A $^{32}$P-Postlabeling Assay for the Oxidative DNA Lesion 8,5’-Cyclo-2’-deoxyadenosine in Mammalian Tissues

EVIDENCE THAT FOUR TYPE II I-COMPOUNDS ARE DINUCLEOTIDES CONTAINING THE LESION IN THE 3’ NUCLEOTIDE*

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8,5’-Cyclopurine-2’-deoxynucleotides, which are strong blocks to mammalian DNA and RNA polymerases, represent a novel class of oxidative DNA lesion in that they are specifically repaired by nucleotide excision repair but not by base excision repair or direct enzymatic reversion. Previous studies using thin layer chromatography of $^{32}$P-postlabeled DNA digests have detected several bulky oxidative lesions of unknown structure, called I-compounds, in DNA from normal mammalian organs. We investigated whether any of these type II I-compounds contained 8,5’-cyclo-2’-deoxyadenosine (cA). Two previously detected type II I-compounds were found to be dinucleotides of the sequence pAp-cAp and pCp-cAp. Furthermore, a modification of the technique resulted in detection of two additional I-compounds, pTp-cAp and pGp-cAp. Each I-compound isolated from neonatal rat liver DNA matched authentic $^{32}$P-labeled cA-containing chromatographic standards under nine different chromatographic conditions. Their levels increased significantly after normal birth. The $^{32}$P-postlabeling technique used here is capable of detecting 1–5 lesions/diploid mammalian cell. Thus, it should now be possible to detect changes of cA levels resulting from low level ionizing radiation and other conditions associated with oxidative stress, and to assess cA levels in tissues from patients with the genetic disease xeroderma pigmentosum who are unable to carry out nucleotide excision repair.

DNA damage, exogenously or endogenously induced, plays a crucial role in human pathology. For example, excessive exposure to short wavelength UV radiation in sunlight results in premature aging of the skin, including an increased incidence of skin cancer. Understanding the mechanisms responsible for these effects required elucidation of the chemical structure of the relevant photoproducts, i.e. the cyclobutane pyrimidine dimer and the 6–4 pyrimidine-pyrimidone dimer (reviewed in Ref. 1). Their identification made possible mechanistic studies leading to the discovery of NER1 (2, 3), which is defective in the inherited disease XP (4). These lesions have also been implicated in sunlight-induced mutagenesis, carcinogenesis, and apoptosis (5, 6).

In contrast to DNA damage caused by exogenous agents such as the short wavelength UV radiation in sunlight, which cannot reach internal organs, cellular DNA is constantly being damaged by oxygen radicals generated as byproducts of endogenous metabolic processes (7, 8). Understanding the chemical structure of this oxidative damage is important because of its likely role in spontaneous carcinogenesis, neurodegeneration, and aging (7–9) and in modulating the cellular response to low levels of ionizing radiation (10).

Studies of oxidative DNA damage have centered around 8-oxo-dG and thymine glycol, non-bulky DNA lesions subject primarily to BER, although NER has been proposed to play a back-up role (11). These lesions often serve as an estimate of oxidative DNA damage in mammalian cells (8, 10). Evidence is accumulating that they play a role in mutagenesis (12), carcinogenesis (13), and Cockayne’s syndrome (14).

Recently, attention has focused also on a novel class of oxidative DNA damage that is resistant to BER but requires NER for removal from DNA (15). The only known members of this class are the cPu, in which C8 of a purine base becomes covalently bound to the C5’ of its own deoxyribose through oxidation by the highly reactive ‘OH (16–20) (Fig. 1). Formation of this covalent bond causes an unusual puckering of the deoxyribose, as well as local distortion of the DNA helix (21). cPu have recently been synthesized as phosphoramidites, permitting their incorporation into synthetic DNA (22, 23).

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1 The abbreviations used are: NER, nucleotide excision repair; BER, base excision repair; cPu, 8,5’-cyclopurine-2’-deoxynucleotides; N, normal deoxynucleoside or deoxynucleotide; p, phosphomonoester or phosphophodiester; Xp, unidentified adducted nucleotide; 8-oxo-dG, 8-oxo-7,8-dihydro-2’-deoxyguanosine; XP, xeroderma pigmentosum; I-compound, endogenous bulky DNA adduct; cA, 8,5’-cyclo-2’-deoxyadenosine; ‘OH, hydroxyl radical; MX, microsomal nuclease; SPD, calf spleen phosphodiesterase; NuP1, nuclease P1; TLC, thin layer chromatography; Nca, pNP-cAp which N is A, C, G, or T; Aca, pAP-cAp; Cca, pCP-cAp; Gca, pGP-cAp; Tca, pTP-cAp; RAL, relative adduct labeling; LOL, level of lesion; RF, recovery factor; MS, mass spectrometry.
Type II I-compounds Contain 8,5'-Cyclodeoxyadenosine

deoxycadenosine (ca) in calf thymus DNA using MS. Using a highly sensitive 32P-postlabeling assay for covalent DNA adducts (26, 27) (Fig. 2), Randerath et al. (26, 27) first produced evidence for a variety of bulky endogenous lesions of unknown structure in normal mammalian tissue DNA. These compounds have been classified into two groups named type I and type II I-compounds (reviewed in Ref. 30). Although type I I-compounds arise by the reaction of unknown metabolic intermediates with DNA and show age, species, tissue, and gender dependence (30), the formation of type II I-compounds has been firmly linked to reactive oxygen species by both in vitro and in vivo experiments including the exposure to pro-oxidant chemicals (29, 31–34). Some but not all type II I-compounds detected in vivo are identical to products generated by oxidizing isolated tissue DNA or synthetic oligonucleotides in vitro under Fenton reaction conditions, which produce oxygen free radicals including ‘OH (29, 32–36).

The Nu P1 version of the 32P-postlabeling assay (27) detects DNA lesions with the character of bulky DNA adducts. Because ca is an oxidative lesion that has biological properties of a bulky adduct, in the present work we attempted to determine whether there was any relationship between type II I-compounds and ca.

**EXPERIMENTAL PROCEDURES**

**Chemical Synthesis of Oligonucleotides Containing ca**—In this paper, the term oligonucleotide refers to a polynucleotide of length n > 2. Oligonucleotides containing a single ca and control oligonucleotides were synthesized and characterized as described (23), and were originally prepared for other experimental purposes (23). Each had a different nucleotide 5’ to the ca. The sequences of the AcA, CaC, TcA, and GcA oligonucleotides were, respectively, as follows: 5’-GCATCTGTTA-AACAGCAATTGTTCAGGAGAC-3’; 5’-AATTCUCGGGTACTGCTC-CAHGGAATAGC-3’; 5’-TGGGAAGCTTCTAT-3’; and 5’-CATAGTTAACAGGTATCGCAT-3’. Oligonucleotides were desalted by passage through Sephadex G-25 spin columns, causing 3’-silyl-ca or 3’-silyl-A-truncated oligonucleotides, formed by incomplete deprotection at the manual desilylation step (23), to be present in the preparations of the oligonucleotides. Digestion products of this truncated material remained at the origin of chromatograms.

**Chemical Synthesis and Purification of Dinucleotide Standards Containing ca**—Dinucleotides containing caP were synthesized as described (23), with the following modifications. Phosphorylation of the dinucleotides containing ca with the corresponding compounds from liver DNA and with synthesized dinucleotide standards. We extracted dinucleotides from two-dimensional TLC maps with 2-propanol/6 n ammonia (1:1, by volume) as described (32, 33). Aliquots containing 0.1–0.3 nCl were separated in one dimension individually or as mixtures in solvents 1, 5, and 7–13, then visualized. It may be noted that the solvents chosen operate according to three different principles, i.e., anion exchange on a protonated (39) stationary medium (nos. 2–4), partitioning between a highly alkaline mobile phase and a mostly unprotonated (39) stationary phase (no. 13). Since LOL values represent the number of lesions in 109 N and 109 values represent the number of lesions in 109 N and 109 DNA nucleotides (cpm)

\[
\text{RAL} = \frac{\text{DNA lesion (cpm)}}{\text{Normal DNA nucleotides (cpm)}}
\]

(Eq. 1)

The specific activity of ATP was determined as described (27). Because 100% adenosine recovery was not achieved, the RAL value for each lesion was divided by a RF to account for losses inherent in the procedure. Accurate level of lesion (LOL) values were then calculated according to Ref. 27.

\[
\text{RF} = \frac{\text{Observed recovery (cpm)}}{\text{Theoretical recovery (cpm)}}
\]

(Eq. 2)

\[
\text{LOL} = \text{RAL} \times \text{RF}^{-1}
\]

(Eq. 3)

The theoretical recovery was derived from the molecular weights of the ca dinucleotides and the specific activity of ATP. The observed recovery was determined experimentally in triplicate by carrying known amounts of each adduct through the entire procedure. Since LOL × 10^9 values represent the number of lesions in 10^9 N and
Type II I-compounds Contain 8,5'-Cyclodeoxyadenosine

**RESULTS**

Type II I-compounds (Spots 2 and 5) Resembled Dinucleotides Containing 3'-cAp—Type II I-compounds have been defined (30, 40) as endogenous bulky oxidative DNA lesions of unknown structure that are present in untreated mammalian tissues including neonatal tissues. Their levels increase in conditions of oxidative stress in vivo (30, 32–34). We detected these I-compounds by digesting DNA with nucleases followed by 5'-labeling of the products with carrier-free [γ-32P]ATP and multidimensional TLC, as shown schematically in Fig. 2 (upper left). Typical L and C map profiles are illustrated in Fig. 2 (upper right) with six spots numbered. We showed previously that each of the numbered spots is related to oxidative stress, but only spots 2, 3, and 5 can be generated by oxidation of DNA in vitro via the Fenton reaction (29, 32, 33). We detected spots 2 and 5 in all newborn tissues examined (Fig. 2, lower) including skin (data not shown). The intensities of all the spots varied among tissues (Fig. 2).

Given this information, we asked whether some of these spots contained cA (Fig. 1). To this end, we synthesized four modified oligonucleotides, each containing a different normal nucleotide, i.e., Ap, Cp, Gp, or Tp, 5' to a single cA. We also synthesized the corresponding dinucleotides containing cA (i.e., AcA, CcA, GcA, TcA). Nuclease treatment of oligonucleotides or dinucleotides containing cA, followed by one-dimensional development with solvent 1 (Fig. 3A) or 6 (Fig. 3, B and C), resulted in a single major 32P-labeled spot from each compound depending on the nucleotide 5' to the lesion. Di- and oligonucleotides containing normal A in place of cA were completely digested to monomers (27), which moved close to the solvent fronts in solvents 1 and 6 and thus were absent from the maps. On the other hand, omission of enzymatic digestion resulted in a single 32P-labeled spot. In the case of oligonucleotides, the labeled material remained at or near the origin (data not shown), whereas the synthesized dinucleotides containing cA gave the same spots without digestion as the corresponding digested oligonucleotides (Fig. 3). Thus, under our conditions, the cA dinucleotides were resistant to further enzymatic digestion at the 3',5'-phosphodiester and 3'-monoester bonds. These collective results suggested that the end products of the digestion and 5'-phosphorylation of oligonucleotides containing cA were dinucleotides of the sequence 5'-cA-cAp (5', 32P)NcA. Cadet and co-workers (22) also found, using different techniques, that the presence of cPu confers resistance of dinucleotides to enzymatic digestion.

When the initial separation was performed in solvent 1 (Figs. 2 and 3A), the spots denoted AcA and CcA migrated in a manner suggesting their identity to previously identified I-compounds 2 and 5 (also called L2 and C5), respectively (34). The other two spots (GcA and TcA) migrated out of the C area with solvent 1 (Fig. 3A). We eliminated this problem by replacing solvent 1 with solvent 6 (Fig. 3B).

In order to establish more firmly that the four oligonucleotide digestion products matched labeled dinucleotides containing 3'-cAp (i.e., 5'-32P)NcA-cAp, we used synthesized dinucleotide standards (Fig. 3, C and D). These compounds elicited a pattern (C) without digestion in solvent 6, which resembled that given by the digestion products of oligonucleotides containing cA (B). The results were further confirmed by one-dimensional chromatography in nine different conditions (solvents 1, 5, and 7–13; only solvent 7 is shown in D). In each case, the dinucleotide standards matched the labeled oligonucleotide digestion products.

Chromatographic Matching of Four Type II I-compounds (Spots 2, 5, 7, and 8) with Authentic Dinucleotide Standards Containing 3'-cA—We next tested directly the hypothesis that the labeled oligonucleotide digestion products (AcA, CcA, GcA, and TcA) were chromatographically identical to spots 2, 5, 7, and 8. Liver DNA and oligonucleotides containing cA were digested, postlabeled, and chromatographed in parallel. We chose neonatal rat liver for detailed analysis because previous
studied had shown that DNA from this tissue has a high abundance of type II I-compounds and low levels of other endogenous adducts such as type I I-compounds (34). The chromatograms were run in solvent 6 instead of 1 and in solvent 5 instead of 4 (Fig. 2) in order to retain GcA and TcA. As shown in Fig. 4, the two-dimensional pattern of spots 2, 5, 7, and 8 from liver DNA (upper) matched those of AcA, CcA, GcA, and TcA, respectively, from digested oligonucleotide digestion product. Approximately 0.2 nCi of each compound was applied. Identical results were obtained when the dinucleotides were incubated with enzymes before 32P labeling. For solvent identification, see Table I.

We finally established the relationship between the digestion products and in vivo spots 2, 5, 7, and 8 by extensive co-chromatographic analyses. In total, we used nine diverse solvents (nos. 1, 5, and 7–13) for co-chromatography of extracted spots isolated from two-dimensional maps. In each condition, in vivo spots 2, 5, 7, and 8 matched the labeled digestion products (see Fig. 5 for a subset exemplifying solvents 7–9). These collective results showed the four type II I-compounds to be identical, under diverse chromatographic conditions, to the four NcA dinucleotides obtained by direct chemical synthesis or by digestion of oligonucleotides containing cA.

Nonrandom Distribution of cA in Vivo—Visual inspection of Fig. 4 (top) suggested that the intensities of the four dinucleotides containing cA in vivo were not identical. This observation could be due to methodological factors, such as differential labeling efficiencies or differential losses during chromatography, or reflect actual in vivo differences. Studies using the synthesized dinucleotide standards revealed that all four dinucleotides labeled at the 5′ N with >95% efficiency, similar to deoxynucleoside 3′-monophosphates (27). However, significant losses of individual dinucleotide adducts occurred during chromatography, especially during washing after the initial chromatography in concentrated phosphate (solvent 1 or 6). The mean values (± S.D.) for RF (see Equations 2 and 3 under “Experimental Procedures”) were 0.74 ± 0.01 (AcA), 0.37 ± 0.03 (CcA), 0.40 ± 0.05 (GcA), and 0.23 ± 0.01 (TcA). We used these values to calculate the number of individual and total NcA lesions/diploid cell (Table II). The data indicate that major differences existed in the amounts of individual NcA dinucleotides in vivo. Unequal frequencies of normal NA dinucleotide sequences in rat DNA might provide a possible explanation. As shown in Table II, this was not the case, however. For example, the number of AcA lesions in rat liver DNA was 3–5 times lower than those of GcA and TcA, whereas the frequency of AA in genomic DNA of the rat and most other eukaryotes (41) exceeds that of the three other NA dinucleotides (Table II). These results indicated a nonrandom distribution of cA in different sequence contexts.

Postnatal Increases in cA Levels—Consistent with previous observations (34, 42), type II I-compound levels increased significantly during the prenatal–postnatal transition (Table II). The greater postnatal increase of adduct 2 (AcA) has been observed in a previous independent experiment (34). This result could be due to a greater sensitivity of the AA sequence, compared with the other NA sequences, to the 4-fold increase of pO2 occurring at birth (34).

DISCUSSION

In this work, we present evidence that two previously observed type II I-compounds (32, 34) are dinucleotides of the
Lindahl I-compounds are dinucleotides containing cA confirms in previous experiments. Our finding that a subset of type II detection necessitated changes in the chromatographic conditions. Multiple two-dimensional chromatograms (Fig. 4) were prepared, and then the individual spots were extracted and re-chromatographed one-dimensionally as described under “Experimental Procedures.” Lane a, extracted I-compound; lane b, mixture of lanes a and c; lane c, dinucleotide. The radioactivity/compound was 0.1–0.3 nCi. Development in solvents 7, 8, and 9 as indicated. For solvent identification see Table I.

### Table II

| Sequence | Lesions/diploid cell ± S.E.M* | Normal dinucleotide sequences | Frequencyb |
|----------|-------------------------------|-----------------------------|------------|
| AcA      | 14 ± 1                        | 42 ± 3                      | AA         | 8.8  |
| CcA      | 26 ± 2                        | 52 ± 7                      | CA         | 6.8  |
| GcA      | 69 ± 3d                       | 115 ± 8d                    | GA         | 6.6  |
| TcA      | 68 ± 7d                       | 111 ± 8d                    | TA         | 6.3  |
| Total    | 177 ± 10                      | 320 ± 25d                   |            |

* Calculated according to Equation 4 (n = 6).

b Values are incidence of dinucleotide/incidence of all 16 dinucleotides × 100 in rat DNA (41).

c Significantly different from fetal (p < 0.01).

d Significantly different from CcA and AcA (p < 0.001).

sequences AcA and CcA. In addition, we have found two new I-compounds to be the dinucleotides GcA and TcA, whose detection necessitated changes in the chromatographic conditions. This explains why the latter compounds were not noticed in previous experiments. Our finding that a subset of type II I-compounds are dinucleotides containing cA confirms Lindahl’s suggestion (7) that the \(^{32}\text{P}\)-postlabeling method may be suited to reveal bulky oxidative DNA lesions repaired by NER.

Our conclusion that the four I-compounds are dinucleotides containing cA rests on two major points. 1) Digestion of four oligonucleotides, each of which was synthesized with a different normal nucleotide 5’ to cA, resulted in dinucleotides containing cA and a normal 5’ nucleotide; and 2) each of the four isolated I-compounds matched the corresponding cA-containing dinucleotide standards in nine different solvent systems, which separate on the basis of different physical and chemical principles. In essence, the four dinucleotides containing cA represented a four different chemical derivatives of the same lesion. Independently, the recent finding by Dizdaroglu et al. (25) of cA in calf thymus DNA using MS techniques provided evidence that cA is an endogenous lesion in mammalian organ DNA.

Although the classic method for establishing the structure of a DNA adduct is MS, that option was not available to us since the amount of individual I-compounds on a single chromatogram is in the range of 10–100 amol, which is below the limit of detection of mass spectrometers at the present time. For example, Dizdaroglu et al. (25) reported a detection limit of 2 fmol of cA corresponding to 1 lesion in \(10^7\) normal nucleotides, using liquid chromatography MS. In contrast, the \(^{32}\text{P}\)-postlabeling approach detects 1–5 lesions in \(10^{10}\) normal bases, and is therefore 3 orders of magnitude more sensitive than the MS technique. Indeed, our data indicate that the liquid chromatography MS technique of Dizdaroglu et al. (25) would not have detected cA in any of the rat organs we have analyzed. However, as these authors (25) pointed out, it may be possible to modify the MS method to detect lower levels of cA.

Levels of cA in fetal and postnatal liver were on the order of 180–320 lesions/cell (Table II). The levels of cA in calf thymus DNA, reported by Dizdaroglu et al. (25) correspond to 1200 lesions/cell (assuming a mammalian cell contains \(12 \times 10^6\) nucleotides). The difference between the two findings may represent tissue and/or species differences. The data in Fig. 2 show that levels of dinucleotides containing cA vary across different rat tissues, and previous studies have shown that levels of type II I-compounds in pig liver are 4–10-fold greater than those observed in rat liver (43). The levels of cA in rat liver can also be compared with the levels of other endogenous lesions reported for rodent liver. Oxidative DNA lesions such as 8-oxo-dG may be present at 1–2 orders of magnitude greater levels (44, 45) (see below). Compared with DNA adducts resulting from lipid peroxidation, levels of cA were ~1 order of magnitude lower than malondialdehyde (46) and exocyclic propano (47) adducts, but comparable to those reported for the premutagenic exocyclic etheno adducts (48).

Previous studies (34) have demonstrated that normal birth of rats is associated with a sudden severalfold increase in the levels of several type II I-compounds including spots 2 and 5, now identified as AcA and CcA. In liver this increase persists during the entire preweanling period (34). Similar increases have now been documented for GeA and TcA (Table II). Our collective results indicate that, in normal rats (and other mammals), the postnatal period is characterized by elevated steady state levels of cA in several tissues. Furthermore, recent studies (42, 49) have demonstrated that formation in neonatal tissues of the I-compounds we now identify as derivatives of cA is intensified by elevated levels of transition metals, such as iron, copper, and nickel, in the maternal diet. Given the likely in vivo cytotoxicity of cA (24), these findings are relevant to the present controversy over the routine use of iron supplements in...
healthy pregnant women (50).

Our data have potential implications for patients with XP, a genetic disease with defective NER. As NER is the only known pathway for repair of cPu (23, 24), cA would not be able to be removed once formed in tissues of XP patients. If the present results in rats can be extrapolated to humans (see below), XP patients begin life outside the womb with augmented levels in many tissues of an endogenous irreparable toxic DNA lesion. The resulting increased cell death expected to occur in affected organs would provide a novel explanation for the dwarfism, retarded testicular development, and microcephaly that is observed in the most severely affected XP patients (51–54).

The potential role of cPu in the neurodegeneration observed in XP patients has been discussed previously (15, 23, 24), but the present results do not directly address this issue. For several reasons, it is not possible to relate the absolute levels of cA we find in rat organs, or their relative amounts in different rat organs, to what may exist in human tissues. Hanawalt (55) has recently expressed the need for caution in the interpretation of results from rodent systems for human genetic toxicology because of the qualitative differences in NER between rodent and human cells. Specifically, rodent cells are much less efficient at removing thymine dimers from the genome overall than are human cells (56, 57). Since the 32P-postlabeling assay measures lesions in total DNA, this assay predominantly reflects lesion formation and repair in the genome overall. However, at present we do not know how cA lesions are repaired in the genome overall in rat or human cells. Previous host cell reactivation studies, using transfected plasmid substrates, have demonstrated that, whereas cA and thymine dimers are repaired at equivalent rates in CHO cells, cA is repaired significantly faster than thymine dimers in human cells (23). Finally, XPA knockout mice do not develop neurodegeneration (58), in contrast to humans with XP. Given the great sensitivity of the 32P-postlabeling assay, it is possible that in future studies the assay will be able to detect and quantify cA in human organs, where lesion levels may be significantly lower than in rodents. A finding of abnormally increased amounts of cA in the brain of XP patients with neurodegeneration would support the hypothesis (23, 24) that cA is at least one of the free radical-induced lesions that cause neurodegeneration in XP patients.

Although cPu are complete blocks to the replicative DNA polymerase δ (24), the recent discovery of DNA polymerases capable of bypassing highly distorting DNA lesions in an error-prone manner (59) raises the possibility that cA may be mutagenic under some circumstances. Such an effect would be relevant to the reported 10–20-fold increase in internal cancers in XP patients (60), as well as to the increased rate of mutation accumulation and tumors in XP knockout mice (61, 62).

The DNA lesion most commonly assayed as a measure of oxidative damage is 8-oxo-dG (44, 45). However, this lesion can be formed by mechanisms involving metabolic activation of chemicals (e.g., carcinogenic secondary nitroalkanes; Ref. 63), which is relevant for analysis of tissue samples from humans who may be exposed to such chemicals (64). The problem of artifactual generation of this lesion during sample preparation and work-up (44) has represented another drawback of using 8-oxo-dG as a measure of oxidative DNA damage, although recent methodological improvements have largely solved this problem (45). In contrast, the formation of cPu is inhibited by O2 (18), so artifactual generation during experimental manipulations and storage is not of concern. The other commonly measured oxidative DNA lesion is thymine glycol. A recent paper (65) described an ultrasensitive assay for detecting this lesion in DNA at a level of 1 lesion/108 normal nucleotides.

Previous work has shown the Nu P1-enhanced version of the 32P-postlabeling assay to have a sensitivity of 1 individual adduct in 1015 normal nucleotides (27). This assay, shown herein to be able to detect cA, thus provides the most sensitive technique currently available for detecting DNA damage from OH. The cA adduct has been found to be completely stable in tissues or DNA solutions stored at -70 °C for >2 years. The ability to estimate exceedingly low levels of DNA lesions caused by OH has important implications for risk assessment of low level ionizing radiation. Risk is customarily estimated by linear extrapolation of high dose effects. The 32P-postlabeling assay described herein should make it now possible to assess directly the levels of DNA damage from very low levels of ionizing radiation, eliminating the problems associated with risk extrapolation (65, 66).

The levels of cA dinucleotides represent an equilibrium between DNA lesion formation and repair. Our finding, therefore, that individual NcA dinucleotide levels were significantly different from what would be expected based on the frequency of their normal counterparts (Table II) implies sequence-specific effects on the formation and/or the repair of the lesion in vivo. Lloyd and Phillips (36) have presented evidence for site-specific mechanisms involved in the formation of oxidative lesions that appear to correspond to what we have now identified as dinucleotides containing cA. Sequence dependence has also been reported for various other types of oxidative DNA damage (67–70). Henle et al. (70) developed models for the patterns of OH-induced DNA strand breaks based on the coordination of iron ions in specific sequences and proposed that such patterns could affect the generation of specific DNA lesions. In addition, sequence context can also alter the efficiency of NER both in vitro (71) and in vivo (72). The role of NER in the pattern of dinucleotides containing cA observed here can possibly be assessed by comparing tissue DNA of mutant mice lacking the XPA gene and wild-type controls.

In summary, we have shown that cA is a component of four type II I-compounds in mammalian cellular DNA in vivo, where its level is enhanced by conditions of oxidative stress. These results have implications for oxidative DNA damage occurring through the reaction of tissue DNA with oxygen free radicals formed endogenously (34) or as a consequence of exposures to ionizing radiation and pro-oxidant chemical mutagens/carcinogens (32). The ultrasensitive assay described herein should be useful in the detection and estimation of oxidative DNA damage in relation to normal development, aging, cancer, and degenerative diseases.

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REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, pp. 24–29, American Society for Microbiology, Washington, D. C.

2. Setlow, R., and Carrier, W. (1963) Proc. Natl. Acad. Sci. U. S. A. 51, 226–231

3. Pettijohn, D., and Hanawalt, P. (1966) Biochim. Biophys. Acta 72, 127–129

4. Cleaver, J. E. (1968) Nature 215, 655–656

5. Hart, R., Setlow, R., and Woodhead, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5574–5577

6. Ziegler, A., Jonassen, A. S., Lefell, D. J., Simon, J. A., Sharma, H. W., Kimmelman, J., Remington, L., Jacks, T., and Brash, D. E. (1994) Nature 372, 773–776

7. Lindahl, T. (1993) Nature 365, 769–714

8. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7915–7922

9. Hanawalt, P. C. (1998) Mutat. Res. 400, 117–125

10. Vileichnik, M. M., and Kaudreason, A. G., Jr. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5381–5386

3 G.-D. Zhou and K. Randerath, unpublished data.
