NON-RANDOM BINDING OF A CHEMICAL CARCINOGEN TO THE DNA IN CHROMATIN

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Received Eebruary 9,1977

SUMMARY - The distribution of a liver carcinogen (N-hydroxy-2-aminofluorene) along the DNA of chromatin has been studied using two nucleases as probes for the structure of chromatin. Rats were injected with the carcinogen and killed at various times after the injection. The nuclei of the liver were prepared and digested with Staphylococcal nuclease or pancreatic nuclease DNAse I. We show that the carcinogen is non randomly distributed along the DNA of chromatin since it binds preferentially to the regions of chromatin digested by the Staphylococcal nuclease whereas it is preferentially bound to the DNAse I resistant fraction. Our results also indicate that the two nucleases do not recognize exactly the same region of chromatin.

The digestion of chromatin by nucleases has been recently used to study the distribution of chemical carcinogenes along the molecule of DNA in chromatin (1-4). Using pancreatic nuclease DNAse I several reports (1-3) have shown the heterogeneous distribution of the DNA-alkylation products in liver chromatin after in vivo administration of the hepato-carcinogen dimethylnitrosamine. Using the same enzyme it has been shown that the distribution of N-hydroxy-2-aminofluorene, another liver carcinogen, was non-random along the DNA in liver chromatin (3). We have used Staphylococcal nuclease (4) to study the distribution of N-acetoxy-N-2-acetylaminofluorene in chromatin modified in vitro and we show that the carcinogen binds preferentially to the region of chromatin sensitive to the enzyme. This is in contrast to the result obtained with DNAse I by R. Ramanathan et al. (3), who show that in vivo this molecule binds preferentially to the DNAse I resistant fraction. However, it is difficult to know whether this difference is due to the fact that we

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ISSN 0006-291X

are using different enzymes or because our study has been made in vitro. In the present study we have thus compared the action of Staphylococcal nuclease and pancreatic nuclease on chromatin modified by the carcinogen in vivo. Our results confirm that the carcinogen binds preferentially to the Staphylococcal nuclease sensitive fractions of chromatin but that it is preferentially bound to the DNAse I resistant fractions. These results also indicate that the two enzymes do not recognize exactly the same regions of chromatin.

MATERIALS AND METHODS

The animals used in this study were male and female rats of the BD 4 strain obtained from the Centre International de Recherches sur le Cancer, Lyon, France. The animals weighed about 150 g to 170 g at the time of sacrifice. 2-acetylaminofluorene (AAF) labelled with ^{14}C in position 9 (Specific activity 26 Ci/mM) dissolved in propanediol was injected intraperitonially to the rats at a dose of 55 µCi/100 g body weight. The animals were killed by decapitation at various times (10 min to 72 hours) after injection of the radioactive carcinogen. Livers were rapidly removed and frozen in liquid nitrogen. Rat liver nuclei were prepared as described by Axel et al. (5) : the tissue was disrupted in 10 mM Tris - HCl (pH 8.0), 0.25 M sucrose, 1 mM $CaCl_2$ in a Potter homogenizer and filtered through five layers of gauze. The nuclei were collected by centrifugation and washed two times in the same buffer containing 0.5% Triton-X-100 (v/v) followed by three washes in the buffer without Triton. Pancreatic DNAse (DNAse I, Worthington) and Staphylococcal nuclease (Worthington) digestions were performed in 10 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 1 mM $CaCl_2$ at 37°C. The digestions were stopped by the addition of a five fold excess of EDTA over the divalent ion concentration. The digestion of the samples was monitored by measuring the amount of acid soluble material (DNA and radioactive carcinogen) in 5% perchloric acid. Values for zero time and endogenous digestion were substracted from the experimental values. The addition of pancreatic ribonuclease did not change the value obtained for the endogenous digestion.

The resistant fractions of DNA and the total DNA were extensively deproteinized with Proteinase K (100 µg/ml) in the presence of 0.4% SDS, 0.8 M NaCl and 5 mM EDTA followed by an extraction with phenol-chloroform-isoamylalcohol (24:24:1 v/v). The DNA was then precipitated with two volumes of 95% ethanol (overnight at -20°C).

RESULTS AND DISCUSSION

Fig. 1 shows the time course of AAF and metabolite uptake by rat liver DNA. The rat liver DNA was isolated after ^{14}C-AAF injection and extensively deproteinized as indicated in materials and methods. We have plotted the percentage of modifi-

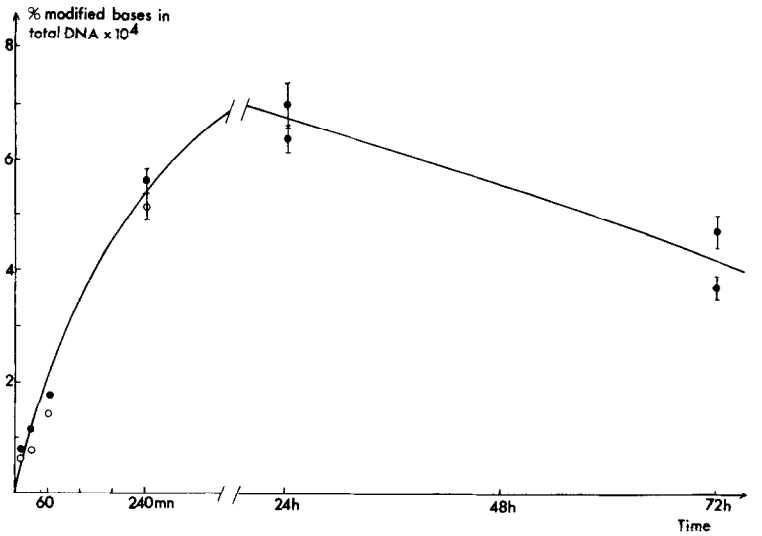

Figure 1

Time course of ^{14}C-AAF uptake by rat liver DNA. At the indicated times the rats were killed and DNA was isolated and purified as described in "Materials and methods". o female rats ● male rats

cation(number of fluorene residues/100 bases) of the DNA as a function of time elapsed between the injection of the carcinogen and the sacrifice. Labelling of DNA occurs within 10 min after administration of the radioactive carcinogen and increases very rapidly during the initial 4 hours. The maximum level of carcinogen binding was found to occur 24 h after injection, then it declined slowly.

In order to study the distribution and the sites of fixation of the carcinogen along the DNA, we have made use of two nucleases (Staphylococcal nuclease and pancreatic nuclease DNAse I) as probes for the structure of chromatin (6-8). Nuclei were isolated from the livers of rats killed at various times after the injection of the carcinogen and immediately sujected to degradation by the nuclease.

Staphylococcal nuclease allows the fractionation of the DNA of chromatin into two zones (6) : about half of the DNA is digested by the enzyme, whereas the remainder, inaccessible to the enzyme, is not digested. The amount of modified base was measured in the digestible and resistant fraction. The results presented in Table I indicate that the carcinogen is bound in

Table I - Percentage of modified bases in digested and staphylococcal nuclease resistant chromatin DNA.

Time between injection and sacrifice	% modification of digested DNA x 10^4	% modification of resistant DNA x 10^4	k
10 min	1.13 ± 0.06	0.52 ± 0.03	2.2 ± 0.2
30 min	0.73 ± 0.07	0.56 ± 0.03	1.3 ± 0.2
1 h	1.74 ± 0.13	1.40 ± 0.08	1.2 ± 0.2
4 h	4.44 ± 0.49	3.29 ± 0.18	1.3 ± 0.2
24 h	7.23 ± 0.42	4.89 ± 0.35	1.5 ± 0.2
72 h	5.70 ± 0.41	4.09 ± 0.22	1.4 ± 0.2

% modification = number of fluorene residues/100 bases

$$k = \frac{\text{\% modification of digested DNA}}{\text{\% modification of resistant DNA}}$$

both regions. The percentage of modification is always bigger in the digestible regions. 10 min after administration of the carcinogen it is two times greater than that of the resistant regions, but after 30 min and later the difference of the modification between the digestible and resistant fractions decreases and the ratio k (see legend to table I) levels out at 1.3. This result is consistent with our previous finding (4) obtained with chromatin modified by the same carcinogen in vitro and where we have shown that the regions of chromatin accessible to Staphylococcal nuclease are 1.8 times more modified than the inaccessible regions. Thus, although there is a difference in the reactivity of the two regions, it is noteworthy that the regions of chromatin tightly covered with histone can fairly well react with the carcinogen, this being true in vitro as well as in vivo. The reactivity of the DNA of chromatin with the carcinogen can be explained in view of the recent finding concerning the structure of nucleosomes, since it has been shown that most of the DNA lies on the outside of the chromatin fiber, wrapped around a protein core (9-11). Thus, the DNA of chromatin

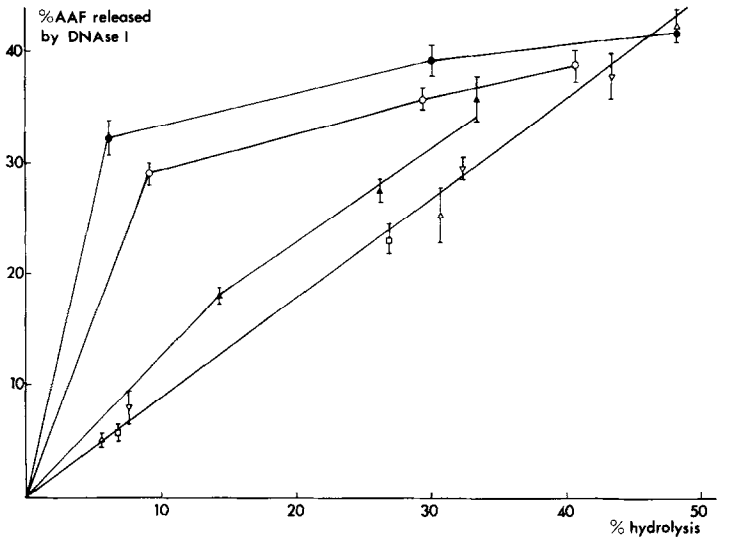

Figure 2

Plot of the carcinogen released as a function of the nucleotides released during the digestion of rat liver chromatin by pancreatic nuclease DNAse I. Rats were killed at different times after injection of the carcinogen | • 10 min o 30 min ▲ 1 hour △ 4 hours ▽ 24 hours □ 72 hours|. The liver nuclei were prepared as indicated in "Materials and Methods" and digested with DNAse I.

must be very accessible to a small molecule like AAF ; furthermore, the DNA of the nucleosomes is constrained (12) and bears perhaps a secondary structure which alters its reactivity with the carcinogen.

That the DNA, tightly bound with proteins, is able to react well with the carcinogen is confirmed by the experiments with DNAse I. A recent paper by R. Ramanathan et al. (3) has shown that when 50% of the total nucleotides of chromatin are digested by DNAse I, the carcinogen, bound to nuclease "inaccessible" regions, is four times greater than that bound to nuclease "accessible" regions. In this last study livers of rats killed 4 hours and more after the injection were used. We wanted to know if this was also true at very short times after the injection (see below).

On the other hand DNAse I is able to digest selectively the active genes of chromatin ; indeed two recent reports (13-

14) have shown that 70% - 80% of the active genes are digested when only 10% of the DNA of reticulocyte or oviduct nuclei are digested with DNAse I. It was thus interesting to digest the chromatin with DNAse I in order to check how much of the carcinogen was bound to the active genes, making the assumption that DNAse I is also able to recognize these genes in rat liver nuclei. The results of DNAse I digestion are presented in Fig. 2 : 10 min and 30 min after the injection, when 8% to 10% of the DNA is digested, 30 to 32% of the carcinogen is removed, but when a further 40% of DNA is digested, only 8 - 10% more carcinogen is released. The resistant fraction of DNA is 1.5 to 1.8 times more modified than the digested fraction when 50% of the total DNA of chromatin is digested. In Fig. 2 the results of the digestion of nuclei of rats killed one hour and later after the injection of carcinogen are also shown. The rapid release of carcinogen at the beginning of the digestion is not found anymore, but we observe a uniform release of the carcinogen during the digestion. However, the heterogeneity of distribution of the carcinogen between the digestible and the resistant fractions remains (i.e. when half of the DNA has been digested we find that the resistant fraction is 1.5 times more labelled than the digestible fraction). Thus, the fraction of DNA resistant to DNAse I seems to be more labelled with the carcinogen at any time after the injection between 10 min and 72 hours. Moreover, R. Ramanathan et al. (3) have shown that this difference of labelling is observed even one week after the injection.

The comparison of the results obtained with the two nucleases shows that the enzymes do not recognize the same regions of chromatin, since in one case (DNAse I) the resistant fraction is more labelled than the digested one, whereas the opposite is true with Staphylococcal nuclease.

Finally, using DNAse I, we can distinguish three zones in rat liver chromatin :

a) a first zone (I) which is rapidly modified by the carcinogen, containing about 10% of the total DNA and perhaps the active genes, since it is digested first by DNAse I. The higher reactivity of this portion of DNA may be due to a greater accessibility of these genes to the carcinogen, probably because they bear a structure different from the rest of the

chromatin. During the cell cycle all the active genes would in turn be able to belong to zone I.

b) a second zone (II) less accessible to the carcinogen but digested by DNAse I and where the fixation of carcinogen occurs only later after the injection.

c) a third zone (III) which is able to bind 1.5 to 1.8 times more carcinogen than the DNAse I sensitive zones (I + II).

This scheme of binding of the carcinogen to chromatin is certainly over-simplified, but the exact mechanism of in vivo fixation of a carcinogen to DNA will be very difficult to ascertain, since there is a competition between its binding and its removal by enzymatic repair mechanism ; further understanding of these mechanisms will thus be necessary.

Nevertheless our results indicate that the carcinogen is non randomly bound to the DNA of liver chromatin in vivo and that there is not a direct relationship between the accessibility of the DNA to nuclease and its reactivity with a carcinogen.

ACKNOWLEDGMENTS

The authors wish to thank Dr L. Tomatis and R.M. Montesano from the "Chemical Carcinogenesis Department of C.I.R.C., Lyon, France" for providing rats and technical facilities. The technical assistance of Miss H. Brésil was greatly appreciated.

We thank Dr M. Daune and Dr H. Ohlenbusch for helpfull discussions.

G.M. was a recipient of a fellowship from the "Ligue Nationale Française contre le Cancer".

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