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# Effects of Neocarzinostatin on Chromatin in HeLa $S_3$ Nuclei

TERRY A. BEERMAN, GAIL MUELLER, AND HYRANNE GRIMMOND

*Department of Experimental Therapeutics and Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York 14263*

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SUMMARY

Neocarzinostatin solubilizes chromatin from HeLa $S_3$ nuclei by introducing strand scissions in linker regions. Multimeric nucleosome patterns are seen on both native and denaturing gel analysis. The mechanism of drug action differs from the type of chromatin digestion seen with micrococcal enzyme in that DNA damage occurs through single-strand breaks and less acid-soluble material is produced. In addition, drug-induced release of soluble chromatin from the nuclei is not very dependent upon the addition of EDTA. The monomer repeat size is larger than that found for micrococcal enzyme and contains linker regions that are partially single-stranded. Core histone proteins as well as histone $H_1$ do not appear to be altered by drug action, although there is clear evidence that DNA damage can occur in nucleosome cores. The chromophore portion of the drug degrades chromatin as effectively as the holoantibiotic.

## INTRODUCTION

Neocarzinostatin is an antitumor agent whose cytotoxic activity seems to involve drug-induced damage to DNA (1). Structurally the drug consists of a 10,700-dalton protein to which a small chromophore is hydrophobically bound (2, 3). The drug's main biological activities, cleavage of cellular and cell free DNA as well as inhibition of cell growth, appear to reside with the chromophore, whereas the protein provides stability (3, 4).

Early studies have characterized in some detail the mechanism of drug-induced DNA damage in cell-free systems (5, 6). Strand scissions are predominantly single-stranded and occur preferentially at thymine (6, 7). The reaction is stimulated by mercaptans and can be inhibited by the absence of oxygen (8). Both single- and double-stranded DNA serve as targets while RNA appears to be resistant to damage (9). Goldberg and collaborators (10) have been able to postulate much of the molecular mechanism of how the chromophore is able to cleave cell-free DNA. In addition, there is abundant evidence relating cellular DNA damage to both the inhibition of DNA synthesis and cell growth (1, 9).

Given the strong relationship between drug-induced damage to DNA and cytotoxicity, it is important to understand how neocarzinostatin alters its cellular DNA target, chromatin. In an earlier paper we stated that neocarzinostatin or its chromophore form caused a disruption of the chromatin repeat structure (11). Experiments with high concentrations of drug suggested that damage to chromatin occurred at both linker and core sites as seen by intranucleosomal spaces being filled in. Drug treatment of nuclei resulted in solubilized chromatin, although no attempt was made to characterize this process.

This paper continues with these studies and shows that the release of chromatin from nuclei is very different for neocarzinostatin and micrococcal nuclease. The chromatin repeat unit in neocarzinostatin-digested chromatin was found to be larger than that produced by enzymatic digestion. In addition, neocarzinostatin cleaves core DNA, although the sites of damage were different than those produced by DNase I (12).

## MATERIALS AND METHODS

Neocarzinostatin (NCS-157365) was provided by the Investigational Drug Branch of the National Institute of Cancer. Before use, it was dialyzed against 10 mM Tris (pH 7.8) and concentrated in a negative-pressure microprotein dialysis concentrator. Chromophore was extracted from lyopholized neocarzinostatin as described elsewhere (11). The concentration of chromophore was expressed in microgram equivalents of native neocarzinostatin per milliliter. Activity of the chromophore was matched to that of the holoantibiotic by measuring the amount of cell-free DNA endonucleolytic activity (11). Micrococcal nuclease was obtained from Bohringer Mannheim (Indianapolis, Ind.). DNase I enzyme was obtained from Sigma Chemical Company (St. Louis, Mo.), and S1 nuclease was from Miles Laboratories (Elkhart, Ind.). All of the chemicals were reagent-grade.

*Radiochemicals.* [*methyl*-$^{14}$C]-Thymidine (51.0 mCi/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.).

*Cell culture.* HeLa $S_3$ cells were grown in Spinner culture in Joklik's medium supplemented with 5% calf

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serum. Cells were maintained at a density of $3.0 \times 10^5$ cells/ml and were labeled overnight with [$^{14}$C]thymidine (0.01 μCi/ml). Nuclei from these cells were isolated as described elsewhere and were resuspended in 10 mM Tris-HCl (pH 7.8)/1 mM $CaCl_2$ (13). The amount of DNA present was measured by its absorbance in 0.1 N NaOH at 260 nm in a Perkin-Elmer *lambda* 3 UV/VIS spectrophotometer. Counts per minute per microgram were determined by adding an aliquot of nuclei to 0.1 N NaOH, neutralizing with 1.0 N HCl, and counting in a Beckman LS 7000.

*Native gels.* Nuclei were incubated in a mixture containing drug or micrococcal enzyme, 10 mM Tris, 1 mM $CaCl_2$, and, unless otherwise noted, 2 mM dithiothreitol and 6.1 μg of DNA in a total volume of 100 μl. To samples containing micrococcal enzyme, dithiothreitol was added after the incubation; whereas in those containing drug, dithiothreitol was present during the incubation. After incubating for the appropriate time at 37°, EDTA was added, except where indicated, to a final concentration of 1 mM. Samples were placed on ice for 10 min and then centrifuged in an Eppendorf Microfuge for 5 min. An aliquot of the supernatant was counted as above to determine DNA concentration. Supernatants containing 1.5 μg of DNA were then treated with sodium dodecyl sulfate, run on native 4% polyacrylamide gels at 30 V for 14 hr, stained, and photographed as described previously (11).

*Determination of acid-soluble material.* The acid-soluble data were obtained by removing an aliquot from each of the incubation mixtures at the end of the incubation periods but just prior to the addition of EDTA. Each sample withdrawn was added to a volume of cold 1.0 M perchloric acid/1.0 M NaCl equal to 9 times that of the aliquot. The samples were incubated on ice for 30 min followed by centrifugation in an Eppendorf Microfuge. The supernatants were added to a xylene/Triton X-100/2,5-diphenyloxazole (PPO)/1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) mixture (xylene, 3 liters; Triton X-100, 1 liter; PPO, 12 g; POPOP, 0.8 g) and the radioactivity was counted.

*Isolation of mononucleosomes.* Mononucleosomes were obtained by incubating nuclei with micrococcal nuclease as described above. EDTA was then added and the samples were placed on ice for 10 min. They were then centrifuged in a Microfuge, and a volume of the supernatant containing the equivalent of 100 μg of DNA was layered onto a gradient of 5–20% sucrose/1 mM EDTA. It was then centrifuged in an SW 41 rotor at 34,000 rpm for 20 hr at 4° in a Beckman L2 65B ultracentrifuge. The gradient was fractionated using a Buchler Instruments Autodensi-Flow IIC and the fractions were counted to determine the position of the mononucleosome peak. Mononucleosomes were concentrated in a negative-pressure microprotein dialysis concentrator against 1 mM EDTA.

*Denaturing gel.* Incubation of nuclei was carried out as described above, and the isolated chromatin was treated with 0.2% sodium dodecyl sulfate/0.1 M NaCl to a final volume of 250 μl. Samples were incubated for 1 hr at 37° with 3 μg of proteinase K. They were then extracted with 24:1 chloroform/isoamyl alcohol and ethanol-precipitated. Samples were centrifuged in a Microfuge for 10 min and the pellet was resuspended in 8 M urea and heated at 90° for 5 min. Bromophenol blue and xylenecyanole FF dyes were added as markers, and samples were loaded on a 6% polyacrylamide [acrylamide/bisacrylamide (19:1)]/6.5 M urea gel. The running buffer was 10 mM Tris-borate/1 mM EDTA (pH 8.3), and the gel was run for 20 hr at 40 V. The gel was stained in ethidium bromide (0.4 μg/ml) and photographed as described above.

*DNase I analysis.* Mononucleosomes were incubated in 6 mM $MgCl_2$/5.3 mM NaAC (pH 6.5) for 5 min at 37°. A microgram amount of DNAse I equal to that of the mononucleosomes (12 μg of DNase I) was then added and the incubation was continued for 10 min. In a separate reaction mixture, drug was added to mononucleosomes (7.5 μg) in the presence of 2 mM dithiothreitol for 30 min at 37°, then incubated in 6 mM $MgCl_2$/5.3 mM NaAc (pH 6.5) for 5 min at 37°. Both samples were then made to 1% sodium dodecyl sulfate/10 mM EDTA/10 mM Tris (pH 8.0)/100 mM NaCl, incubated with 3 μg of proteinase K for 1 hr at 37°, extracted with chloroform/isoamyl alcohol, and ethanol-precipitated. The pellets were incubated in 8 M urea at 90° for 5 min. Marker dye was added and the samples were loaded on an 11.2% polyacrylamide [acrylamide/bisacrylamide (19:1)]/6.5 M urea gel, which was run in 9 mM Tris-borate/0.25 mM EDTA (pH 8.3) for 16 hr at 50 V. Gels were stained and photographed as previously described for denaturing gels.

*Agarose gel electrophoretic analysis of S1 nuclease-treated nucleosomes.* Native gel analysis for S1-treated mononucleosomes was performed on 1.5% agarose gels. Essentially, after S1 treatment, samples were stripped of proteins by incubation at 55° in the presence of 0.1% sodium dodecyl sulfate prior to analysis on a 1.5% agarose gel consisting of 50 mM Tris (pH 8.0)/2 mM NaAc/2 mM EDTA. Electrophoresis was done on a submersion gel run for 24 hr at 30 V. Samples were stained with ethidium bromide (0.15 μg/ml) and photographed as described elsewhere (11).

## RESULTS

Digestion of HeLa $S_3$ nuclei under moderate incubation conditions with micrococcal nuclease produces the classical oligomeric forms of chromatin repeat units (Fig. 1A) (14). More severe digestion produces further breakdown to lower oligomeric forms and more highly trimmed monomers (Fig. 1B). A representative profile of soluble chromatin obtained from neocarzinostatin-treated nuclei is shown in the same figure (C–F). Control samples in the presence or absence of dithiothreitol (dithiothreitol is required for the DNA strand scission activity of the drug) produced no 260 nm absorbing material. Treatment of nuclei at a drug concentration of 100 μg/ml resulted in a substantial increase of 260 nm absorbing material, which was of relatively high molecular weight and gave no discrete bands on agarose gels (Fig. 1C). Greater levels of drug resulted in increased amounts of 260 nm absorbing material, which contained discrete oligo- and mononucleosome structures (Fig. 1D and E). The appearance of discrete nucleosome repeat units suggests that, at least

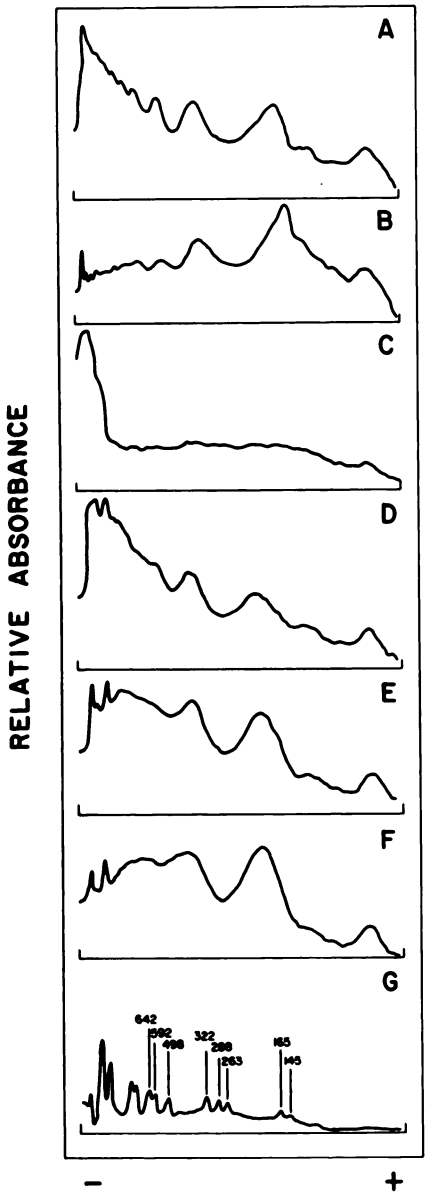


FIG. 1. *Scans of photographic negatives of samples incubated with micrococcal nuclease or neocarzinostatin and separated by native gel electrophoresis*

A, HeLa cell nuclei incubated with 1.83 units of micrococcal nuclease for 20 min; B, nuclei plus 18.3 units of nuclease for 10 min; C–F, nuclei incubated with neocarzinostatin at 100 μg/ml, 500 μg/ml, 1000 μg/ml, and 2000 μg/ml, respectively, for 30 min; G, Hae III digest of PM2 DNA. Control samples with and without dithiothreitol did not release detectable amounts of chromatin.

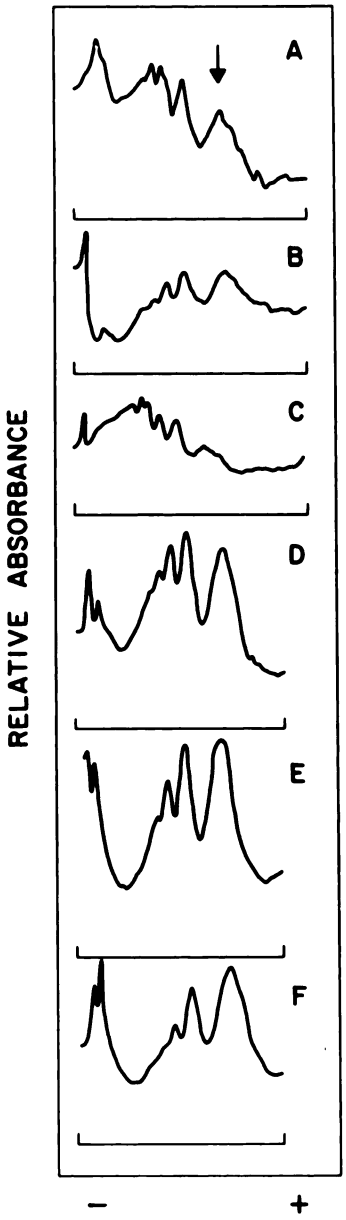


FIG. 2. *Scans of photographic negatives of samples incubated with micrococcal nuclease or neocarzinostatin and separated by denaturing gel electrophoresis*

A, HeLa cell nuclei incubated with 1.83 units of micrococcal nuclease for 20 min; B, nuclei plus 18.3 units of micrococcal nuclease for 10 min; C–F, nuclei incubated with neocarzinostatin at 100 μg/ml, 500 μg/ml, 1000 μg/ml, and 2000 μg/ml, respectively, for 30 min. Control samples with and without dithiothreitol did not release detectable amounts of chromatin. The *arrow* indicates the position of a 165-base pair band.

for early digestion of nuclei by neocarzinostatin, damage is occurring at linker regions. Unlike micrococcal nuclease, where more severe digestion results in further trimming of mononucleosomes linker regions (Fig. 1A and B), increased drug concentrations (while producing more lower molecular weight chromatin) do not further reduce the size of the mononucleosomes (Fig. 1D–F).

Cell-free endonucleolytic activity of neocarzinostatin produces mainly single-strand scissions in DNA (1). Our analysis of chromatin degradation, done under nondenaturing conditions, detects double-strand scissions. The digestion of the chromatin could be the result of true double-strand scissions. More likely, chromatin degradation results from two single-strand scissions occurring in close juxtaposition on opposite DNA strands. Measurement of single-strand scissions in the soluble chromatin was determined by analysis on denaturing acrylamide/urea gels. Chromatin from micrococcal nuclease (cleaves DNA by double-strand scissions)-treated nuclei resembles closely the nucleosome pattern seen on the native gel (Fig. 2A and B). On the other hand, for

TABLE 1

*Comparison of the digestion profile of HeLa $S_3$ nuclear chromatin by micrococcal enzyme and neocarzinostatin*

HeLa nuclei were incubated with enzyme or drug under the conditions listed. Percentages of acid-soluble material and soluble chromatin were measured as described under Materials and Methods. Nucleosome sizes were calculated from acrylamide gel analysis using an HAE III PM2 digest for size markers. Experiments were performed several times, and the averages are presented.

| Incubation agent and concentration | Incubation time | % Acid-soluble | % cpm in supernatant | | Monomer size (base pairs) ± 5 | Dimer size (base pairs) ± 9 |
|---|---|---|---|---|---|---|
| | | | −EDTA | +EDTA | | |
| | *min* | | | | | |
| Micrococcal enzyme (units/ml) | | | | | | |
| 1.83 | 2 | 0.5 | — | — | — | — |
| 1.83 | 10 | 2.0 | 11.4 | 3.43 | 165 | — |
| 1.83 | 20 | 4.5 | 28.5 | 67.6 | 155 | 335 |
| 18.3 | 10 | 21.0 | 29.5 | 69.5 | 143 | 319 |
| Neocarzinostatin (μg/ml) | | | | | | |
| 100 | 30 | 0.7 | 8.3 | 15.8 | — | — |
| 500 | 30 | 1.5 | 19.5 | 33.8 | 177 | 348 |
| 1000 | 30 | 2.8 | 49.5 | 70.0 | 180 | 350 |
| 2000 | 30 | 4.9 | 47.3 | 71.0 | 179 | 365 |

neocarzinostatin there was an increase in the amount of low molecular weight oligomeric nucleosomes at drug levels which produced no such material when analyzed on native gels (compare neocarzinostatin in 100 μg/ml in Figs. 1C and 2C). HeLa cell DNA treated with drug and analyzed under both native and denaturing conditions produced smeared electrophoretic patterns (data not shown). The digestion of nuclear chromatin to multimeric units is dependent upon chromatin structure.

Production of micrococcal nuclease-generated acid-soluble material (DNA less than 10–20 base pairs) and the accompanying increased solubilization of chromatin from digested nuclei are presented in Table 1. Micrococcal nuclease solubilization of chromatin from nuclei is stimulated by the presence of EDTA. Chromatin released in the absence of EDTA is thought to be enriched in actively transcribing material (15). The ratio of the percentage of chromatin released in the presence and absence of EDTA ranged from 2.4 to 3.0 (Table 1). For neocarzinostatin, there was a significant release of soluble chromatin in the absence of EDTA (Table 1). The ratio of EDTA-solubilized chromatin to material isolated in the absence of EDTA ranged from 1.4 to 1.9. Under conditions where micrococcal nuclease and neocarzinostatin solubilize similar amounts of chromatin, there was much less acid-soluble DNA produced by the drug. Also, the chromatin solubilized by neocarzinostatin contains a higher percentage of smaller oligomeric sizes (Fig. 1). The sizes of the monomer and dimer peaks from Fig. 1 are listed in Table 1. Soluble chromatin from drug-treated nuclei has a repeat length greater than enzyme-solubilized chromatin. There was no evidence that further digestion by drug affected the repeat size.

Neocarzinostatin solubilizes chromatin by producing single-strand scissions in linker DNA regions. The large-size monomer repeat unit could be attributed to the presence of single-strand tails on the linker regions. To test this hypothesis we first purified fractions of micrococcal enzyme and drug-solubilized mononucleosomes on sucrose gradients. The fractions used for further analysis were 165 base pair mononucleosomes from the micrococcal-solubilized chromatin and 174 base pair size units from drug-solubilized chromatin. As expected, mononucleosomes from the micrococcal digest were not affected by S1 nuclease, an enzyme that digests single-strand DNA (16) (Table 2). The size of the mononucleosomes from the neocarzinostatin digests were reduced by treatment with S1 nuclease, indicating a trimming of single-strand DNA from linker regions. The decrease in size was limited, and the mononucleosomes never were reduced to the size of the micrococcal repeat length.

The endonucleolytic activity of neocarzinostatin as well as its other biological activities resides with the chromophore portion of the drug. Experiments were done to compare the nature of chromatin digestion with intact drug and isolated chromophore. At equal levels of DNA strand scission activity (see Materials and Methods), both drug forms had a similar effect on nuclear chromatin (Fig. 3). The electrophoretic pattern of the oligomeric units seemed quite comparable, and there was a similar dependence on dithiothreitol for maximal endonucleolytic activity (data not shown).

The data presented to this point address the nature of drug action on the DNA linker regions of chromatin. An

TABLE 2

*Effects of S1 nuclease on micrococcal nuclease and neocarzinostatin-generated mononucleosomes*

Mononucleosomes were collected from sucrose gradients as described under Materials and Methods. Incubations were done in a total volume of 25 μl, which consisted of 0.75 μg of the appropriate mononucleosome, and a final concentration of 1 mM $ZnCl_2$, 30 mM NaAc (pH 4.6), 50 mM NaCl, 5% glycerol, and S1 nuclease at the indicated level. The reaction was terminated by addition of EDTA, and the samples were analyzed by agarose gel electrophoresis as described under Materials and Methods. The data presented are an average of three experiments.

| S1 nuclease treatment | Micrococcal mononucleosomes (base pairs ± 2) | Neocarzinostatin mononucleosomes (base pairs ± 2) |
|---|---|---|
| *units/ml* | | |
| 0 | 165 | 174 |
| 1.65 | 164 | 170 |
| 3.30 | 164 | 170 |
| 9.90 | 165 | 170 |

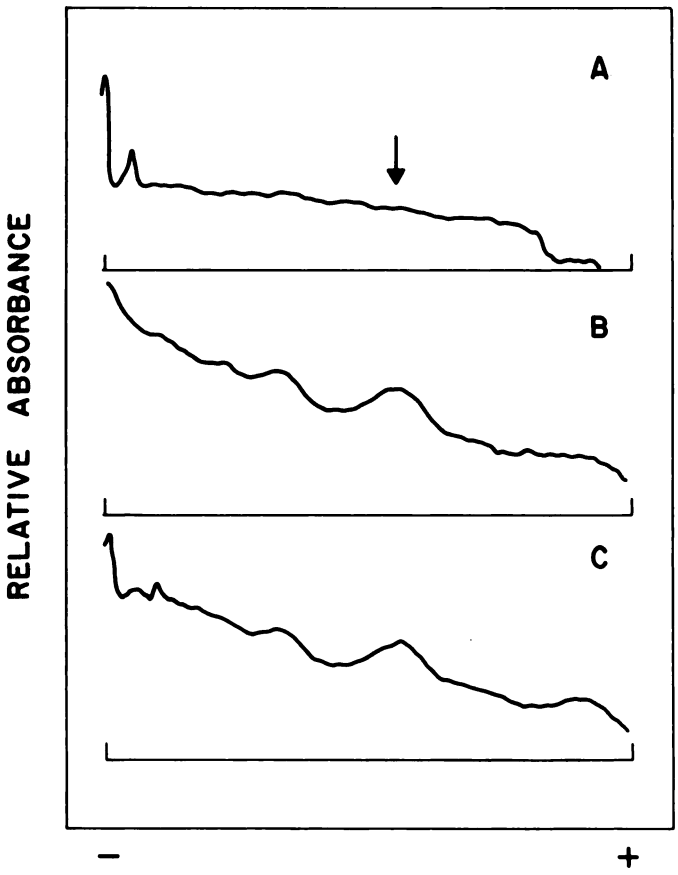


FIG. 3. *Degradation of nuclear chromatin by neocarzinostatin and its chromophore*

The experimental procedure was done essentially as described in Fig. 1. Chromophore was isolated and characterized as described under Materials and Methods. Samples: A, control; B, neocarzinostatin at 1000 μg/ml; C, chromophore at 1000 μg/ml. A control was also run with the equivalent amount of methanol used in the chromophore incubation and showed no change from the control sample in A. The *arrow* indicates the position of a 180-base pair band.

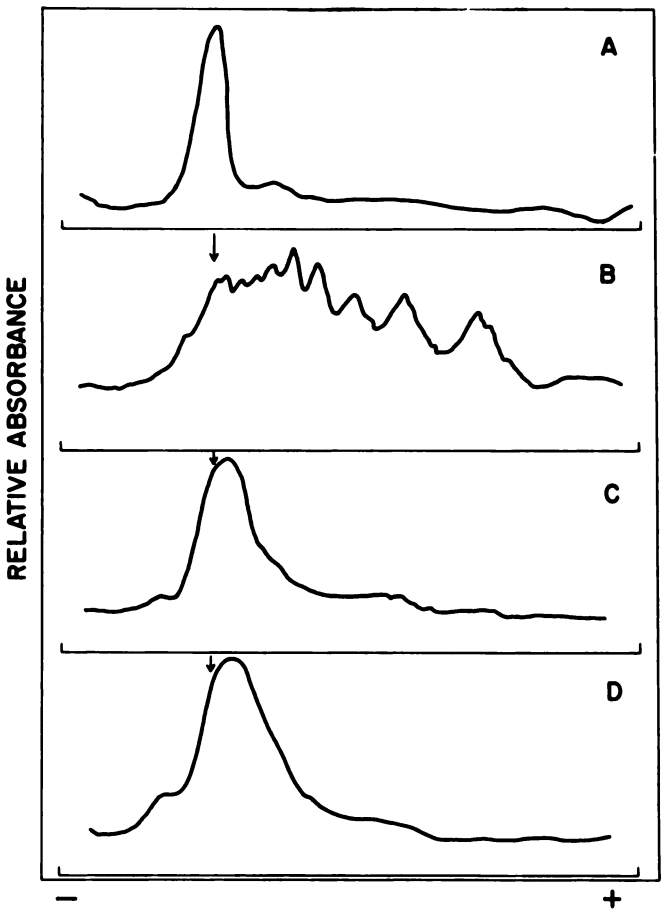


FIG. 4. *Degradation of mononucleosomes by neocarzinostatin: denaturing gels*

Mononucleosomes were purified from HeLa $S_2$ chromatin as described under Materials and Methods. Samples were then treated with other neocarzinostatin or enzyme and analyzed on acrylamide/urea gels as described under Materials and Methods. A, control; B, DNase I; C and D, neocarzinostatin at 1.0 and 7.5 mg/ml. The *arrows* indicate the position of the control mononucleosome peak.

earlier investigation gave evidence that neocarzinostatin can also cleave intranucleosomal regions of isolated chromatin (11). Treatment of isolated mononucleosomes with drug produces clear evidence that DNA damage can occur in DNA core sites (Fig. 4). The DNA strand scission pattern is quite different from that seen with DNase I (Fig. 4B), which cleaves nucleosomal DNA at regular intervals of 10.4 base pairs (12). The damage to core DNA occurs at random sites, as seen by the gradual broadening of the mononucleosome band with increasing levels of drug (Fig. 4C and D). Neocarzinostatin digestion of core DNA can be seen at even lower drug levels if the amount of DNA is reduced.

## DISCUSSION

Neocarzinostatin degrades nuclear chromatin by a mechanism quite different from that of the classical probe used to study chromatin structure, micrococcal nuclease. This enzyme degrades chromatin by placing double-strand scissions in linker DNA (14). As digestion proceeds, increasing amounts of acid-soluble DNA are produced from further enzyme action on the tail regions of the nucleosomes. Although neocarzinostatin also preferentially cleaves chromatin at linker DNA regions, it releases little acid-soluble DNA. This is consistent with the observation that the nucleosome repeat sizes (177–180 base pairs) of drug solubilized monomer or dimer chromatin remain constant even at higher concentrations of drug. Any trimming of DNA tails from small-sized nucleosomes would have a substantial effect on the observed repeat length.

These data suggest that neocarzinostatin solubilizes chromatin by placement of single-strand scissions in the DNA of linker regions. We confirmed this hypothesis by comparing the degree of breakdown of drug-solubilized chromatin on native and denaturing electrophoresis gels (Figs. 1 and 3). From these data we estimate that the ratio of drug-induced single- to double-strand scissions is about 5:1. An earlier study reports that neocarzinostatin causes equal amounts of single- and double-strand scissions in chromatin solubilized from CHO nuclei (17). Our data are consistent with those of a very recent study in which neocarzinostatin treatment of rat liver nuclei resulted in a 7:1 ratio of single- to double-strand scissions

(18). There is no obvious explanation of the difference between these results. The data obtained with CHO nuclei could be influenced by the fact that it required very high levels of drug. Chromatin digestion comparable to our data with HeLa $S_3$ nuclei (500 μg/ml of drug for 60 min) required roughly 11 times more drug incubated for 8 hr.

Neocarzinostatin damage of cell-free DNA produces a ratio of approximately 30:1 single- to double-strand scissions. The drug's preference for cleaving nuclear chromatin at linker DNA regions coupled with the relatively small size of these regions (40–60 base pairs) would tend to introduce single-strand scissions in close proximity. This would certainly increase the probability of single-strand scissions occurring sufficiently close to each other (10 base pairs) on opposite DNA strands to produce a double-strand cleavage. The presence of single-strand DNA tails on drug-solubilized mononucleosomes, indicated by the results of the S1 nuclease digestion experiment, further supports the hypothesis that double-strand scissions are created by a close juxtaposition of multiple single-strand scissions. Digestion of the single-strand tails with S1 nuclease was only partially complete. In a similar analysis with bleomycin, another of the DNA strand scission drugs, S1 nuclease digested all of the single-strand DNA contained on the nucleosome tails (19). Both neocarzinostatin and bleomycin cleave chromatin preferentially in linker DNA regions, but the resulting mononucleosome structures would appear to be different.

It is interesting to note that neocarzinostatin solubilization of chromatin from nuclei is not as dependent upon EDTA as is micrococcal nuclease. Actively transcribing chromatin is known to be released preferentially from micrococcal nuclease-treated nuclei in the absence of EDTA (15). Although these data provide far from conclusive evidence that neocarzinostatin also has a preference for transcribing regions of chromatin, it is an observation that warrants further examination. The report on the effects of neocarzinostatin reactivity with rat liver nuclei concluded that the drug did not preferentially cleave transcribing chromatin (18). Recent data on bleomycin demonstrated that this drug does have a preference for transcribing chromatin over that of bulk chromatin (20). Unlike the study with bleomycin, in which a single gene known to be actively transcribed was analyzed, the effects of neocarzinostatin on the total population of transcribing chromatin were measured. To improve the resolution of the assay, we are now analyzing neocarzinostatin activity on a number of individual inactive and actively transcribing cloned genes.

The data depicting neocarzinostatin effects on mononucleosomes devoid of linker DNA (core DNA) demonstrate clearly that DNA damage does occur in the more highly ordered core domain of chromatin. The average size of the 146 base pair core is shifted to a smaller-sized unit (Fig. 4C and D) with a significant increase in the breadth of the band. It should be noted that the high levels of drug required to observe degradation of core DNA are necessitated by the fact that electrophoretic analysis of the DNA requires large amounts of material. Neocarzinostatin produces a constant frequency of single-strand scissions in cell-free DNA regardless of the amount of DNA present (21). Thus, if the amount of DNA is doubled, the number of DNA scissions per molecule is halved. Given the small size of the mononucleosomes, their concentration in the assay would be quite high. A substantial level of drug would be needed to produce sufficient breaks per nucleosome to be observed by the gel analysis.

Whether the drug cleaves core DNA at preferential sites like DNase I is still unclear, and awaits a more detailed analysis. To our knowledge, this type of drug-induced damage to core areas of chromatin is the first demonstration that DNA strand scission drugs can cleave this domain of chromatin. Bleomycin is reported not to cut core DNA, nor was such an effect observed previously with neocarzinostatin (17, 19).

Comparisons between the effect of neocarzinostatin on isolated chromatin and nuclear chromatin indicate differences in the drug's mode of action. In an earlier study, treatment of soluble chromatin with high levels of drug caused strand scissions in both core and linker regions (11). Neocarzinostatin damages nuclear chromatin in CHO cells at linker regions (17). Our data with HeLa $S_3$ nuclei show that drug damage also occurs primarily at linker regions. There are significant differences between the assays using soluble chromatin or nuclei. In nuclei, chromatin is quite condensed and is packaged as a high-ordered structure (22, 23). Nucleosomal DNA in HeLa $S_3$ cells is associated not only with histone proteins but also with various nonhistone proteins such as HMG 14 and 17 (15). The nonhistone proteins bind to linker regions which are also the primary site of action of neocarzinostatin. The amount and size of the chromatin will affect the number of strand scissions that occur per unit size of chromatin. These factors could influence the specificity of drug for a particular region of nuclear chromatin and point out the necessity of examining drug action with both soluble chromatin and nuclei.

In addition to examining how neocarzinostatin damages nucleosomal DNA, studies were conducted to determine whether the drug alters chromatin-bound histone proteins. Soluble chromatin from drug-treated nuclei was run on electrophoretic gels as DNA and nuclear protein complex (data not shown). The nuclear protein complex bands were similar to the pattern seen on DNA gels, indicating that the drug does not cause profound changes between DNA-protein associations. Similar accounts of core histones were isolated from both micrococcal enzyme- and neocarzinostatin-generated mononucleosomes as well as linker-bound $H_1$ histone (data not shown).

DNA strand scission drugs such as neocarzinostatin and bleomycin demonstrate a strong relationship between damage to cellular DNA and cytotoxicity (6, 24). It is not clear how the interaction between these drugs and the cellular form of DNA—chromatin—influences drug reactivity. A common feature of both neocarzinostatin and bleomycin is that they preferentially cleave nucleosomes at linker regions. Compared with cell-free DNA, neocarzinostatin damage to chromatin is more likely to produce a double-strand scission. The positioning of double-strand scissions at unique sites on chromatin could be a major factor contributing to the extreme cytotoxicity of this drug. Neocarzinostatin also cleaves

core DNA. Further investigations are needed to determine whether there are preferential drug-cutting sites in core DNA, and whether this relates to DNA interactions with core histones.

Another area to investigate is whether DNA-reactive drugs cleave differently chromatin which is being replicated or transcribed. Both of these types of chromatin have an altered nucleosomal structure which affects their interaction with nucleases such as micrococcal or DNase I (25, 26). Transcribing chromatin, which has unique associations of nonhistone proteins with its linker DNA, could very well provide altered target sites for drugs which preferentially cleave at DNA linker regions.

Finally, drug-chromatin reactivity has potential use as a system for studying synergism between DNA-reactive agents. Our laboratory is currently investigating how the presence of intercalation drugs which bind preferentially to linker DNA can affect the strand scission activity of drugs such as neocarzinostatin and bleomycin.

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**Send reprint requests to:** Dr. Terry A. Beerman, Department of Experimental Therapeutics and Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, N. Y. 14263.