Release of 3-Methyladenine from Linker and Core DNA of Chromatin by a Purified DNA Glycosylase

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ABSTRACT

Oligonucleosomes were isolated from [14C]thymidine-labeled HeLa cells by digestion of the nuclei with micrococcal nuclease and were then alkylated with [3H]methyl nitrosourea. Nucleosome core particles were also prepared by further digestion of the oligonucleosomes. The distribution of 3H-labeled methyl groups in the linker versus the core DNA was established by a determination of 3H:14C ratios in oligonucleosome and core DNA. The ratios in the core DNA of 145 and 165 base pair DNA fragments were 5.2 and 5.4, respectively, while the ratio in the oligonucleosomal DNA was 8.2. Assuming an equal mixture (as determined) of 145 and 165 base pair fragments of DNA in the 185 base pair repeat, the relative concentration of 3H methyl groups in the linker versus the core DNA was 4.2. Thus, 45% of the 3H methyl groups were in the linker DNA, and 55% were in the core DNA. Some shielding of the DNA was evident during alkylation. The concentrations of alkyl groups on the linker and core DNA were 67 and 12% of that found on free DNA alkylated under comparable conditions. No evidence for preferential shielding of the major or minor groove was observed. The purified 3-methyladenine DNA glycosylase I of Escherichia coli released approximately 37% of the 3-methyladenine from the linker DNA and 13% from the core DNA. The limited enzymatic removal of 3-methyladenine in vitro compared to the efficient removal in vivo suggests that conformational changes of the oligonucleosome and core structure must occur for total repair.

INTRODUCTION

Recent advances in the understanding of chromatin structure have led to investigations of its role in the distribution of damage in DNA by carcinogens and in its repair. The DNA exists in nucleosome core particles and in linker regions. In the core particle, 146 base pairs of DNA are wound 1.75 times around the histone octamer (15, 17, 21). The binding of the DNA to the histones prevents micrococcal nuclease from degrading the core DNA under conditions when it can degrade the linker DNA, and the binding also restricts the action of DNase I to sites at every 10 base pairs in the core DNA (18, 30).

The distribution of carcinogens as adducts on linker versus core DNA has been studied by the use of micrococcal nuclease and DNase I to degrade the linker DNA. A number of in vivo studies have shown that the linker DNA reacts more extensively than does core DNA with a variety of chemicals, including dimethylsulfoxamidase (26), MNNU (31), and benzo(a)pyrene (12). However, UV-induced pyrimidine dimers were found to be uniformly distributed between linker and core DNA (36), and similar results were observed with dimethylnitrosamine (23). Contrary to these studies, adducts from N-hydroxy-2-acetylaminofluorene (25) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (31) were found preferentially in the core DNA.

More accurate procedures to study the in vivo distribution of carcinogens in linker versus core DNA is the determination of adduct concentration in oligonucleosomes and mononucleosomes and then calculation of the concentration in linker DNA. Previously, activation of linker DNA by carcinogens as adducts in linker versus core DNA for N-acetoxy-2-acetylaminofluorene was 4:1 to 5:1 (13) and, for different benzo(a)pyrene adducts, it was 3:1 to 10:1 (9, 11, 14, 16). Similarly, the ratio for aflatoxin B was 5:1 (1). Thus, this series of experiments is consistent and shows a 3- to 10-fold higher extent of reaction with linker versus core DNA.

The repair in vivo of linker and core DNA has been examined by measuring the incorporation of labeled nucleotides into micrococcal nuclease-sensitive and -resistant DNA in chromatin. After exposure to UV (28, 29, 36) or to chemical agents (13, 22, 26), repair synthesis was located in DNA which was initially nuclease sensitive. This was interpreted as evidence that initial repair synthesis occurrence in the linker DNA. However, nucleotide incorporation is a step which follows recognition and removal of the altered bases. When the actual removal of altered bases from linker and core DNA was studied, the results were inconsistent. The removal of adducts produced by dimethylsulfoxamide (26), N-acetoxy-2-acetylaminofluorene (13), and benzo(a)pyrene diol(epoxide I (14) was found to occur more rapidly from the linker DNA than from the core DNA. However, pyrimidine dimers (36) and 7-bromomethylbenz(a)anthracene adducts (22) were removed from linker and core DNA at similar rates.

Because the nucleotides incorporated in repair in these experiments were initially nuclease sensitive, because, in pulse-chase experiments, some of these nucleotides become resistant (3), and because lesions were removed from both linker and core DNA, it was suggested that the repair process induced a conformational change in the core particle which caused it to become temporarily nuclease sensitive (3, 22). This "unfolding" of the DNA would make altered bases in the core DNA more available to repair enzymes as well as to micrococcal nuclease, but this process would be difficult to define from in vivo studies.

The first in vitro experiment concerning the availability of altered bases was by Ishiwata and Oikawa (10). From experiments with reconstituted chromatin and an extract from human...
lymphoblastoid cells, they concluded that the m^3Ade DNA glycosylase was unable to release any m^3Ade from core DNA. More detailed studies in this laboratory by Price et al. (24) have shown that one-third of the m^3Ade residues on chicken erythrocyte core particles were released by a purified m^3Ade DNA glycosylase. There are no data available from other laboratories on the in vitro release of methylated bases from linker and core DNA by a defined enzyme.

We have investigated the limitations of DNA repair in chromatin by using [14C]thymidine-labeled oligonucleosomes isolated from HeLa cells and alkylated with [3H]MNU. Core particles were prepared from oligonucleosomal fractions by digestion with micrococcal nuclease, and the DNA was isolated by gel electrophoresis. The alkylation of linker and core DNA by MNU was determined by the ratios of H/3H in the oligonucleosomal DNA and the core DNA. A purified DNA glycosylase, specific for m^3Ade, was then used to determine the limits of the enzymatic release of m^3Ade from oligonucleosomal DNA and core DNA. From these data, enzymatic release from linker DNA could be calculated.

MATERIALS AND METHODS

Materials. Joklik's modified Eagle's medium and horse serum for suspension culture were obtained from M. A. Bioproducts, Walkersville, Md. Penicillin G and streptomycin sulfate were purchased from ICN Nutritional Biochemicals Div., Cleveland, Ohio. [3H]MNU (specific activity, 1.6 Ci/mmol) and [methyl-14C]thymidine (specific activity, 51 mCi/mmol) were from New England Nuclear (Boston, Mass.). Micrococcal nuclease and RNase A were purchased from Sigma Chemical Co. (St. Louis, Mo.), and proteinase K was obtained from E. M. Biochemicals (Darmstadt, Germany). Glass-distilled high-performance liquid chromatography solvents were purchased from E. M. Science Inc. (Cincinnati, Ohio), and potassium phosphate (Ultrex) was obtained from J. T. Baker Chemical Co. (Phillipsburg, N. J.). DEAE-cellulose (DE 52) was obtained from Whatman, Ltd. (Maidstone, Kent, England), and hydroxyapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories (Richmond, Calif.).

Haelli fragments of X 174 DNA and HindIIl fragments of X DNA, used as molecular weight markers in electrophoresis, were from Bethesda Research Laboratories, Gaithersburg, Md. Histones from calf thymus were obtained from Sigma Chemical Co. [3H]Thymidine SV40 Form III DNA (specific activity, 8.6 x 10^4 dpm/μg), used as a marker for sucrose gradient experiments, was purchased from Bethesda Research Laboratories.

Cell Culture. HeLa S3 cells, a gift from Dr. S. Penman, were maintained in suspension cultures at 37° in Joklik's modified Eagle's medium supplemented with 7% horse serum, penicillin G (250 units/ml), and streptomycin sulfate (0.25 mg/ml). Cells were generally harvested at densities of 7 to 10 x 10^6/ml by centrifugation at 500 x g for 10 min, washed with phosphate-buffered saline, and stored at -20°. All steps were performed at 0-4°, and all buffers washed with phosphate-buffered saline, and stored at -20°. For labeling densities of 7 to 10 x 10^5/ml by centrifugation at 500 x g for 10 min, the supernatant fraction, designated S-I, was removed. The nuclear pellet was resuspended in 3 ml EDTA, pH 8.0, at 2 x 10^6 nuclei/ml, allowed to lyse for 10 min at 37°, and then centrifuged for 10 min at 5000 x g. This supernatant fraction, designated S-II, contained the chromatin to be used as substrate. The test digestion was monitored by gel electrophoresis of 20-μl aliquots of S-I and S-II. A time point, usually less than 3 min, was then chosen for the digestion of the remaining suspension of nuclei to yield oligonucleosomes which contained no less than 6 nucleosomes. The supernatant fraction was dialyzed against 10 mM Tris-HCl (pH 8.0); 1 mM EDTA; 5 mM NaCl; 0.5 mM PMSF (TENP). The dialysis buffer was changed daily to minimize proteolytic degradation of the chromatin samples.

Protein and DNA Determination and Characterization. The protein concentration of the S-II fraction was determined by the method of Bradford (4) using bovine serum albumin as a standard. DNA concentration was determined by the absorbance at 260 nm using an extinction coefficient of 20 A mug per mg of DNA.

The proteins of the oligonucleosomal material were analyzed by SDS-polyacrylamide gel electrophoresis by the method of Weintraub et al. (35), with minor modifications. The separating gel contained 18% acrylamide, while the stacking gel contained 6% acrylamide.

The molecular weight of the DNA isolated from high-molecular-weight chromatin (S-II) was determined by electrophoresis on a 2% polyacrylamide gel and 0.5% agarose slab gel by the method of Compton et al. (8).

Photographic negatives of gels were scanned with a Transidyne 2510 densitometer. This system was also used for determination of the repeat length of the nucleosomal DNA. The size of the DNA of S-II after digestion to monomer DNA fragments was determined on 8% polyacrylamide gels (18) which were stained with toluidine blue and scanned with the densitometer. A HindII digest of X DNA and a Haelli digest of φX 174 were used as markers.

Alkylation of High-Molecular-Weight Chromatin. The oligonucleosomal material of S-II, at a DNA concentration of 0.7 to 1.0 mg/ml, was dialyzed against 10 mM Tris (pH 8.3); 1 mM EDTA; 0.5 mM PMSF for 3 hr. One mCi of [3H]MNU in ethanol was evaporated to dryness under nitrogen. Oligonucleosomal material (2 ml) was preincubated at 30° for 5 min and then added directly to the MNU. The mixture was incubated at 30° for 30 min. Immediately following the incubation, dialysis of the sample against TENP buffer was started and was continued with several changes of buffer for at least 2 days. Free DNA was alkylated under similar conditions. Approximately 1 H methyl group was present on core particles. The extent of alkylation of the proteins compared to the nucleic acids was determined by their separation on a hydroxyapatite column (20).

Preparation and Isolation of Core DNA. Core DNA, with approximately 145 and 165 base pairs of DNA, was first obtained by a test digestion of the S-II fraction. Alkylated oligonucleosomes were incubated at 37° for 2 min. CaCl2 was added to a final concentration of 0.1 mM, and then micrococcal nuclease (100 units/ml) was added. At varying times up to 10 min, aliquots were taken out and placed on ice, and the reactions were terminated by addition of EDTA at a final concentration of 15 mM. By gel electrophoresis, the optimum digestion time for the
maximum yield of core particles was determined for the main preparation. Core monomers were then isolated in 5 to 20% sucrose gradients, containing 10 mM Tris-HCl, pH 8.0, by centrifugation in a Sorvall TV 865 vertical rotor at 65,000 rpm for 1.25 hr at 4°C. The DNA yield was 12% of the oligonucleosomal DNA. 3H-Labeled SV40 Form III DNA was used as a standard. The 11S peak was pooled and placed in dialysis with TENP buffer.

Purification and Analysis of Double-labeled DNA from Oligonucleosomes and Core Particles. DNA from the S-11 oligonucleosome fraction and from core particles, both labeled by [3H]thymidine and by [14C]MNU, was incubated first with RNase A (0.25 mg/ml) for 30 min at 37°C and then with proteinase K (0.2 mg/ml) for 30 min. After addition of SDS and NaCl to final concentrations of 0.1% and 1.0 M, respectively, the mix was extracted twice with phenol-isobutyl alcohol:chloroform [24:1:25 (v/v)], and the DNA was precipitated by addition of 2 volumes of cold ethanol. After centrifugation, the precipitate was washed and then dissolved in sterile 10 mM Tris-HCl (pH 8.0):1 mM EDTA. In one aliquot, the 3H and 14C content was determined by a Packard Tri-Carb sample oxidizer. In a second aliquot, the DNA was hydrolyzed at 70°C for 30 min in 0.2 M HCl, and neutralized, and the hydrolysate was applied to a 1-ml DEAE-cellulose column to remove pyrimidine-containing oligonucleotides and nucleotides (7). The peak column fractions were pooled and analyzed by HPLC. The apparatus for HPLC was from Waters Associates and included a Bondapak fatty acid analysis column. The Program A solvent system described previously by Thomas et al. (34) was used for isolation of the various alkylated bases.

Enzyme Assays. The enzyme used to remove alkylated bases from oligonucleosomal DNA was the m3Ade DNA glycosylase I of Escherichia coli, fraction Vla, purified by Thomas et al. (34). The conditions for the reaction are indicated in the legend for Chart 3. The reaction was stopped by the addition of 0.05 ml of 2.5 M sodium acetate, pH 5.0, 0.5 mg of carrier DNA, and 2 volumes of cold ethanol. The DNA was pelleted by centrifugation, and an aliquot of the ethanol supernatant fraction was counted. Another aliquot of the ethanol supernatant fraction was evaporated to dryness, resolubilized, and analyzed by HPLC as noted above. The total m3Ade in the DNA was determined by first subtracting the m3Ade present in RNA. The latter was obtained by treatment of the original oligonucleosomes with RNase A to determine the percentage of total counts in RNA (28%) and then by HPLC analysis to determine the percentage of counts in the RNA due to m3Ade (2.6%). Thus, 0.007% of the total counts was in m3Ade in RNA. The RNA correction was determined once and applied to the different experiments. The total m3Ade in the DNA was then the sum of that isolated in the ethanol supernatant fraction and in the precipitate fraction after correction for the RNA.

The m3Ade DNA glycosylase was used to determine the amount of m3Ade released from linker versus core DNA after a 60-min incubation with and without enzyme. The additions to the reaction mixtures were made as indicated in the legend for Table 2. The reaction was terminated by placing the samples on ice. Purified free DNA from oligonucleosomes was prepared, hydrolyzed, and analyzed by HPLC as described above. Core particles were isolated by sucrose gradient centrifugation after digestion with micrococcal nuclease. The 11S peak was pooled, and purified free DNA was prepared, hydrolyzed, and analyzed by HPLC as noted above.

Determination of MNU Adduct Concentration in Linker and Core DNA. The adduct concentration in the linker DNA was determined from the [3H]:[14C] ratio in the total oligonucleosomal DNA and in the core DNA as outlined by Kaneko and Cerutti (13, 14). For example, a repeat length of DNA of 185 base pairs, a core length of 165 base pairs, and a linker length of 20 base pairs were used in the calculation. The following equation was used:

\[ C_L = \frac{185}{20} C_T - \frac{165}{20} C_C \]

Here, \( C_L \) is the lesion concentration in the linker DNA, \( C_T \) represents the lesion concentration in the total DNA, and \( C_C \) represents the lesion concentration in the core DNA.

RESULTS

Isolation of Oligonucleosomes from HeLa Cells. The size of the oligonucleosomal material and the yield were dependent on the digestion with micrococcal nuclease and the subsequent lysis of the nuclei. Digestion of micrococcal nuclease of [14C]-thymidine-labeled HeLa cells released a soluble fraction (S-I) primarily of mononucleosomal material, which contained 3 to 14% of the total [14C]. A fraction of the remainder of the [14C] (S-II), was released after addition of EDTA and incubation at 37°C. From 22 to 46% of the total [14C] was recovered in the S-II fraction. A major assumption in this work is that the oligonucleosome and core material isolated and analyzed were representative of the total chromatin.

Size of the Oligonucleosomes. The size of the purified DNA of the S-II fraction was determined by electrophoresis on gels containing 2% acrylamide and 0.5% agarose. Twenty-five % of the material was from 23.3 to 9.5 kb, 25% was from 9.5 to 4.2 kb, 25% was from 4.2 to 1.9 kb, and the remaining 25% was from 1.9 to 1.1 kb. The smallest DNA of the S-II fraction was found on an 8% polyacrylamide gel to be equivalent to approximately 6 nucleosomes. The repeat length of the S-II fraction, determined from the peaks of the oligonucleosomes containing 2 to 8 nucleosomes (8), was found to be 184.9 ± 1.1 (S.D.) base pairs.

Proteins of Oligonucleosomes. The proteins of the S-II fraction were electrophoresed on SDS:polyacrylamide gels, and the staining of these gels showed that there was approximately 1 histone H1 plus H1° molecule present for every nucleosome. There was no evidence of proteolysis of the core histones during the experiments as judged from SDS gels. The proteins remained intact for several weeks with daily changes of the TENP storage buffer which contained fresh PMSF. The protein:DNA ratio (g/g) of the S-II fraction was 2.45.

Alkylation of Oligonucleosomes. The ratio of methylation by [14C]MNU of protein compared to DNA in the oligonucleosomal fraction was 0.9 (Table 1). The ratios of m3Ade to m5Gua and O°-methyguanine to m5Gua for oligonucleosomal DNA versus nucleosomal DNA are also presented in Table 1. From this data, there appears to be no preference for the major or minor groove of the DNA in oligonucleosomes compared to nucleosomes.

Distribution of MNU Adducts in Linker and Core DNA. The distribution of alkylated bases in linker and core DNA was determined from analysis of the relative specific activity of DNA adducts in total oligonucleosomal DNA versus core DNA. As measured by the following equation:

\[ \frac{\text{Relative alkylation of oligonucleosomal DNA vs. proteins}}{\text{DNA}} \]

Relative alkylation of oligonucleosomal DNA vs. proteins

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<th>Proteins</th>
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<td>1.00</td>
<td>0.90</td>
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Ratio of alkylated bases in oligonucleosomal DNA vs. nucleosomal DNA

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<th>Oligonucleosomal DNA</th>
<th>Nucleosomal DNA</th>
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<tbody>
<tr>
<td>m3Ade:m5Gua</td>
<td>0.096 ± 0.002</td>
</tr>
<tr>
<td>O°-methyguanine:m5Gua</td>
<td>0.129 ± 0.008</td>
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</table>

* Mean ± S.D.
shown in Chart 1, the ratio of $^{3}H:^{14}C$ of the total purified oligonucleosomal DNA was 8.2, and this ratio remained constant after a second ethanol precipitation of the oligonucleosomal DNA. Alkylated oligonucleosomes were then digested by micrococcal nuclease for isolation of nucleosomal core monomers. Core DNA was separated by gel electrophoresis into fragments containing 145 or 165 DNA base pairs; 48% of the fragments were 145, and 52% were 165 base pairs. These fragments were excised separately from the gel and analyzed in the Packard Tri-Carb sample oxidizer. The $^{3}H:^{14}C$ ratios of these were 5.2 and 5.4, respectively. Sucrose gradient centrifugation was also used to isolate a mixture of the 145 and 165 DNA base pair fragments, as shown in Chart 2. The peak fractions of the core had a relative S-value of 10.7. The fractions shown by the bar were oxidized, and the $^{3}H:^{14}C$ ratio was 5.4 (Chart 1). Thus, the ratios obtained from samples separated by gel electrophoresis and by sucrose gradient centrifugation were similar.

Assuming 165 base pairs for the core DNA with a $^{3}H:^{14}C$ ratio of 5.4 and 185 base pairs for the linker plus core DNA with a ratio of 8.2, then the calculated ratio for a 20-base pair linker is 31.3. The ratio of $^{3}H$ methyl groups in linker versus core DNA is 5.8:1. Assuming 145 base pairs for the core DNA with a $^{3}H:^{14}C$ ratio of 5.2, the linker versus core DNA ratio is 3.7:1. In the DNA of core particles, the ratio of 165 to 145 base pair fragments was approximately 1:1. In core particles isolated in a sucrose gradient, the $^{3}H:^{14}C$ ratio was 5.4. In this case, assuming the 1:1 ratio of 165 and 145 fragments, the linker:core ratio is 4.2 and represents an average value for the S-II fragment. Purification of DNA was essential in these experiments, since the ratio in the starting material was high because of $^{3}H$ label in proteins and RNA as well as in low-molecular-weight material that was poorly dialyzable. The variability in $^{3}H:^{14}C$ ratios for the analysis of one purified DNA sample was 5.28, 5.25, and 5.16.

**Determination of Maximum Release of m$^{3}$Ade from Oligonucleosomal DNA by a m$^{3}$Ade DNA Glycosylase of E. coli.**

The availability of alkylated bases to a repair enzyme was examined as a function of time using the purified m$^{3}$Ade DNA glycosylase I from *E. coli* (34) as a probe. This enzyme preparation recognized primarily m$^{3}$Ade residues; a contaminating activity liberated m$^{5}$Gua at approximately one-two-hundredth the rate and was insignificant in these experiments. The m$^{3}$Ade DNA glycosylase was incubated at 37°C with alkylated oligonucleosomes, aliquots were removed at varying times, the DNA was purified, and the ethanol supernatant and precipitated fractions were analyzed by HPLC. The results are shown in Chart 3. For each incubation time with enzyme, a control incubation without enzyme was done because of spontaneous release of alkylated bases (Chart 3A). When this spontaneous release was subtracted from the total release in the presence of the enzyme, a plateau was observed by 45 min (Chart 3B). This represents the maximum amount of m$^{3}$Ade released by the *E. coli* enzyme and, at 45 min, it was 19% of the total m$^{3}$Ade residues in DNA. In a second experiment, this plateau was reached between 30 and 45 min with release of approximately 22% of the total m$^{3}$Ade residues. The enzyme is active after incubation for 60 min as noted previously (24). There was no inhibition of the glycosylase activity by the oligonucleosomal preparation as determined with enzyme, labeled free DNA, and unlabeled oligonucleosomes. The slow release observed from 45 to 60 min in both the control and the sample with enzyme (Chart 3A) was due to spontaneous hydrolysis of 3%/hr and also to release by endogenous glycosylase. In a second preparation, this release was reduced to approximately one-half of the rate shown in Chart 3A.

**Release of m$^{3}$Ade from Linker and Core DNA by a m$^{3}$Ade DNA Glycosylase of E. coli.**

The availability of alkylated residues in oligonucleosomal and core DNA to the purified m$^{3}$Ade DNA glycosylase I was examined. To measure release from the total DNA, the glycosylase and alkylated oligonucleosomes were incubated for 60 min and, from 1 aliquot, the DNA was purified and analyzed by HPLC. From a second aliquot, the oligonucleosomal material was digested with micrococcal nuclease, and core particles were isolated by sucrose gradient centrifugation. The peak fraction had a relative S-value of 11.0 and contained a mixture of 145 and 165 DNA base pair fragments by gel electrophoresis. This material was purified and analyzed by HPLC. The
results are shown in Table 2. For all samples, there were control incubations done without enzyme. The m3Ade released by the purified glycosylase from the oligonucleosomal DNA was 24% of the total m3Ade residues, while that released from the core DNA was 13% of its total m3Ade residues. This represents the difference between the release with and without enzyme. These values were obtained by determining the 3H counts in m3Ade as a percentage of the total 3H counts in DNA in the sample analyzed (Table 2). Analyses were done in duplicate, and the differences between the values obtained were between 4 and 8%. Another method to calculate the percentage of m3Ade released by the glycosylase was based on the ratios of m3Ade to m7Gua in the precipitated DNA. In this case, the amount of m3Ade released by the enzyme from the total DNA was 25% and, from the core DNA, it was 17%. This method of calculation is considered to be less accurate than the previous one.

The data which allow a calculation of the percentage of the m3Ade released by the glycosylase from the core and the percentage from the linker DNA are shown in Table 3. Results are shown first based on the assumption that all core particles contain 165 base pairs of DNA. The relative specific activity of 3H methyl groups in the linker DNA was 5.8 times that of the core DNA. The sizes of the core and the linker DNA were 165 and 20 base pairs, respectively. Therefore, in the alkylated oligonucleosomal fraction, 59% of the total 3H counts were in the core DNA, and 41% were in the linker DNA. From Table 2, it was shown that 13% of the m3Ade residues were released enzymatically from the core DNA, and 24% of the m3Ade residues were released from the oligonucleosomal DNA. The amount of m3Ade residues released enzymatically from the linker DNA was then calculated and found to be 40%. If the assumption is made that the core DNA is an equal mixture of fragments of 165 and 145 base pairs as was determined, then the amount of m3Ade released by the enzyme from core DNA is 13%, and the amount released from linker DNA is 37%.

DISCUSSION

In investigating the role of chromatin structure in the repair of DNA, it was first necessary to define the distribution of damage occurring in the linker and core DNA. The isolation of oligonucleosomes and nucleosome core fractions revealed that [3H]MNU-induced DNA adducts exhibited a 4.2-fold preference for linker DNA compared to core DNA. This data is comparable to results of others noted in the introduction. Of the total alkylated sites, 55% were in the core DNA, while 45% were in the linker DNA.

**Table 2**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>m3Ade as a % of</th>
<th>% of m3Ade released by glycosylase</th>
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<tr>
<td>Oligonucleosomal DNA</td>
<td>6.64</td>
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<tr>
<td>Nucleosomal DNA</td>
<td>6.95</td>
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**Table 3**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Relative specific activity (H:1°C)</th>
<th>Relative DNA base pairs</th>
<th>% of total m3Ade in either core or linker DNA released by glycosylase</th>
<th>Relative specific activity (H:1°C)</th>
<th>DNA base pairs (av.)</th>
<th>% of total 3H counts released by glycosylase</th>
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<tbody>
<tr>
<td>Core DNA</td>
<td>1.0</td>
<td>165</td>
<td>59</td>
<td>13</td>
<td>1.0</td>
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</tr>
<tr>
<td>Linker DNA</td>
<td>5.8</td>
<td>20</td>
<td>41</td>
<td>40</td>
<td>4.2</td>
<td>30</td>
</tr>
</tbody>
</table>

**Chart 3.** Enzymatic release of m3Ade from oligonucleosomal DNA at 37°C. In A, the reaction mixture contained oligonucleosomes with 25 μg of DNA with 290 dpm of 14C per μg of DNA and 9390 dpm of 3H per μg of DNA. 1.35 units of m3Ade DNA glycosylase I, 25 mM Tris-HCl (pH 8.0), 0.4 mM EDTA, 1.9% glycerol, 0.2 mM dithiothreitol, and 2 mM NaCl in a final volume of 85 μl. Incubations were at 37°C for 60 min. All reactions were run in duplicate. The amount of glycosylase added was adequate to release from alkylated free DNA, 3 times the total m3Ade residues present on the oligonucleosomal DNA. The assay is described in "Materials and Methods."
The methylation of the linker DNA was approximately 67% of the comparable methylation of free DNA, while the core DNA was approximately 12%. This suggests that there is some shielding from alkylation of the linker DNA and extensive shielding of the core DNA. Previous studies of alkylation of isolated chicken erythrocyte nucleosomal DNA showed that the alkylation was 48% as effective as with free DNA (24). The lower relative alkylation of DNA of oligonucleosomal core particles compared to free core particles must represent constraints in the structure of the former as noted above. There was no evidence obtained to show preferential alkylation in the major or minor groove of the DNA in oligonucleosomes compared to nucleosomes. This agrees with an earlier report (24).

The ratio of alkylation of proteins compared to oligonucleosomal DNA was higher in the oligonucleosome preparation (0.9) than in purified chicken erythrocyte nucleosomes, where it was approximately 0.1 (24). However, the protein:DNA ratio of the oligonucleosomes was 2.5 compared to a ratio of 1.0 in the purified nucleosome core particles (24). A large number of proteins were visualized by gel electrophoresis, and no attempt was made to determine the degree of binding of these to the DNA and core proteins.

The availability of alkylated bases in the linker and core DNA for enzymatic removal was examined by use of a purified m$^3$Ade DNA glycosylase of E. coli (34). In previous experiments, the E. coli enzyme was more active with a nucleosome core substrate than was a partially purified rat liver DNA glycosylase (24) and, since the E. coli enzyme was more easily obtained, it was used as a probe for in vitro repair of the DNA of chromatin.

The enzyme released only 13% of the m$^3$Ade residues present in the core DNA of the oligonucleosomes. This amount of enzyme could have released from free DNA 3.4 times the total m$^3$Ade residues that were present in the core DNA. This 13% released from the core DNA can be compared to 30 to 40% of the m$^3$Ade released from alkylated core particles of HeLa cells$^5$ and to 33% released from alkylated core particles of chicken erythrocytes (24). Thus, the core DNA in oligonucleosomal fractions is less accessible to alkylation and to repair than that in free core particles. The enzyme released only 37% of the m$^3$Ade residues in the linker DNA. This demonstrates that the linker DNA is not as accessible as is free DNA to repair enzymes in this oligonucleosomal structure. This may be related to the large number of proteins which are isolated with oligonucleosomes and are presumably associated with the linker DNA, as they are not observed when core particles are isolated. The mechanism of the slow release of m$^3$Ade in the control samples to which no enzyme was added is not understood at present.

The limited enzymatic removal of altered bases in the linker and core DNA of the oligonucleosomes may be due to a compact structure similar to that proposed by Worcel et al. (38). Here, alternating nucleosomes interact in a "2-track" array with the linker DNA to form a zigzag pattern. In the helical repeat of the ribbon structure, the alternating nucleosomes are not symmetrical, due to the crossover pattern of the linker DNA. This may be the structure of the 250-Å chromatin fiber observed by electron microscopy (32, 33). In this twisted helical ribbon model, the linker DNA is less exposed than it is in a previous model (37).

The new model could account for the limited enzymatic release from both the linker and core DNA in the oligonucleosome material.

The release of m$^3$Ade, described from in vivo experiments, has been shown to be quite rapid and efficient. The half-life of m$^3$Ade was found to be approximately 2 hr in human fibroblasts (5), Chinese hamster cells (27), and rat liver (19), and 80% of the m$^3$Ade residues were removed from the murine lymphoma cell line L5178Y DNA by 5.5 hr.$^5$ This rapid removal of m$^3$Ade suggests that some form of lability occurs in the chromatin structure to make areas of damaged DNA more accessible for repair. Migration of nucleosomes on the DNA appears to be an unlikely explanation (13), although it is not ruled out (39). Conformational changes in the nucleosomal structure that allow for greater accessibility of the DNA for removal of lesions have been proposed (3, 22). Conformational changes in nucleosomes, induced chemically by the addition of ethidium bromide or acetic anhydride, can enhance the enzymatic release of m$^3$Ade residues from nucleosome core DNA (24). The loosening of the DNA-histone interaction seems necessary for DNA repair of nucleosome core DNA in vivo. Thus, mechanisms must exist in the cell to unravel the 250-Å chromatin fiber and also to loosen DNA associated with the core histones. In the in vitro system described here, m$^3$Ade groups are protected in both linker and core DNA. The system apparently lacks other factors which are active within the cell. It is hoped that such a system can be used to identify these factors.

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Release of 3-Methyladenine from Linker and Core DNA of Chromatin by a Purified DNA Glycosylase

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