Vol. 13, No. 5, November 1986 BIOCHEMISTRY INTERNATIONAL
Pages 903-913

DIGESTION OF REPAIR SITES IN RAT LIVER DNA BY ENDOGENOUS NUCLEASES

Bernard W. Stewart, Michelle Haber and Ewan J. Ward*

Children's Leukaemia and Cancer Research Unit
Prince of Wales Children's Hospital, Randwick 2031, New South Wales, Australia

Received August 4, 1986

SUMMARY

The proportion of sheared rat liver DNA recovered from benzoylated DEAE-cellulose in the final stage following stepwise elution with NaCl and caffeine solutions was dependent upon the DNA isolation procedure. An increase in the proportion of DNA containing single stranded regions, consequent upon delay or addition of Mg^{2+} prior to phenol extraction, suggested nuclease mediated degradation. Administration of methyl methanesulphonate to rats resulted in a consistent proportional increase in the caffeine-eluted fraction. The results of caffeine gradient elution of control and alkylated DNA from benzoylated DEAE-cellulose were consistent with repair-associated single stranded regions being substrates for endogenous single strand-specific exonucleases.

INTRODUCTION

The effects of endogenous nucleases on DNA in isolated nuclei have been known for some time (1). Recognition of the structure of chromatin was a consequence of the digestion pattern observed during autodigestion mediated by endogenous DNases (2). In addition, chromatin contains regions of DNA which are hypersensitive to DNase I and which are located near the 5', and sometimes the 3', boundaries of transcribed genes (3). We report here evidence of a further class of sites within DNA which are degraded by endogenous nucleases, namely regions of DNA repair.

During excision repair of DNA, single stranded regions are generated. Structural analysis of DNA undergoing repair permits characterisation of this intermediate stage in terms of the capacity of derivatized DEAE-celluloses to bind single stranded regions in otherwise double stranded DNA (4,5). Double

---

*Present address: Queensland Institute of Medical Research, Bramston Terrace, Herston 4006, Queensland, Australia.

0158-5231/86/110903-11$01.00/0

Copyright © 1986 by Academic Press Australia.
All rights of reproduction in any form reserved.

stranded DNA is eluted from benzoylated DEAE-cellulose (BD-cellulose) by 1.0M NaCl whilst retained DNA is recovered following addition of caffeine to the NaCl solution (6). Recent investigations have established that single stranded regions may be generated in rat liver DNA during nuclei isolation (7). It thus appears likely that artefactual findings may be generated as a consequence of single strand-specific autodigestion during DNA isolation. We have investigated this possibility in relation to structural change consequent upon the repair of rat liver DNA exposed to methyl methanesulphonate (MMS) in vivo.

METHODS

Female Wistar rats were obtained and caged as previously described (8). Hepatic DNA was radiolabelled in vivo by subjecting 120g animals to partial hepatectomy and administering [^{3}H]thymidine (50 uCi) 21 and 29 hours after surgery. Animals were then maintained under normal conditions for a minimum of 2 weeks before being used for experimental purposes. MMS in aqueous solution was administered by intraperitoneal injection, control animals receiving an equal volume of physiological saline. Rats were killed by a sharp blow to the head followed by jugular vein exsanguination. The liver was homogenised in 5 volumes of 0.15M NaCl-0.015M trisodium citrate, pH 7.0 (SSC) and centrifuged at 575g for 10 minutes. The crude nuclear pellet was then resuspended in SSC (8ml) and DNA isolated by phenol extraction before overnight dialysis against 10mM Tris-HCl-1mM EDTA, pH 8.0 (TE buffer) (7). Where indicated, phenol extraction was preceded by digestion with proteinase K ('Proteinase K method'). This involved adding 2ml of the crude nuclear suspension to a lysing solution (16ml) containing 20mM Tris-HCl (pH 8.0), 0.5mM EDTA, 1M NaCl and 0.5% sodium dodecyl sulphate. After addition of proteinase K (3mg), the lysate was incubated for 40 minutes at 37°C in a shaking water bath. Following addition of an equal volume of phenol mixture (7) the preparation was stirred at room temperature for 15 minutes prior to centrifugation at 9,225g for 10 minutes. The aqueous layer was removed and extracted twice with ether prior to dialysis against TE buffer. When phenol was not employed, nuclei suspended in SSC were lysed at room temperature for 60 minutes in 8M urea, 0.24M sodium phosphate, 1mM EDTA, 1% sodium dodecyl sulphate and 0.02% NaN_3, pH 7.0; final volume 10ml ('Cell lysis method'). A 1ml aliquot of lysate was diluted to 3ml with lysing buffer and the DNA sheared by passage 6 times through a 25 gauge needle before loading onto a column of Biogel P-6DG desalting gel (12ml packed gel bed). Fractions were monitored for both absorbance (260nm, 1 absorbance unit equals 50ug DNA/ml) and radioactivity, peak DNA-containing fractions being pooled.

Preparations of DNA, sheared either in the course of preparation or, in most cases, immediately prior to chromatography, were subject to stepwise or gradient elution from BD-cellulose as previously described (7).

RESULTS

In the course of isolating DNA as chromatin, it was noted that purification of nuclei in the presence of Mg^{2+} was associated with the generation of extended single stranded regions in DNA (7). That such structural change was attributable to Mg^{2+}-activated nuclease activity was confirmed by addition of Mg^{2+} to the SSC solution in which liver was homogenised prior to phenol extraction. Stepwise elution of respective DNA preparations from BD-cellulose indicated that the proportion exhibiting single stranded character was responsive to the presence of Mg^{2+} in concentrations as low as 0.1mM (Table 1). In the absence of added Mg^{2+}, addition of chelating agents to the aqueous phase had only a slight effect on the proportion of caffeine-eluted DNA, ethyleneglycol-bis(-aminoethyl ether)N,N'-tetraacetic acid (EGTA) being the most effective. Apart from the effects described in Table 1, manipulation of the phenol extraction procedure, either by reducing

Table 1: Manipulation of Nuclease Activity During Phenol Extraction of DNA

Addition	Proportion of Caffeine-eluted DNA
None	0.067 $\pm$ 0.006
Mg^{2+}	
1.0mM	0.247
0.1mM	0.084
Chelating agents	
5mM EDTA	0.068
20mM EDTA + 5mM EGTA	0.068
10mM EGTA	0.047

Mg^{2+} or chelating agents, at the concentrations indicated, were added to the SSC solution used for homogenization prior to phenol extraction. The resultant DNA preparations were sheared and fractionated by stepwise elution from BD-cellulose (7), results being expressed in terms of the proportion of caffeine-eluted DNA. The result shown for the unmodified procedure is the mean of five experiments, other results being from a single experiment or the mean of two determinations.

the number of extractions from two to one, or by varying the pH of the aqueous phase between 5.5 and 8.0 had, at most, a marginal effect on the proportion of caffeine-eluted DNA after BD-cellulose chromatography.

The degree of single stranded character exhibited by rat liver DNA, as indicated by BD-cellulose chromatography, was found to be primarily dependent upon the isolation procedure. A range of procedures, involving lysis of liver nuclei in 8M urea, invariably resulted in a marked increase in the proportion of caffeine-eluted DNA by comparison with DNA isolated by immediate phenol extraction in the absence of Mg^{2+}. When lysis in urea was employed, least degradation occurred using the procedure outlined in the Methods section, which resulted in retention of approximately double the amount of DNA isolated by phenol extraction. Digestion with proteinase K, prior to phenol extraction, also caused an increase in the amount of caffeine-eluted DNA. Such an increase was dependent upon the period for which the preparation was maintained at 37°C for digestion purposes (Fig. 1).

Use of different DNA isolation procedures resulted not only in an increase in the caffeine-elutable fraction of DNA from control animals, but a parallel increase in the amount of this fraction in DNA preparations from MMS-treated animals (Table 2). The implication, of a proportional relationship independent of isolation procedure, between control and MMS-exposed DNA, was examined further. Whilst some differences were apparent, a similar pattern, of increased binding to BD-cellulose by DNA induced by MMS, was observed irrespective of the method of DNA isolation. Thus, maximum effect was recorded 30 minutes after treatment, whilst structural change was not evident in DNA isolated 2 hours after administration of the alkylating agent (Fig. 2).

The affinity of various preparations of DNA containing single stranded regions for BD-cellulose was determined by caffeine-gradient elution, a proportional relationship having been established between nucleotide length and eluting caffeine concentration (9). DNA preparations containing a high

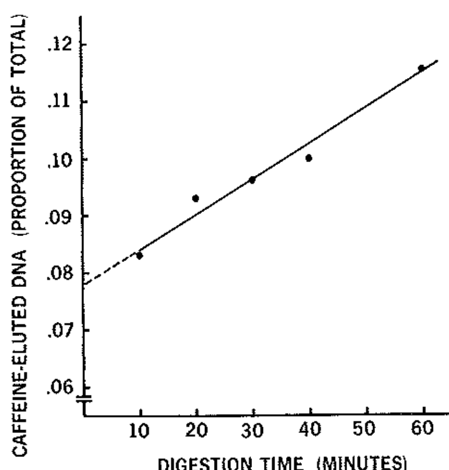

Figure 1. Change in the proportion of rat liver DNA eluted with caffeine from BD-cellulose as a consequence of incubation with proteinase K prior to phenol extraction as described in the 'Methods' section. After shearing (6 passages through 25-gauge needle) the DNA preparations were subjected to stepwise elution from BD-cellulose in the presence of 0.3M NaCl, 1.0M NaCl and 1.8% caffeine respectively.

Table 2: Effect of Different Isolation Procedures on BD-cellulose Binding Characteristics of Hepatic DNA from Control and MMS-treated Rats

In Vivo Treatment	Isolation Procedure		
	Phenol extraction	Cell Lysis	Proteinase K
Control	0.070	0.138	0.091
MMS	0.168	0.220	0.230

DNA was isolated both from control rats, and from rats receiving 120 mg/kg body weight MMS 3 hours before sacrifice, by procedures described in the Methods section. After shearing, DNA was subject to stepwise elution from BD-cellulose (7), results being expressed in terms of the proportion recovered during caffeine elution.

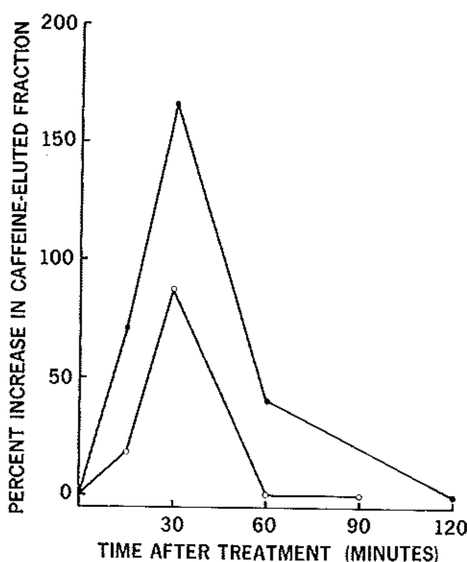

Figure 2. Patterns of MMS-induced increase in the proportion of caffeine-eluted DNA as determined using different DNA isolation procedures. Rats were killed at intervals following injection of 60 mg/kg body wt. MMS and DNA prepared either by immediate phenol extraction of the crude nuclear pellet (O) or by lysis of the nuclei in 8M urea (●) as described in the Methods section. Following stepwise elution of each sheared preparation from BD-cellulose, the proportion of caffeine-eluted DNA was expressed relative to that using DNA isolation from control animals by the respective procedures.

proportion of caffeine-elutable DNA also exhibited extensive degradation as indicated by gradient elution insofar as DNA was recovered throughout the 0-0.8% gradient. The results indicated single stranded regions from 10 to greater than 20,000 nucleotides, with no evidence of size specificity. On the other hand, binding of phenol-extracted preparations to BD-cellulose could be better characterised despite the smaller proportion of caffeine-eluted DNA and the consequent low levels of radioactivity in individual gradient fractions. In control preparations (Fig. 3a), the amount of DNA containing single stranded regions increased early in the caffeine gradient (fractions 21-30), after which the level of radioactivity fell slowly to background level. The single fraction containing greatest radioactivity (fraction 30) corresponded

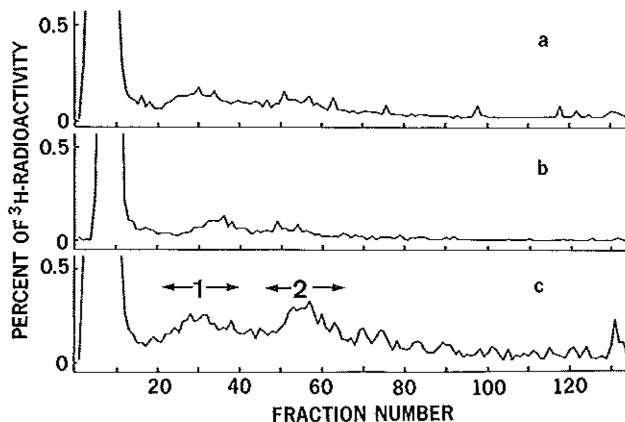

Figure 3. Effect of MMS treatment in vivo on chromatographic behaviour of rat liver DNA subjected to caffeine gradient elution from BD-cellulose. DNA was isolated by immediate phenol extraction of the crude nuclear pellet from control rats (a), rats killed 30 minutes after injection of 60 mg/kg body wt. MMS (b) and 3 hours after injection of 120 mg/kg body wt. MMS (c). After elution with 0.3 and 1.0M NaCl (fractions 1-5, 6-10 respectively), DNA containing single stranded regions was recovered during elution in the presence of 0 - 0.8% caffeine (fractions 11-129), the column being finally washed with 2% caffeine (fractions 130-135). Radioactivity per chromatogram was 3-7 x 10^4 dpm, and each result illustrated is typical of at least two chromatograms made using DNA isolated from different animals after the respective in vivo treatments. For details regarding designated peaks '1' and '2', see text.

to the binding of single stranded DNA 200 nucleotides in length (9). DNA from rats receiving 60 mg/kg body weight MMS and killed when binding to BD-cellulose was maximal, likewise resulted in a broad spread of DNA early in the caffeine gradient (Fig. 3b). Administration of 120 mg/kg body weight MMS permitted two broad peaks, designated '1' and '2' in Fig. 3c, to be discerned. The larger (peak 2) exhibited a local maximum between fractions 53 and 58, corresponding to the binding of DNA from 1200 to 1800 nucleotides (9). A further conspicuous feature of this chromatogram was the recovery of DNA throughout the gradient and in particular beyond fraction 100.

DISCUSSION

In monitoring alkylation-induced single stranded regions in DNA *in vivo*, the effectiveness of particular isolation procedures to preserve DNA structure can be rationalised in terms of the need to limit endogenous nuclease activity (10,11). Mammalian nucleases are generally activated by cations (12) and the effect of adding Mg^{2+} to SSC buffer on the yield of caffeine-eluted DNA (Table 1) is consistent with such an increase being nuclease-mediated. Nucleolytic activity has been ascribed to the non-histone protein component of chromatin (13,14). The enzyme(s) reponsible for increased binding of DNA to BD-cellulose would appear to be tightly integrated within chromatin, since addition of proteinase K failed to inactivate it and paradoxically permitted further degradation during the incubation (Fig. 1).

Excision repair of DNA necessitates strand breakage. Stewart (5) suggested that the alkylation-induced binding of DNA to BD-cellulose was a consequence of the polymerization and ligation stages of repair being rate-limiting thereby permitting accumulation of repair intermediates. In control preparations, binding of a small proportion of mammalian DNA to BD-cellulose in the absence of cell replication is attributable to transcription (15,16). Previous work (7) was consistent with this class of structural lesion being susceptible to endogenous nucleases. Data included in Table 2 and Fig. 2 suggest that less satisfactory procedures of DNA isolation result in a higher proportion of transcription- and repair-associated sites binding to BD-cellulose than in preparations of DNA obtained by immediate phenol extraction in which nuclease activity is either absent or minimal. An increase in the number of BD-cellulose binding sites is attributable to nucleolytic attack upon any repair- or transcription-associated lesions which would not otherwise be retained during 1M NaCl elution. Such effects would include enlargement of single stranded regions shorter than 5 nucleotides (16).

Weintraub (17) has concluded that DNase I hypersensitive sites are regions of DNA 200 to 400 base pairs in length and having certain sequence characteristics. The caffeine-gradient chromatogram of DNA from control animals is consistent with such configurations being responsible for retention of a minor fraction of the total DNA when double stranded DNA was eluted with 1.0M NaCl. Structural characteristics of lesions associated with alkylation repair require more detailed consideration. In the first instance, the size of repair 'gaps' might be related to 'patch' size, which, in the case of MMS, has been estimated to an upper limit of 40 nucleotides (18). However, asynchrony between the initial and final stages of DNA repair, at least when produced by inhibitors, causes an increase in the length of repair patches (19). In the chromatogram from MMS-exposed DNA (Fig. 3c), peak 1 may thus be attributable to the structure of repair lesions _in vivo_. However, peak 2 is indicative of 'gap' sizes too large (up to 1800 residues) to be associated with repair (19). One explanation for the apparently extended 'gaps' is that shorter single stranded regions have been subject to nucleolytic attack. Similar degradation may be responsible for recovery of DNA at higher caffeine concentrations (fractions 80-120) suggestive of even more extensive degradation of MMS-exposed DNA. Finally it should be noted that the data do not permit definitive characterization of single stranded regions because a single sheared fragment may contain more than one such site.

The structural changes detected in this investigation, in terms of their dependence on incubation time (Fig. 1) and cation concentration (Table 1) and on their physical characteristics (Fig. 3) are compatible with nucleolytic activity. More specifically, the increase in size of repair-associated single stranded regions is compatible with an exonucleolytic activity acting upon gaps or nicks presumably introduced into DNA initially by repair-associated endonucleases. Such activity may be distinguished from

the endonuclease characterised by Machray and Bonner (20) in rat liver chromatin. The latter nicked, but did not degrade, supercoiled DNA. Chow and Fraser (21) have described an extranuclear endo-exonuclease of Neurospora crassa which senses single strand-like regions in superhelical DNA. An immunochemically similar endo-exonuclease occurs in nuclei, the activity of which is responsive to DNA damage (MJ Fraser, personal communication); this enzyme is believed to have a role in DNA repair. By analogy, degradation of repair lesions during isolation of DNA may be a consequence of nuclease activity which, in vivo, has a role in the repair process. In this regard, Cleaver (22) has postulated that control of Ca^{2+}, Mg^{2+}-dependent nucleases is mediated by poly(ADP-ribosyl)ation. Relationships between these various observations remain to be clarified. Meanwhile, utilisation of isolation procedures which minimise nuclease activity would appear to be essential to the structural analysis of DNA during transcription and replication as well as during repair.

ACKNOWLEDGEMENTS

We are grateful to Maria Kavallaris for expert technical assistance. This research was supported by a grant from the National Health and Medical Research Council (Australia). The Children's Leukaemia and Cancer Research Unit is supported by the Children's Leukaemia and Cancer Foundation (Australia).

REFERENCES

1. Hyodo, M. and Ono, T. (1970) Biochim. Biophys. Acta 213, 228-230.
2. Hewish, D.R. and Burgoyne, L.A. (1973) Biochem. Biophys. Res. Commun. 52, 475-481.
3. Elgin, S.C.R. (1981) Cell (Cambridge, Mass.) 27, 413-415.
4. Strauss, B.S. (1981) in DNA Repair. A Laboratory Manual of Research Procedures (Friedberg, E.C. and Hanawalt, P.C., Eds.) pp 319-339 Marcel Dekker, New York.
5. Stewart, B.W. (1981) Cancer Res. 41, 3238-3243.
6. Caffin, N.A. and Mackinlay, A.G. (1975) Anal. Biochem. 63, 442-451.
7. Ward, E.J., Haber, M., Norris, M.D. and Stewart, B.W. (1985) Biochemistry 24, 5803-5809.
8. Stewart, B.W. and Haski, R. (1984) Chem.-Biol. Interact. 52, 111-128.
9. Haber, M. and Stewart, B.W. (1981) FEBS Lett. 133, 72-74.
10. Paul, I.J. and Duerksen, J.D. (1976) Biochem. Biophys. Res. Commun. 68, 97-105.

11. Vanderbilt, J.R., Bloom, K.S. and Anderson, J.N. (1982) J. Biol. Chem. 257, 13009-13017.
12. Sierakowska, H. and Shugar, D. (1977) Prog. Nucl. Acid Res. 20, 59-130.
13. Vinter, V.G., Belyaeva, M.I. and Zotkina, N.L. (1974) Biochemistry (Engl. Transl.) 39, 765-769.
14. Lambert, M.W. and Studzinski, G.P. (1979) Biochem. Biophys. Res. Commun. 91, 1481-1487.
15. Tapiero, H., Leibowitch, S.A., Shaool, D., Monier, M.N. and Harel, J. (1976) Nucleic Acids Res. 3, 953-963.
16. Stewart, B.W., Huang, P.H.T. and Brian, M.J. (1979) Biochem. J. 179, 341-352.
17. Weintraub, H. (1983) Cell (Cambridge, Mass.) 32, 1191-1203.
18. Francis, A.A., Snyder, R.D., Dunn, W.C. and Regan, J.D. (1981) Mutat. Res. 83, 159-169.
19. Cleaver, J.E. (1985) Cancer Res. 45, 1163-1169.
20. Machray, G.C. and Bonner, J. (1981) Biochemistry 20, 5466-5470.
21. Chow, T.Y.K. and Fraser, M.J. (1983) J. Biol. Chem. 258, 12010-12018.
22. Cleaver, J.E. and Morgan, W.F. (1985) Mutat. Res. 150, 69-76.