Vol. 95, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1980 Pages 816-821

IN VIVO REPLICATION OF CARCINOGEN-MODIFIED RAT LIVER DNA: INCREASED SUSCEPTIBILITY OF O^6-METHYLGUANINE COMPARED TO N-7-METHYLGUANINE IN REPLICATED DNA TO S_1-NUCLEASE.*

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Received May 9,1980

Summary

In order to characterize rat liver DNA replicated in vivo on a carcinogen-damaged template, the replicated DNA was treated with S_1-nuclease and the release of (^{14}C)-dimethylnitrosamine induced O^6-methylguanine, a lesion associated with miscoding and N-7-methylguanine, a lesion that does not miscode were monitored. The results indicated that both the methylated guanines became susceptible to S_1-nuclease upon replication. However, a greater percentage of O^6-methylguanine (22% of the total O^6-methylguanine present in the DNA) compared to N-7-methylguanine (4% of the total N-7-methylguanine present in the DNA) was rendered acid soluble by S_1-nuclease. The preferential release of O^6-methylguanine compared to N-7-methylguanine from replicated DNA was interpreted to indicate its occurrence in local denatured regions probably generated as a result of misbase pairing.

Cell proliferation appears to be essential in the initiation of carcinogenesis, and replication of DNA with carcinogen-induced lesions, prior to their repair, has been suggested as a mechanism by which the original damage in the parental DNA may be fixed in the DNA of the daughter cell (1-4).

However, very little is known about the replication of carcinogen-damaged DNA in vivo, particularly with respect to the types of lesions that permit replication, and virtually nothing is

*This work was supported in part by U.S. PHS research grants CA-23958, CA-21157 from the National Cancer Institute and from the National Cancer Institute of Canada and the Medical Research Council of Canada.

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Abbreviation: PH, partial hepatectomy.

0006-291X/80/140816-06$01.00/0

known about the nature and fate of the DNA replicated on such damaged templates. These aspects are of considerable importance in the light of the results obtained in in vitro studies demonstrating that modified bases like O^6-alkylguanine and O^4-methylthymine when present in DNA templates cause miscoding by both RNA (5) and DNA polymerases (6, 7). If such modified bases do in fact permit replication in vivo, it raises an important consideration whether such replicated DNA exhibits altered characteristics compared to normal replicated DNA.

In order to answer this question, the following experimental approach was employed: (i) methylate the parental DNA strand with (^{14}C)-dimethylnitrosamine (^{14}C)-DMN); (ii) 4 hr later, at a time when free circulating DMN is no longer present in the system (8), induce DNA replication by the application of a cell proliferative stimulus like partial hepatecomy (PH); (iii) isolate the hepatic DNA 44 hr later, at a time when the daughter strand attains the same size as that of the DNA synthesized on an undamaged normal template (2); (iv) treat the isolated DNA with S_1-nuclease an enzyme that attacks single stranded regions of DNA as well as DNA containing mismatched base pairs (9, 10) and (v) monitor the release of O^6-methylguanine (O^6-MeG), a lesion associated with miscoding and N-7-methylguanine (N-7-MeG) a lesion that does not miscode.

The results indicated that upon replication, O^6-MeG, compared to N-7-MeG, exhibited an increased susceptibility towards S_1-nuclease.

Experimental Procedures

Male, albino rats of Wistar strain weighing 150g were injected intraperitoneally with (^{14}C)-DMN, sp. activity 46 mCi/mmole (New England Nuclear, Montreal, Canada), at a dose of 0.5 mg/50 μCi/100g body wt. After 4 hr, the animals were either partially hepatectomized or sham-operated and sacrificed 44 hr later. The liver taken out during surgery was used as the 4 hr sample. The DNA was isolated from all the liver samples by extensive deproteinization with pronase (100 μg/ml) followed by Marmur's procedure (11).

S_1-nuclease digestion: The reaction mixture for S_1-nuclease digestion contained the following in μmoles/ml: sodium chloride,15; sodium acetate, pH 5.0, 40; zinc chloride, 0.2; DNA, 2.5-2.7 A_{260} units and 6250 units of S_1-nuclease (Miles Elkhart, Indiana). After incubation at 37°C for 1 hr, the non-digested DNA was precipitated with a final concentration of 0.5 N PCA. The digested and non-digested fractions were hydrolysed with 0.1 N PCA at 70°C for 45 min or at 37°C for 16 hr and the methylated bases were determined

by fractionation on Sephadex G-10 columns (12). Under these conditions less than 5% of the native rat liver DNA and greater than 95% of the heat-denatured DNA were rendered acid-soluble by S_1-nuclease.

Results and Discussion

The data presented in Table 1 show that although the DNA sample taken 4 hr after DMN administration has a higher O^6-MeG and N-7-MeG content compared to the sample taken 48 hr later, yet less than 1% of these methylated bases are rendered acid-soluble by treatment with S_1-nuclease. This is of interest because administration of DMN has been

Table 1

Susceptibility of O^6-methylguanine containing regions in replicated and non-replicated liver DNA towards S_1-nuclease

Source of liver DNA	% Methylated bases released by S_1-nuclease[a]		Ratio of O^6-MeG/N-7-MeG in[c]	
	O^6-MeG[b]	N-7-MeG[b]	S_1-nuclease digested DNA	S_1-nuclease resistant DNA
4 hr after (^{14}C)-DMN[d]	0.6 ± 0.31	0.5 ± 0.19	0.12 ± 0.06	0.10 ± 0.06
48 hr after (^{14}C)-DMN[d] (with replication)	22.2 ± 9.3	3.9 ± 2.0	0.52 ± 0.18	0.08 ± 0.06
48 hr after (^{14}C)-DMN[e] (without replication)	7.0	3.0	0.18	0.07

[a]The N-7-MeG and O^6-MeG content of the liver DNA used for S_1-nuclease digestion was (in pmoles/mg DNA): 916 and 91 in 4 hr sample; 370 and 34 in 48 hr preparation with replication; and 360 and 27 in 48 hr sample without replication.

[b]These are expressed as percentage of methylated base present in the total DNA. Values are corrected for the methylated nucleotides rendered acid soluble in the absence of S_1-nuclease. The average blank values are (%): for O^6-MeG: 13.6 ± 2.6; and for N-7-MeG: 4.2 ± 0.8. The blank values were essentially the same irrespective of the source of the DNA.

[c]The ratio of O^6-MeG/N-7-MeG in total DNA was 0.10 ± 0.01 in 4 hr sample; 0.09 ± 0.06 in 48 hr preparation with replication; and 0.08 in 48 hr sample without replication.

[d]Values are the average ± S.E. of 4 to 6 experiments and in each experiment livers of 2 or 3 rats were pooled.

[e]Values are the average of 2 experiments and in each experiment livers of 2 or 3 rats were pooled.

shown to induce alkali-sensitive lesions, particularly single-strand breaks, as measured by sedimentation of DNA in alkaline sucrose gradients (13) or binding of DNA polymerase I (14); single-stranded regions also have been demonstrated by fractionation on benzoylated DEAE-columns (15). The results of the present study, however, clearly indicate that alkylation per se does not create any large segments of single-strand DNA, but may cause only single-strand breaks; because if there were to be any single-stranded regions one would expect a significant extent of release of the two methylated guanines by S_1-nuclease.

In contrast, in the DNA isolated 44 hr after replication, about 22% of the O^6-MeG was released in the enzyme digest and under identical conditions less than 4% of the N-7-MeG was digested by the S_1-nuclease. This preferential release of O^6-MeG was also reflected in the higher ratio of O^6-MeG/N-7-MeG in the S_1-nuclease digests of the replicated DNA. However, in the absence of an exogenous cell proliferative stimulus at 48 hr after the administration of DMN 7% instead of 22.2% of O^6-MeG and 3% instead of 3.9% of N-7-MeG were released by S_1-nuclease. Although it is difficult to explain at the present time, this increased susceptibility to S_1-nuclease of methylated guanines in the non-replicated DNA, it is logical to suppose that it may be due to the repair of carcinogen-modified DNA which may result in S_1-nuclease-sensitive lesions such as apurinic sites and strand interruptions.

Increased susceptibility of O^6-MeG and N-7-MeG towards S_1-nuclease in the replicated DNA compared to that in the 4 hr sample was not totally unexpected, because during replication single-strandedness of the DNA increases and at 44 hr after PH there will be some cells still undergoing replication and in addition as pointed out above, repair of carcinogen-modified DNA may also create S_1-nuclease-sensitive lesions. However, what was surprising is the preferential release by S_1-nuclease of O^6-MeG compared to N-7-MeG from the replicated DNA.

The study raises an important question why some of the O^6-MeG containing regions in the DNA methylated in vivo, which were non-digestible by S_1-nuclease become susceptible

to the enzyme when the methylated DNA undergoes replication. Two possible mechanisms were considered to explain the results. According to the first possibility, O^6-MeG in the parental strand acts as a terminator for DNA polymerases, thus creating gaps in the daughter strand. This was considered less likely for the following reasons: Firstly, in vitro studies using O^6-MeG containing polynucleotides as templates have shown that this modified base is not a terminator for either RNA polymerase (5) or DNA polymerase (6), although it caused miscoding. Secondly, the size of the DNA synthesized in vivo on DMN methylated liver DNA was the same as that made on control rat liver DNA, as measured by sedimentation of either the DNA obtained by lysing the cells (2) or purified liver DNA (Columbano, A., unpublished observations) in alkaline sucrose gradients. In addition, recently, we have demonstrated that both O^6-MeG and N-7-MeG permitted in vivo replication (16). The second and more probable explanation considered was that although O^6-MeG in the parental strand permits replication some of these are defective and give rise to destabilized regions thereby rendering them digestible by S_1-nuclease. Although the nature of the defect has not been characterized, one can visualize a situation where, during replication, a wrong base for example cytosine is incorporated opposite to a O^6-MeG; and such a mismatched base pair may create a potentially destabilized region in the replicated DNA.

The reason why only a fraction of O^6-MeG in the replicated DNA becomes susceptible (see Table 1) may in part reside in the reasoning that during replication not all O^6-MeG are paired with cytosine, instead some may be paired with thymine. Such a pairing will be a perfect match and thus does not become S_1-nuclease-susceptible. Further, the relative susceptibility of O^6-MeG in the replicated DNA may also depend on its location in the neighbouring environment, for example AT or GC-rich regions of DNA.

The present study thus clearly identifies the presence of a secondary lesion generated as a result of in vivo replication of DNA containing carcinogen-induced O^6-MeG which can be monitored by S_1-nuclease. A precise characterization of the nature of this secondary lesion requires further experimentation.

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