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Detection of Neocarzinostatin Chromophore–Deoxyribose Adducts as Exonuclease-Resistant Sites in Defined-Sequence DNA†

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ABSTRACT: A 5′-end-labeled DNA restriction fragment was treated with the nonprotein chromophore of neocarzinostatin under anoxia in the presence of dithiothreitol, conditions known to maximize formation of chromophore–deoxyribose adducts. Under conditions where unmodified DNA was digested to completion, chromophore-treated DNA was highly resistant to digestion by exonuclease III plus the 3′ → 5′ exonucleolytic activity of T4 DNA polymerase and partially resistant to digestion by exonuclease III plus snake venom exonuclease. The electrophoretic mobilities of the products of exonucleolytic digestion suggested that (i) digestion by exonuclease III or T4 polymerase terminated one nucleotide before the nucleotide containing the adduct, (ii) the remaining nucleotide directly adjacent to the adduct (3′ side) could be removed by snake venom phosphodiesterase, but at a slow rate, (iii) the covalently linked chromophore decreased the electrophoretic mobilities of the digestion products by the equivalent of approximately three nucleotides, and (iv) adducts formed under anaerobic conditions occurred at the same nucleotide positions as the strand breaks formed under aerobic conditions (primarily at T and, to a lesser extent, A residues). The close similarity in sequence specificity of adducts and strand breaks suggests that a common form of nascent DNA damage may be a precursor to both lesions. A chromophore-induced free radical on C-5′ of deoxyribose, subject to competitive fixation by addition reactions with either oxygen or chromophore, is the most likely candidate for such a precursor. The base specificity of adduct formation does not reflect the reported base specificity of neocarzinostatin-induced mutagenesis, suggesting that lesions other than adducts may be responsible for at least some neocarzinostatin-induced mutations, particularly those occurring at G·C base pairs.

At least four types of lesions are formed in DNA by the antitumor antibiotic neocarzinostatin (or its isolated nonprotein chromophore), in the presence of sulfhydryl cofactors. The predominant lesion is a strand break, resulting from selective, oxygen-dependent oxidation of deoxyribose to a 5′-aldehyde (Kappen et al., 1982). The next most frequent lesion is release of free base, presumably associated with some as yet undetermined form of sugar oxidation (Kappen & Goldberg, 1983). In addition, at least two types of adducts between DNA and the nonprotein chromophore of neocarzinostatin have been detected. In the presence of 2-mercaptoethanol and oxygen, a labile adduct containing an oxidized deoxyribose is formed, although with a very low yield (Povirk & Goldberg, 1982a,b). In the presence of dithiothreitol, a much more stable adduct species is produced, with a somewhat higher yield (Povirk & Goldberg, 1984). Under anaerobic conditions, the yield of this type of adduct is further increased (while strand breakage and base release are suppressed), so that stable adducts become the predominant DNA lesion. This stable adduct species, and probably the labile adduct species as well, involves covalent linkage of the chromophore to deoxyribose in DNA.

Chromophore–DNA adducts were originally isolated as two or three base long fragments after extensive nucleolytic digestion of chromophore-treated DNA (Povirk & Goldberg, 1982a, 1984). The resistance of these chromophore–oligonucleotide adducts to further nuclease digestion suggested the possibility that adducts might, like pyrimidine dimers, block exonucleolytic digestion of DNA (Setlow et al., 1964). The sequence specificity of adduct formation could then be determined by mapping adducts as exonuclease termination sites in defined-sequence DNA (Tullius & Lippard, 1981; Royer-Poroka et al., 1981). We sought to compare the sequence specificity of adduct formation with that of strand breakage, as well as with the specificity of neocarzinostatin-induced mutagenesis (Foster & Eisenstadt, 1983).

Experimental Procedures

Preparation of Labeled Restriction Fragments. DNA of plasmid pMC1 (obtained from E. Eisenstadt) was isolated and digested with *Hin*cII. The 789 and 935 base pair (bp) fragments, containing sequences of the *lacI* gene (Calos et al., 1978), were separated on a 5% polyacrylamide gel and electroeluted. The 789-bp fragment was 5′-^{32}P end labeled by the exchange reaction (Maxam & Gilbert, 1980) and digested with *Dde*I, and the resulting 66-bp fragment was isolated on an 8% polyacrylamide gel. The sequence of the labeled strand of this fragment is 5′-AACCACCATC$_{10}$AAACAGGATT$_{20}$TTCGCCTGCT$_{30}$GGGGCAAACC$_{40}$AGCGTGGACC$_{50}$GCTTGCTGCA$_{60}$ACTCTC. A fragment containing the first 49

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bases of this sequence, but labeled in the same strand at the 3′ end, was prepared by digesting the 789-bp fragment with *Ava*II, filling in the recessed 3′ ends with dATP, dGTP, and [α-^{32}P]dCTP (Maniatis et al., 1982), and isolating the 49-bp *Hinc*II/*Ava*II fragment. The unlabeled 5′-phosphate was removed with calf intestinal alkaline phosphatase (Maniatis et al., 1982).

To prepare other 5′-end-labeled DNA fragments, the 789- or 935-bp fragments were digested with a second restriction enzyme, and the resulting fragments were either end labeled as a mixture or separated on an 8% polyacrylamide gel and end labeled. The labeled DNA was then digested with a third enzyme, and the desired fragment was isolated.

Chromophore–DNA Reactions. Neocarzinostatin chromophore (250 μM in 80% methanol/20% water containing 16 mM sodium citrate, pH 4) was prepared as described (Povirk et al., 1981). A reaction vessel for small-volume anaerobic reactions was formed by sealing two perpendicular ends of a 0.25 in. diameter glass T joint, leaving one end open for evacuation. A reaction mixture containing 500 μM DNA (an excess of sonicated calf thymus DNA and a trace of labeled restriction fragment), 60 μM chromophore in 2 mM sodium citrate, and 4 mM ethylenediaminetetraacetic acid (EDTA), pH 4, was lyophilized to remove methanol and redissolved in an equal volume of water at 0 °C. Immediately after addition of dithiothreitol to 1 mM, the mixture (100 mL) was loaded on ice into one sealed end of the reaction vessel, and 5 μL of 2.5 M tris(hydroxymethyl)aminomethane (Tris), pH 8, was placed in the side arm. The vessel was then attached to a three-way valve and 10 times alternately evacuated by aspiration and filled with argon (or air for aerobic controls), while the reaction mixture was constantly vortexed. The vessel was then tipped to mix the reaction mixture with the Tris buffer and again vortexed. Tris buffer raises the pH of the mixture to 7.8, initiating activation of the chromophore by dithiothreitol, with resulting DNA damage (Povirk & Goldberg, 1983). After a 20-min incubation at 20 °C under positive argon pressure, the solution was removed, and the DNA was ethanol precipitated after addition of sodium acetate to 0.3 M.

Enzymatic Digestions. Chromophore-treated 5′-end-labeled DNA was dissolved in 66 mM Tris-HCl, pH 7.4/0.66 mM $MgCl_2$ (twice the original reaction volume) and incubated at 37 °C in the presence of exonuclease III (New England Biolabs), as indicated in the legends. In some cases snake venom phosphodiesterase (Sigma) or T4 DNA polymerase (New England Nuclear) was added, and the incubation was continued. The $MgCl_2$ concentration was increased to 8 mM before addition of T4 polymerase. In some cases the time course of enzymatic digestion was followed by removing 2-μL aliquots and determining the acid-precipitable radioactivity (Povirk, 1977). After enzyme treatments, samples were ethanol precipitated in the presence of 0.3 M sodium acetate, 20 mM EDTA, and 40 μg/mL tRNA.

Chromophore-treated, dephosphorylated 3′-end-labeled DNA was dissolved in 0.15 M sodium acetate/5 mM EDTA, pH 5 (4 times the original reaction volume), denatured by heating for 2 min at 90 °C, and cooled on ice for 30 s. Spleen phosphodiesterase (Sigma) was then added, and the sample was incubated at 37 °C for 5 min and ethanol precipitated in the presence of 0.3 M sodium acetate and 40 μg/mL tRNA.

Precipitated DNA samples were dissolved in 40 μL of water, lyophilized, dissolved in 10 μL of loading solution, denatured, and run on 20% polyacrylamide, 35 × 40 × 0.08 cm DNA sequencing gels as described by Maxam & Gilbert (1980), usually for 11 h at 1000 V. In some cases, specific bands were located by autoradiography, cut out from the gel, and crushed. DNA was eluted by stirring for 16 h at 37 °C in 4 mL of 0.2 M NaCl containing 5 μg/mL tRNA and 20 mM Tris-HCl, pH 7.4. The solution was frozen and evaporated to 0.8 mL, and the DNA was ethanol precipitated.

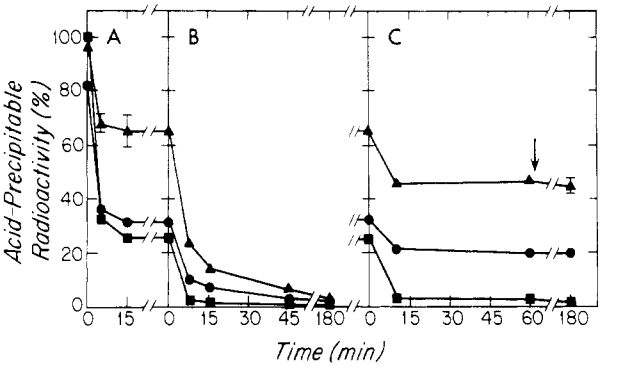

FIGURE 1: Digestion of chromophore-treated DNA with 3′→5′-exonucleases. A 5′-end-labeled 66-bp DNA restriction fragment was treated with chromophore plus dithiothreitol under aerobic (●) or anaerobic conditions (▲) or treated with dithiothreitol alone (■). The treated DNA was incubated in the presence of 3000 units/mL exonuclease III (A) for 15 min. Snake venom exonuclease at 30 μg/mL (B) or T4 DNA polymerase at 50 units/mL (C) was then added and the incubation continued. After 1 h, an additional 50 units/mL T4 polymerase was added (arrow in C). At the indicated time, duplicate 2-μL samples were removed and assayed for acid-precipitable radioactivity.

RESULTS

Resistance of Chromophore-Treated DNA to 3′→5′-Exonucleases. Exonuclease III of *Escherichia coli* digests double-stranded DNA by first removing any terminal 3′-phosphates and then releasing single nucleoside 5′-phosphates from the 3′ ends (Kornberg, 1980). Digestion continues until only single-stranded DNA, which is relatively resistant to the enzyme and has 5′-phosphate and 3′-hydroxyl ends, remains. However, both snake venon phosphodiesterase and the correcting 3′→5′-exonuclease of T4 DNA polymerase act efficiently on such single-stranded substrates, again removing nucleoside 5′-phosphates from the 3′ end (Kornberg, 1980). Hence, sequential treatment with exonuclease III plus either venom phosphodiesterase or T4 polymerase should digest normal DNA to completion. This is shown for [5′-^{32}P]DNA in Figure 1. Treatment with exonuclease III rendered about 75% of the radioactivity acid soluble, and subsequent treatment with either T4 polymerase or venom exonuclease solubilized the remainder within 15 min.

DNA treated with neocarzinostatin chromophore under aerobic conditions was digested with exonuclease III to about the same extent as normal DNA. Subsequent treatment with venom exonuclease digested the chromophore-treated DNA to completion. However, at short digestion times, a fraction of DNA was present that was partially resistant to digestion. This resistant fraction was about 2-fold greater in DNA that had been treated with chromophore under anaerobic conditions so as to maximize adduct formation. Again, the resistant fraction of DNA was more than 2-fold greater when the chromophore treatment was performed anaerobically.

These results strongly suggest that chromophore-induced DNA lesions acted as blocks to the 3′ → 5′ exonucleolytic activity of T4 polymerase and as partial blocks to venom exonuclease. Since exonuclease-resistant DNA is maximized under conditions that maximize adduct formation (Povirk & Goldberg, 1984) and suppress formation of other lesions (i.e.,

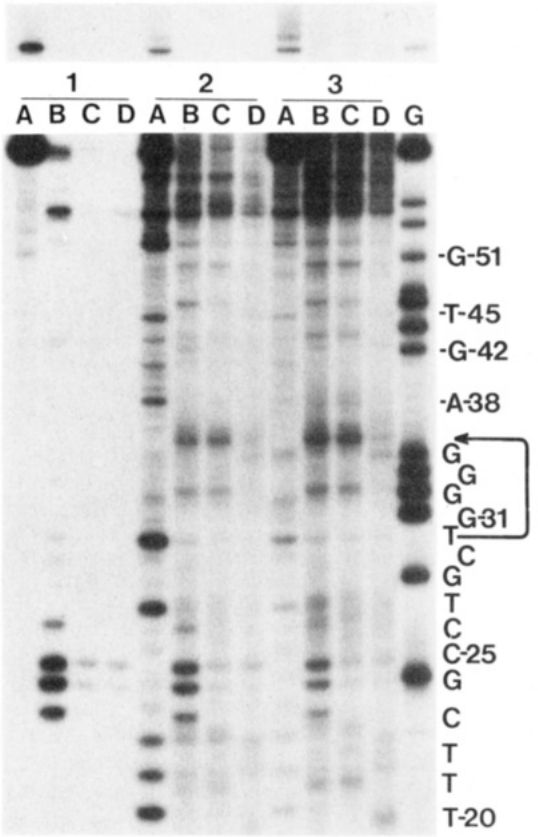

FIGURE 2: Sequencing gel analysis of chromophore-treated DNA after 3′→5′-exonuclease digestion. A 5′-end-labeled 66-bp DNA restriction fragment was treated with chromophore in the presence of dithiothreitol, digested with exonucleases, and subjected to analysis on a DNA sequencing gel. Chromophore treatments were (1) no treatment, (2) treatment under aerobic conditions, and (3) treatment under anaerobic conditions. Subsequent enzyme treatments were (A) no treatment, (B) exonuclease III alone (15 min), (C) exonuclease III (15 min) followed by T4 polymerase (1 h), (D) exonuclease III (15 min) followed by snake venom exonuclease (8 min), and (G) Maxam–Gilbert reaction for cleavage at G residues. Enzyme concentrations were the same as in Figure 1. Arrow shows the putative adduct band migrating near position C_{35}, the equivalent of five nucleotides more slowly than the corresponding strand-break band at T_{30}. At the top of the figure is a shorter autoradiographic exposure of the region of the gel corresponding to the full-length DNA fragment.

under anoxia), it is likely that these exonuclease-resistant lesions are chromophore–DNA adducts.

Mapping of Exonuclease-Resistant Sites in Defined-Sequence DNA. In order to determine termination sites for exonuclease digestion, chromophore-treated, exonuclease-digested [5′-^{32}P]DNA was run on sequencing gels. As shown in Figure 2, digestion of unmodified DNA with exonuclease III produced a series of well-defined bands (lane 1B). These presumably represent natural pause sites for the enzyme, where digestion is terminated due to loss of the complementary strand (Tullius & Lippard, 1981; Royer-Pokora et al., 1981). As expected, subsequent digestion of this single-stranded DNA with T4 polymerase or venom exonuclease virtually eliminated these bands (lanes 1C and 1D).

Treatment with chromophore alone (in the presence of dithiothreitol) produced the characteristic bands corresponding to chromophore-induced strand breaks, occurring primarily at T residues and to a lesser extent A residues (Hatayama et al., 1978; D'Andrea & Haseltine, 1978), and bearing a 3′-phosphate end (lane 2A). As expected, the strand-break bands were greatly reduced in intensity when the reaction was performed anaerobically (lane 3A). When chromophore-treated DNA was digested with exonuclease III (lane 2B), the strand-break bands were virtually eliminated, as expected, and a new series or bands appeared. In addition to the natural pause sites seen in untreated DNA, several rather more diffuse, chromophore-dependent bands were seen, presumably corresponding to termination of exonuclease digestion at sites of chromophore-induced DNA lesions. Subsequent treatment with T4 polymerase eliminated the bands corresponding to exonuclease III pause sites, leaving only chromophore-dependent bands, which for the most part did not show any change in mobility after T4 polymerase treatment. Virtually identical results were obtained when the large fragment of *E. coli* DNA polymerase I, which has a similar 3′→5′-exonuclease activity (Kornberg, 1980), was used in place of T4 polymerase (not shown). These results suggest that both these enzymes were blocked by the same DNA lesions as exonuclease III.

When chromophore treatment was performed anaerobically, the bands corresponding to blockage of exonuclease digestion were intensified (lanes 3B and 3C), suggesting that the blocking lesions were chromophore–DNA adducts. Confirmation of adduct formation and its enhancement under anoxia was obtained by examining a short autoradiographic exposure of the region of the gel corresponding to full-length DNA. The sample treated with chromophore alone (lane 2A) showed a minor band that migrated more slowly than control DNA, suggesting a full-length DNA fragment with a chromophore molecule covalently attached at some point in the fragment; this band was intensified in the sample treated anaerobically (lane 3A).

Examination of data from experiments with several restriction fragments revealed that bands resulting from exonuclease termination sites in chromophore-treated DNA samples consistently appeared to migrate the equivalent of five nucleotides more slowly than bands resulting from chromophore-induced strand breaks. This pattern was most clearly seen at very prominent and well-separated cleavage sites, such as position T_{30} in the fragment shown. Corresponding to the intense strand-break band at T_{30} of the sample treated aerobically (lane 2A), an intense band, presumably representing termination of exonuclease digestion at the site of an adduct, appeared in the anaerobically treated sample near position C_{35} (lane 3C, arrow). Likewise, the less prominent strand-break band at position T_{27} can be paired with a less intense putative adduct band near position G_{32}, and minor strand-break bands at positions A_{38}, A_{41}, and T_{45} can be paired with minor adduct bands near positions 43, 46, and 50, respectively (interestingly, the single C residue at which cleavage occurs, C_{43}, shows no corresponding adduct band). These results suggest an oxygen-dependent competition between strand breakage and adduct formation, at the same sites (primarily T and A residues) in DNA.

When DNA treated anaerobically with chromophore was digested with exonuclease III followed by venom exonuclease, the adduct band near position 35 was split into a doublet near positions 35 and 34 [although a minor band near position 34 also appears in the undigested DNA sample (lane 3A), it is clear from comparison of lanes 2D and 3A that it does not comigrate with the lower band of the doublet]. Similar results were seen at several other prominent adduct sites. These results suggest slow removal of one additional nucleotide near the adduct site by venom exonuclease (see Figure 4). Since studies with various isolated chromophore–DNA adducts show that this enzyme can remove the nucleotide directly adjacent to the adduct site (Povirk & Goldberg, 1982b, 1984), it appears that exonuclease III and T4 polymerase terminate di-

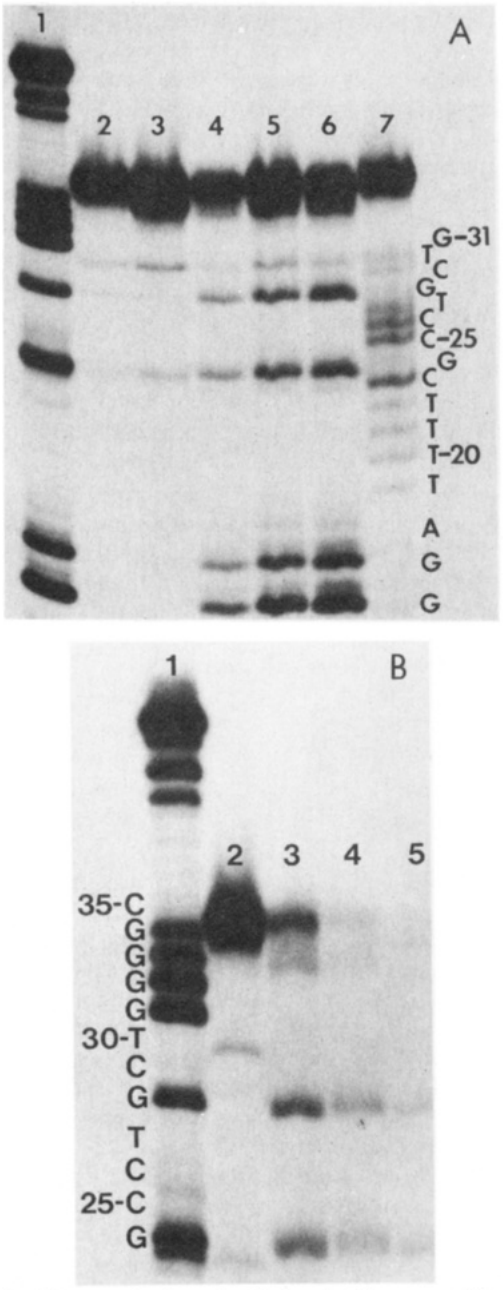

FIGURE 3: Characterization of an isolated adduct-containing oligonucleotide. A 5′-end-labeled fragment containing the first 46 bp of the 66-bp fragment was treated with chromophore (120 μM) anaerobically in the presence of dithiothreitol, and the DNA was digested with exonuclease III and T4 polymerase. The putative adduct band migrating near the position of C_{35} (see Figure 2) was isolated and subjected to the Maxam–Gilbert reactions for base-specific chemical cleavage of DNA. Lane 1 is the 46-bp fragment treated with the Maxam–Gilbert G reaction (dimethyl sulfate plus piperidine). In (A) lanes 2–7 are the isolated adduct band subjected to the following treatments: (2) piperidine alone; (3) the G+A reaction (pH 2 plus piperidine); (4) the G reaction; (5) the G>A reaction (dimethyl sulfate plus heat plus piperidine); (6) the G>A reaction, but with a longer dimethyl sulfate treatment; (7) the C+T reaction (hydrazine plus piperidine). In (B) lane 2 is the adduct band treated with piperidine alone, and lanes 3–5 are the adduct band subjected to modified G reactions consisting of increasingly vigorous 20-min treatments with 1%, 2%, or 4% dimethyl sulfate, followed by piperidine. Electrophoresis was 9 h at 1000 V in (A) and 14 h at 1000 V in (B).

gestion one nucleotide before the adduct site. In retrospect, then, it is not surprising that bands representing exonuclease termination at adduct sites migrate the equivalent of five nucleotides more slowly than the corresponding strand break bands. The 5′-end-labeled fragments generated by strand

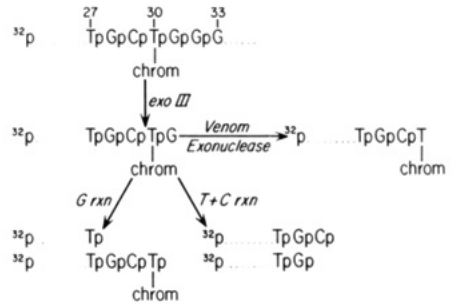

FIGURE 4: Model for the behavior of adduct-containing DNA. Digestion of DNA from the 3′ end by exonuclease III (or exonuclease III plus T4 polymerase) is blocked at G_{31}, one nucleotide before the adduct at T_{30}. The resulting 31 base long adduct containing 3′-hydroxyl-ended oligonucleotide migrates near position C_{35} on the sequencing gel. Treatment of this oligonucleotide with the G or C+T chemical cleavage reaction results in elimination of the appropriate base and sugar moieties. Unmodified oligonucleotides are produced by sequencing reactions at positions 1–30, giving a normal sequencing ladder, but elimination of the G_{31} base and sugar does not remove the adduct, and the resulting oligonucleotide migrates near position G_{34} on the sequencing gel, just ahead of the starting material. Snake venom exonuclease also produces a band migrating just ahead of the starting material, by removing G_{31} enzymatically.

breaks have lost completely the nucleotide at which the break occurs (e.g., T_{30}, as illustrated in Figure 4), while fragments associated with the adduct bands are two nucleotides longer, retaining this nucleotide as well as one additional nucleotide (e.g., G_{31}) on the 3′ side. Furthermore, the enzyme-digested fragments have a 3′-hydroxyl rather than a 3′-phosphate end, which decreases their mobility slightly. Thus, a decrease in mobility of only about 2.5 to 3 nucleotide positions is attributable to the presence of the chromophore.

In order to examine the sites of adduct formation more directly, the putative adduct band migrating near position 35 was cut out from the gel, and the DNA was isolated and subjected to the chemical cleavage reactions described by Maxam & Gilbert (1980). The reactions for cleavage at C+T residues gave a normal sequencing ladder up to and including T_{30} (Figure 3A). The reaction for cleavage at G residues (dimethyl sulfate plus piperidine) gave a normal ladder up to G_{28}, but the bands corresponding to G_{31} through at least G_{34} were absent. Instead, a single shadow band appeared just below the original adduct band, which was not present in samples treated with the C+T reaction, or with piperidine alone (the band generated by piperidine alone, appearing between positions C_{29} and T_{30}, is of unknown origin). By increasing the electrophoresis time, the shadow band could be clearly separated from from the starting material (Figure 3B). Increasing the extent of dimethyl sulfate treatment increased the relative intensity of the shadow band, compared to the band of starting material, suggesting that it resulted from methylation and removal of a G residue.

The most reasonable interpretation of these results is shown schematically in Figure 4. The site of adduct formation is at position T_{30}, probably on the deoxyribose moiety (see Discussion). Digestion with exonuclease III or T4 polymerase is blocked at position G_{31}, giving the 31-base-long, adduct-containing fragment that migrates near position 35 on the gel. A normal hydrazine-plus-piperidine cleavage reaction occurs at T_{30}, in spite of its having a covalently attached chromophore; the thymine base and sugar are removed, giving a normal band in the sequencing ladder at this position. Likewise, a normal dimethyl sulfate plus piperidine reaction occurs at G_{31}, and the guanine base and sugar are removed, but the adduct at T_{30} remains attached; the resulting fragment migrates only slightly faster than the starting material, resulting in the

shadow band, but no band appears at the normal G_{31} position. Strictly speaking, the results do not exclude the possibility that the adduct is on the sugar of the G_{31} residue, and the adduct is retained even after removal of the guanine base and cleavage of the phosphodiester backbone only on the 3′ side of the modified sugar. However, the fact that venom exonuclease can remove an additional nucleotide, presumably G_{31}, from the exonuclease III digestion product strongly suggests that the site of adduct formation is T_{30}, not G_{31}. By analogy, it is likely that all putative adduct bands, which migrate five nucleotides more slowly than bands generated by chromophore-induced strand breaks at T and A residues, result from formation of adducts at the same T and A residues.

As noted above, there was a strong correlation between the intensities of individual adduct bands and those of the corresponding strand break bands, both in the fragment shown and in several other restriction fragments. T residues in the trinucleotide sequences ATG, CTG, and ATC were particularly prominent sites of both strand breakage and adduct formation (data not shown). One fragment examined contained *lacI* sequences corresponding to two previously reported hot spots for neocarzinostatin-induced mutagenesis in vivo, i.e., the ochre 21 and amber 19 mutations resulting from G·C to A·T transitions at base pairs 566 and 569 of the gene, respectively (Coulondre & Miller, 1977). Although no adducts were detectable at these or any other G·C base pairs, T residues in the noncoding strand of the A·T residues directly adjacent (base pairs 567 and 570) were prominent adduct sites. Involvement of these lesions in neocarzinostatin-induced base substitution mutagenesis at the adjacent base pair cannot be excluded, although it seems unlikely.

When glutathione, the most likely in vivo cofactor for activation of neocarzinostatin, was substituted for dithiothreitol, a similar pattern of exonuclease-resistant sites was observed. However, the bands observed were less prominent and more diffuse and migrated even more slowly than those seen with dithiothreitol (data not shown). The putative adduct band seen in samples of chromophore-treated, undigested DNA (migrating more slowly than full-length DNA) also showed a greater retardation in mobility when glutathione was used. These results suggest that adducts are formed at the same sites in DNA with the two reducing agents but that somewhat different forms of the chromophore are present in the adducts formed; this may be due in part to the covalent linkage of the sulfhydryl to the chromophore in the initial activation reaction [see Povirk & Goldberg (1983)].

Digestion of Chromophore-Treated DNA with 5′→3′-Exonucleases. A shorter DNA fragment containing bases 1–49 of the same sequence was labeled at the 3′ end, treated with chromophore, and digested with λ-exonuclease, or with the gene 6 exonuclease of phage T7 (generous gift of J. White and C. C. Richardson). These enzymes release nucleoside 5′-phosphates from the 5′ ends of double-stranded DNA (Kornberg, 1980). Although partial digestion products of various lengths could be generated by treatment with either enzyme, chromophore-treated DNA was no more resistant to digestion than normal DNA, and no chromophore-dependent bands such as those seen with exonuclease III were detected on sequencing gels (data not shown). Thus, chromophore adducts apparently do not act as significant blocks to the action of these enzymes.

The same 3′-end-labeled fragment was dephosphorylated, treated anaerobically with chromophore, denatured, and digested with spleen phosphodiesterase. This enzyme acts as an exonuclease at the 5′-hydroxyl ends of single-stranded DNA, releasing nucleoside 3′-phosphates (Kornberg, 1980). High concentrations of spleen phosphodiesterase digested both chromophore-treated and untreated DNA to completion. However, under conditions where DNA was partially digested, sequencing gels showed chromophore-dependent bands that did not comigrate with any of the partial digestion products of untreated DNA (not shown). The most prominent chromophore-dependent band migrated the equivalent of 6.5 nucleotides more slowly than the prominent strand-break band at position T_{30} (numbering from the 5′ end as in Figure 2). This result is consistent with termination of exonuclease digestion near the prominent chromophore adduct site at position T_{30}, giving a fragment analogous to that generated by exonuclease III. The mobility of this fragment is presumably retarded by the presence of both the covalently bound chromophore and two to three nucleotides adjacent to the adduct site that were resistant to removal by the enzyme.

Discussion

Although the majority of neocarzinostatin-induced lesions in DNA are of a single type, i.e., strand breaks resulting from specific oxidation of DNA sugar moieties to form nucleoside 5′-aldehydes (Kappen & Goldberg, 1983), a complex spectrum of minor lesions is also present, including various types of adducts between the chromophore and DNA (Povirk & Goldberg, 1982a,b, 1984). Both the extent of adduct formation and the nature of the adducts formed are dependent on the specific sulfhydryl cofactor used for chromophore activation. Use of dithiothreitol maximizes adduct formation, and several very stable adduct species are formed. One of these, the major adduct species found in chromophore-treated poly(dA-dT)·poly(dA-dT), has been isolated and characterized in detail (Povirk & Goldberg, 1984). The site of covalent linkage was clearly shown to be the sugar moiety of deoxyadenosine. The methodology employed in these studies, i.e., extensive endonucleolytic digestion of chromophore-treated poly(dA-dT)·poly(dA-dT) followed by isolation of individual adduct species by high-pressure liquid chromatography, proved ineffective when natural DNA substrates were used; the distribution of products was so complex that no single adduct species could be resolved.

In the present study, an alternative approach has been used, i.e., examination of adducts in defined-sequence DNA, using modifications of DNA sequencing techniques. As in previous studies (Povirk & Goldberg, 1984), a competition was seen between direct strand breakage, which is almost completely oxygen dependent, and adduct formation, which is maximized under anoxia. This competition suggests that the two lesions share a common precursor. In addition, the detailed sequence specificity of adduct formation was found to be very similar to that of strand breakage, in terms of both the general preference for T and A residues and the relative prominence of individual T and A sites. This similarity in specificity suggests that the same activated chromophore species is responsible for the generation of both lesions and that the divergence in the pathways leading to strand breakage and to adduct formation occurs after the production of some common form of nascent DNA damage. The recent demonstrations of (i) transfer of ^3H label from [5′-^3H]thymidine-labeled DNA into a nonexchangeable site on the chromophore, under both aerobic and anaerobic conditions (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985), and (ii) incorporation of ^{18}O from dioxygen into chromophore-induced thymidine 5′-aldehyde (Chin et al., 1984) suggest a chromophore-induced free radical on the C-5′ position of deoxyribose as the most logical possibility for the postulated DNA intermediate. Such a deox-

yribose free radical would be expected to react rapidly by addition with molecular oxygen, leading eventually to formation of the 5′-aldehyde, but under anoxia could react by addition with the chromophore, forming a stable adduct. Thus, although the adducts observed as exonuclease-resistant sites in defined-sequence DNA have not been chemically characterized in detail, it appears highly likely that they are analogous in structure to the major adduct species formed under similar conditions in poly(dA-dT)·poly(dA-dT), involving a stable covalent linkage of the chromophore to deoxyribose in DNA, probably at the C-5′ position.

Essentially nothing is known of the possible biological consequences of neocarzinostatin chromophore–DNA adducts. It is believed that chromophore-induced strand breaks are responsible for the lethal effects of the drug. However, in contrast to the preference for T and A residues seen in chromophore-induced strand breakage (Hatayama et al., 1978; D'Andrea & Haseltine, 1978), the mutational spectrum of neocarzinostatin in the *lacI* gene of *E. coli* shows a proportion of G·C to A·T transitions, suggesting that mutagenesis results from a lesion, other than strand breaks, which is formed with significant frequency at G or C residues. The present study was conducted in part to determine whether chromophore–DNA adducts exhibited such a specificity. The observed occurrence of adducts almost exclusively at T and A residues does not suggest a role for adducts in neocarzinostatin-induced mutagenesis. Since chromophore adducts involve modification of DNA sugars, rather than bases, it is possible that normal base pairing can still occur during replication and that little or no base substitution mutagenesis occurs at adduct sites. One hypothesis that cannot at present be excluded, however, is that adducts on T or A residues may result in mutations at adjacent G·C base pairs. In fact, the T residues (in the noncoding strand) directly preceding the G residues associated with two G·C to A·T transition hot spots (ochre 21 and amber 19) were found to be prominent sites of adduct formation. On the other hand, the neocarzinostatin chromophore is an intercalator (Povirk et al., 1981), and other covalently bound intercalators, such as the acridine mustards (Creech et al., 1972), are potent frameshift mutagens. Thus, adducts may be involved in neocarzinostatin-induced frameshift mutagenesis (Eisenstadt et al., 1980), rather than base substitution mutagenesis. The sequence specificity of neocarzinostatin-induced frameshifts is unknown.

Recently, we have detected alkali-labile sites in DNA treated with neocarzinostatin chromophore in the presence of glutathione (Povirk & Goldberg, 1985). This lesion is selectively formed at C residues and appears to be some form of apyrimidinic site, but different in structure from heat-induced apurinic sites. Since this lesion was detected at several sites in *lacI* corresponding to G·C to A·T transition hot spots, it now appears a more likely candidate for producing base substitution mutations, especially G·C to A·T transitions. The mutagenicity of apurinic sites, in single-stranded phage DNA, is well established (Schaaper et al., 1982).

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