertain the generality of this phenomenon. Further, given the resemblance in the structural features of HIV-1 transactivation response region (TAR) RNA to those of the competent DNA substrates, in having a folded structure with an apical loop and a bulge with flanking double-stranded regions stabilized by Watson−Crick base-paired regions (Dingwall et al., 1989, 1990; Roy et al., 1990; Weeks et al., 1990; Weeks & Crothers, 1991, 1992; Hamy et al., 1993; Slim et al., 1991), TAR RNA and its DNA analogue appear to be good candidates as potential substrates. Results of these studies are reported in this paper.

MATERIALS AND METHODS

Nucleic Acid Substrates. Oligodeoxyribonucleotides were synthesized using an Applied Biosystems DNA synthesizer by phosphoramidite chemistry or purchased from Chemgenes. Oligoribonucleotides were similarly synthesized and purified on a denaturing polyacrylamide gel. The 29-mer TAR RNA fragment was a generous gift from Michael Gait. It was also prepared using T7 RNA polymerase by the method of Milligan et al. (1987). RNA sequencing kit was purchased from United States Biochemical Corp. Radioactive materials and enzymes were purchased from New England Nuclear and New England Biolabs, respectively. Oligomers were 5′-end-labeled with ^{32}P by standard procedures (Maniatis et al., 1982). 13-Mer having deuterium at T_{25} was prepared by chain extension of ^{32}P-end-labeled 8-mer (GCCAGATT) using Klenow fragment in a reaction containing the appropriate template, deoxynucleoside triphosphates, and ^{2}H-labeled thymidine 5′-triphosphate as previously described (Kappen et al., 1990, 1991). The labeled oligomers were purified by electrophoresis on a 15% sequencing gel. Neocarzinostatin powder was obtained from Kayaku Antibiotics (Tokyo).

Drug Reaction. NCS-chrom was extracted from the holoantibiotic by cold methanol in the presence of citrate (Kappen & Goldberg, 1985). When duplex DNA was the substrate, the ^{32}P-end-labeled (+) strand was annealed to excess (1.8−2.5-fold) of the (−) strand in 2× reaction buffer by heating at 90 °C for 2 min and slow cooling. Single-stranded DNA substrate was subjected to the same heating and cooling step. Adjustment to the final reaction volume was made by addition of water. Drug reactions with single-stranded RNA substrate were performed without or with prior heating (70 °C for 2 min) and cooling of the RNA. A standard reaction contained 25 mM Tris-HCl, pH 8.5, 1 mM EDTA, oligomer, and NCS-chrom at concentrations indicated in the figure legends. In some reactions EDTA was not present; this did not alter the strand cleavage specificity or intensity. The mixture of components was chilled in ice for 15 min prior to the addition of the drug. Maximum final

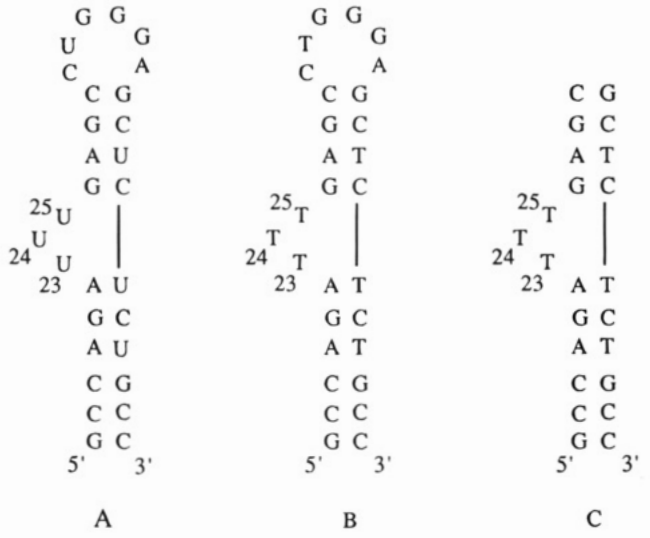


FIGURE 1: Nucleic acid substrates for NCS-chrom, (A) TAR RNA, (B) DNA analogue of TAR RNA, and (C) bulged DNA duplex made up of two linear oligomers, a 13-mer and a 10-mer. The bases are numbered according to Slim et al. (1991).

methanol concentration was 10%. The reaction was allowed to proceed in the dark for 1 h in ice. Portions of the reaction mixture were dried, and the sample pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes and analyzed on a 15% sequencing gel. The gel band intensities were quantitated on a phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Cleavage of RNA by ribonucleases was performed according to the method given by the supplier. Alkali cleavage was in sodium phosphate, pH 12.5, for 1 h at 50 °C.

RESULTS AND DISCUSSION

Strand Cleavage in TAR RNA. A 29-mer oligoribonucleotide (Slim et al., 1991) or a 27-mer (Weeks & Crothers, 1991) corresponding to the Tat (transactivator protein) binding portion of the 59-residue TAR RNA was studied as a potential substrate in the thiol-independent NCS-chrom cleavage reaction. The effect of treatment of 5′-^{32}P-end-labeled 29-mer TAR RNA (Figure 1A) with NCS-chrom is shown in Figure 2. Addition of the drug generates a distinct but weak band (Figure 2, lanes 2 and 3, arrow) over and above the background. This band has a mobility slightly faster than that of the band generated by enzyme cleavage at U_{24}; in comparison with the alkali-induced cleavage, it appears to have a 3′-phosphate end. Absence of this band on inclusion of glutathione in the reaction (lane 4) can be expected in view of the fact that the active intermediate species of the drug (cumulene species) that binds to DNA bulges is different from that formed in the thiol reaction (Hensens et al., 1994). Further, the reaction of thiol with NCS-chrom is much faster than the intramolecular activation of drug in the bulge-dependent reaction (Kappen & Goldberg, 1993). The protein component of native NCS (apoNCS) does not cause cleavage at U_{24} (lane 6), suggesting that the cleavage obtained on addition of NCS-chrom is not due to contaminating apoprotein or ribonuclease. Inhibition of strand cleavage at U_{24} on addition of apoNCS to the NCS-chrom reaction (lane 5) can be explained on the basis that apoNCS binds NCS-chrom very tightly (K_d of 10^{-10} M) (Goldberg, 1991) to form holoNCS, which has little or no activity at 0 °C (Kappen & Goldberg, 1993b). Further

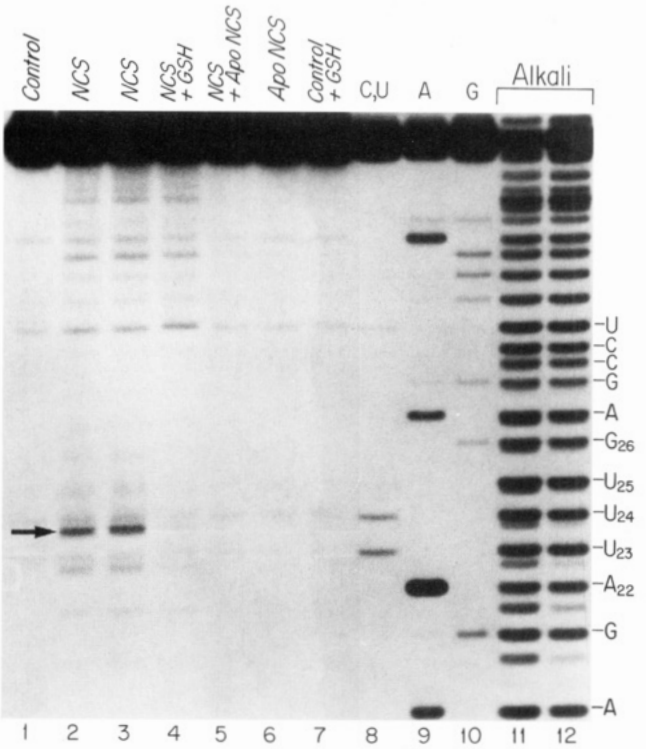


FIGURE 2: NCS-chrom treatment of 5′-^{32}P-end-labeled 29-mer TAR RNA. RNA (12 μM nucleotide) was treated with NCS-chrom (29 μM) under standard conditions. Glutathione (2.2 mM) or apoNCS (2-fold excess) was present in the reaction mixture prior to the addition of NCS-chrom, as indicated. Lanes C,U, A, and G represent base-specific cleavage by *Bacillus cereus*, U_2, and T_1 ribonucleases, respectively. NCS represents neocarzinostatin chromophore in this and subsequent figures.

evidence that the strand cleavage at U_{24} is NCS-chrom specific comes from the finding that NCS-chrom, inactivated by preincubation at room temperature for 1 h in Tris-HCl buffer, pH 9, prior to the addition to RNA does not generate the band at U_{24} (data not shown). An overexposed film in several experiments showed very weak bands corresponding to cleavage at U_{23} and U_{25}. Some of the very weak cleavage sites (with or without glutathione) seen on the top of the gel appear to be in the hairpin loop. Control experiments (lanes 4−6) exclude the possibility of some spurious nuclease involvement in the strand cleavage observed in the RNA bulge region. An RNA duplex made by annealing a 5′-^{32}P-end-labeled 18-mer and a 15-mer corresponding to TAR RNA but lacking the apical loop (similar to Figure 1C) with five extra base pairs (one at the 5′ and four at the 3′ side of the 18-mer) added to enhance its stability was tested as a potential substrate. Treatment of this duplex RNA, which presumably forms the same bulge as in the 29-mer TAR RNA, with NCS-chrom also produced a band, albeit very weak, at a U residue corresponding to U_{24} (data not shown). There was no cleavage with the 18-mer alone. Since both the 18-mer and the 15-mer are needed to make a duplex containing the bulge, this result is consistent with the above findings that NCS-chrom causes bulge-specific cleavage in RNA.

A time course of the generation of the band at U_{24} showed that it is a slow reaction (maximal cleavage in about 1 h) similar to that reported for cleavage in DNA bulges (Kappen & Goldberg, 1993a,b). Furthermore, as in the case of DNA bulges, the strand cleavage at U_{24} is also pH-dependent; the intensity of the band at U_{24} being much less at pH 7.5 than

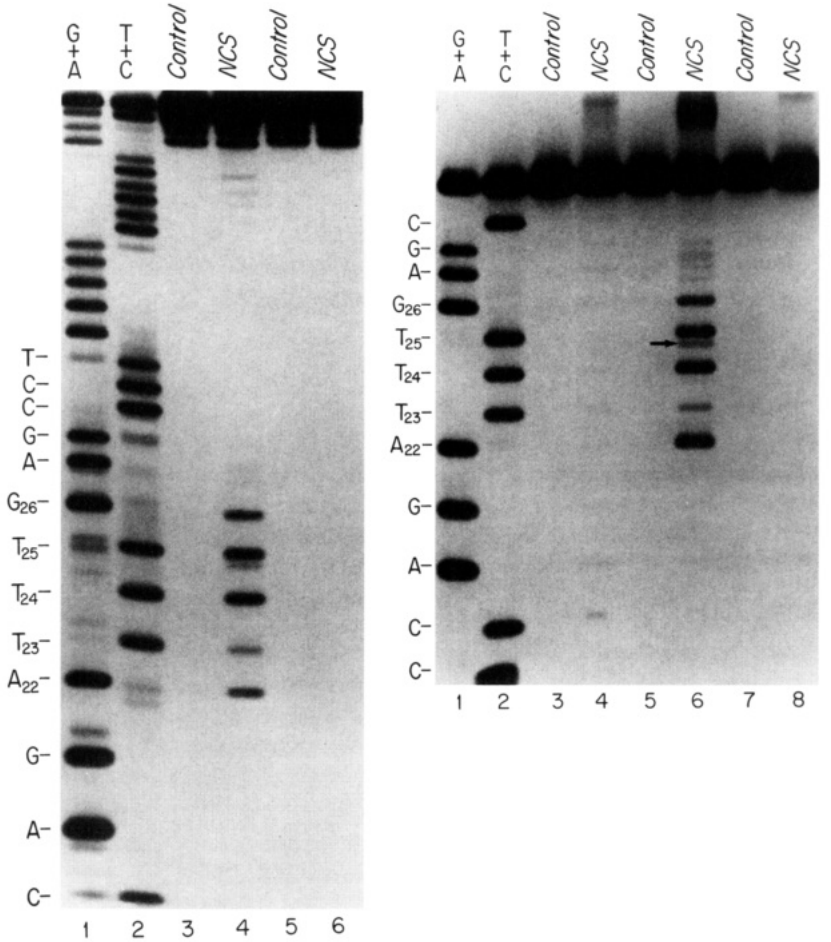


FIGURE 3: (Left) Strand cleavage in 5′-^{32}P-end-labeled 29-mer DNA by NCS-chrom. The 29-mer (225 μM nucleotide), in single-stranded or duplex form, was treated with NCS-chrom (19 μM), as described in Materials and Methods: lanes 3 and 4, single-stranded; lanes 5 and 6, Watson−Crick duplex. (Right) 5′-^{32}P-End-labeled 13-mer (225 μM nucleotide) treated with 45 μM NCS-chrom. Lanes: 3 and 4, single-stranded 13-mer; 5 and 6, bulge-containing duplex (13-mer and 10-mer) (Figure 1C); 7 and 8, Watson−Crick duplex (13-mer and complement). Arrow indicates band of 3′-phosphoglycolate-ended fragment.

that at pH 8.5. Absence of EDTA or presence of 20 mM KCl in the drug reaction or heating the RNA in the buffer at 70 °C for 2 min followed by slow cooling prior to the drug addition had no effect on the cleavage at U_{24} in the 29-mer TAR RNA. On the other hand, addition of NaCl (70 mM), spermine, or spermidine (0.1−5 mM) inhibited the reaction. In addition, when TAR RNA was annealed to excess complementary DNA, there was no cleavage at U_{24}. Further, neither of the enediynes calicheamicin γ_1^I (125 μg/mL) nor esperamicin A_1 (167 μg/mL) caused any cleavage in TAR RNA with or without glutathione.

Strand Cleavage in the DNA Analogue of TAR RNA. Given that TAR RNA appeared to be a poor, but real, target for NCS-chrom in the thiol-independent reaction, it was of interest to test its DNA analogue (Figure 1B) as a substrate in this reaction. In the single-stranded 29-mer TAR DNA, strand breakage occurs selectively in the bulge region; of the three T residues in the bulge, attack at T_{24} and T_{25} (Figure 3, left, lane 4) is much stronger than that at T_{23}. In addition, lesser strand cleavage also occurs at the bases flanking the bulge (A_{22} and G_{26}). In striking contrast the Watson−Crick-base-paired duplex form (29-mer with its complement) is not cleaved at all (lane 6). These results are different from those obtained with previously studied bulged DNA substrates (Kappen & Goldberg, 1993a,b), where NCS-chrom induced strand cleavage exclusively at a single residue on the 3′ side of the bulge with an efficiency far exceeding (about 10 times) that obtained with the 29-mer in Figure 3, left. Further confirmation that the strand breakage in the single-stranded 29-mer is due to bulge recognition comes from experiments designed to create a similar bulge in a duplex substrate by annealing a 13-mer and a 10-mer. But for the absence of the apical loop, this duplex substrate (Figure 1C) has the same sequence and bulge as in the 29-mer TAR DNA. As shown in Figure 3, right, only in the duplex made up of the 13-mer and the 10-mer does strand cleavage occur with the same base specificity (lane 6) as in the single-stranded 29-mer. Neither the 13-mer alone (lane 4) nor its Watson−Crick-base-paired duplex (lane 8) is a substrate in the thiol-independent reaction. With both bulge substrates, a band with a mobility slower than that of the starting material (possibly a drug−DNA adduct) is produced in the drug reaction (Figure 3, right, lane 6).

Chemistry of Damage in DNA. The coincident mobility of NCS-chrom-induced bands and Maxam−Gilbert markers (Figure 3) suggests that the drug-produced fragments have a phosphate at their 3′ ends. This is consistent with the 5′ attack chemistry established for NCS-chrom-induced site-specific cleavage in the previously studied DNA bulge substrates, where cleaved fragments had a nucleoside 5′-aldehyde at their 5′ ends and a phosphate at the 3′ ends (Kappen & Goldberg, 1993a,b). Unlike in the previously studied bulge substrates, however, the cleavage at T_{25} (Figure 3) produces, in addition to the phosphate band, a minor faster

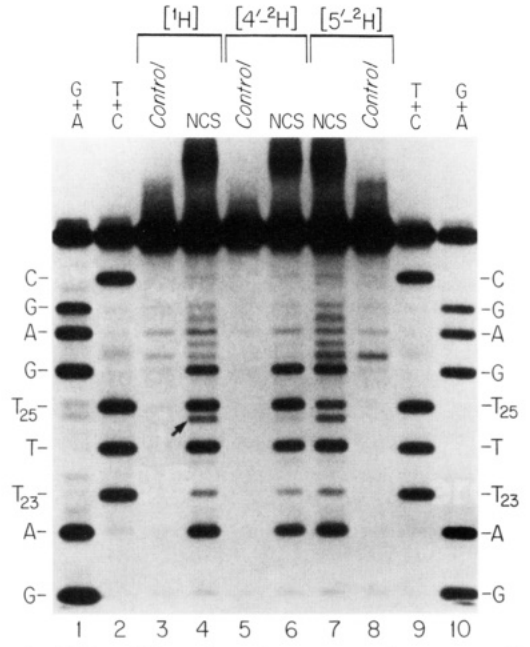


FIGURE 4: Effect of deuterium placement at the target residue T_{25} on strand cleavage by NCS-chrom. Reaction conditions are similar to those in Figure 3, right. 5′-^{32}P-End-labeled 13-mer (182 μM nucleotide) having protium (^{1}H) or deuterium (^{2}H) at the 4′ of 5′ position of T_{25}, annealed to 10-mer (Figure 1C), was the substrate for NCS-chrom (37 μM). Arrow indicates 3′-phosphoglycolate band. Maxam−Gilbert markers (G+A, T+C) for [^{1}H]-13-mer and 5′-^{2}H-labeled 13-mer are shown in lanes 1, 2 and 9, 10, respectively. Not shown are the G+A and T+C markers for 4′-^{2}H-labeled 13-mer, which were identical to those for ^{1}H-labeled and 5′-^{2}H-labeled 13-mers.

moving band (Figure 3, right, lane 6, arrow), with a mobility characteristic of 3′-phosphoglycolate-ended fragments resulting from 4′ chemistry. In order to confirm the 4′ chemistry at T_{25}, we used the deuterium isotope labeling technique which has been shown to be very sensitive to detect minor lesions in DNA (Kozarich et al., 1989; Kappen et al., 1990, 1991; Frank et al., 1991). In experiments shown in Figure 4 the target residue T_{25} in the bulge duplex (Figure 1C) has protium (^{1}H) or deuterium (^{2}H) at its 4′ or 5′ position. It can be seen that only with the substrate having deuterium at the 4′ position is there a selective inhibition of the faster moving band (compare T_{25} in lanes 4 and 6), which confirms 4′ chemistry at T_{25}. Quantitation of the bands at T_{25} gave an isotope effect of 3.1 with deuterium substitution at C-4′. Introduction of deuterium at the 5′ position, on the other hand, enhances the glycolate formation but causes a marked reduction in the phosphate band with an isotope effect of 2.7 (lane 7). A similar shuttling between 4′ and 5′ chemistry on introduction of deuterium has previously been observed in NCS-chrom-induced damage at T residues in Watson−Crick-base-paired duplex DNA in the thiol-dependent reaction (Frank et al., 1991; Kappen et al., 1991). It is of note that in the thiol-dependent reaction with the bulge-containing duplex or Watson−Crick-base-paired duplex there is no phosphoglycolate band at T_{25} (data not shown).

Effect of Sequence Changes on Strand Cleavage in the Bulge. Sequence changes in the bulge-containing duplex (Figure 5, C) affect the specificity and/or the relative intensity of strand cleavage. Duplex D, where the base pair (A_{22}-T_{34}) on the 5′ side of the bulge is replaced by a C-G, is as good a substrate as C with the same base specificity for cleavage, but the cleavage at C_{22} is less. When the base

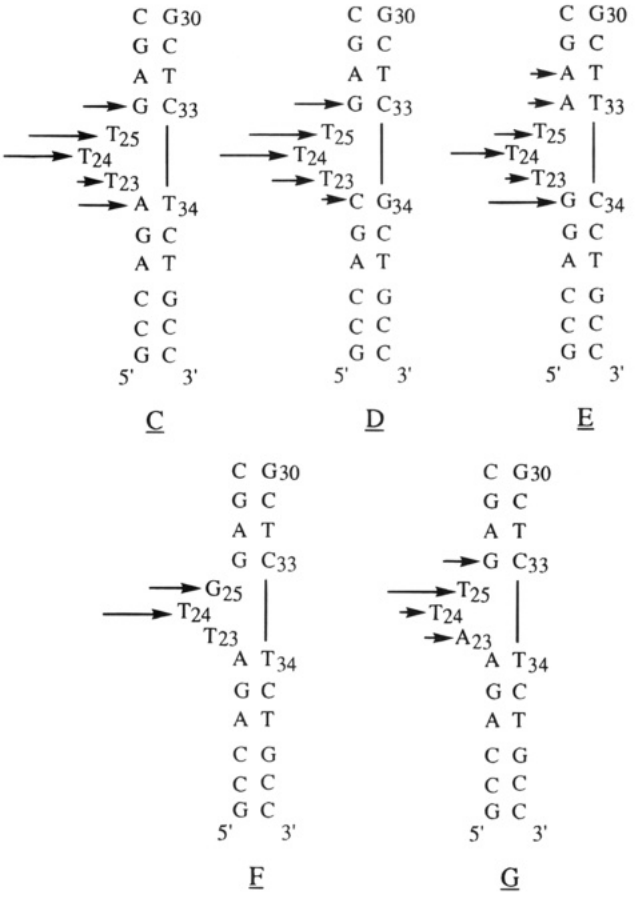


FIGURE 5: Effect of sequence changes on NCS-chrom-induced strand cleavage in the absence of thiol. Standard reactions were performed with the indicated DNA duplexes. Duplex C is the same as in Figure 1C, and the bases are numbered the same way. Arrows point to the site of cleavage. The length of arrows indicates approximately the relative intensity of the bands in the particular duplex as viewed on the autoradiogram. The radioactivity present in C for all the cleavage bands combined was 5−6% of the total.

pairs flanking the bulge (A_{22}-T_{34} and G_{26}-C_{33}) are interchanged (duplex E), G_{22} becomes the most favored cutting site. Sequence changes in the bulge alter the specificity of cleavage as shown in F and G. In duplex F, where T_{25} in the bulge is substituted with a G, cleavage occurs only at T_{24} and G_{25}. Furthermore, G_{25} cleavage does not produce a band corresponding to a 3′-phosphoglycolate-ended fragment (data not shown) as found for the parent duplex C. Substitution of T_{23} with an A (duplex G) suppresses the cleavage at T_{24} and A_{22}. These variations in the specificity and the extent of cleavage with sequence changes may be attributed to the differences in the stability and/or the microstructure of the drug−DNA complex.

CONCLUDING REMARKS

These studies extend the repertoire of lesions produced by NCS-chrom in the thiol-independent reaction at a bulge site in nucleic acids. In earlier work cleavage was restricted to a single nucleotide at the 3′ side of a specific bulge in DNA and the chemistry was entirely 5′-hydrogen abstraction. Here, we show that at a bulge structure based on TAR RNA there may be multiple attack sites in or adjacent to the bulge in the DNA analogue and that the intensity of the lesion at a particular site is sequence-dependent. Further, at a particular site, 4′ as well as 5′ chemistry occurs. To our knowledge this study provides the first reported evidence

that RNA can be a substrate for an enediyne antibiotic; specific cleavage by NCS-chrom is shown to occur in the absence of thiol at mainly a single site within the bulge of TAR RNA. Given the likely three-dimensional structural difference between the RNA and its DNA analogue, it is not surprising that TAR RNA is a much poorer substrate than its DNA analogue. Perhaps only some small fraction of the TAR RNA bears a structure in space similar to that of the bulged DNA. It is likely that reaction conditions optimal for the formation of this species remain to be elucidated. For that matter, even the DNA analogue of TAR RNA is a relatively poor substrate in the thiol-independent reaction, being only about 10% as effective as the bulged DNA studied earlier (Kappen & Goldberg, 1993a,b).

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