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ALKYLATION OF INDIVIDUAL GENES IN RAT LIVER BY THE CARCINOGEN N-NITROSODIMETHYLAMINE

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A method for quantifying alkylated bases in defined gene sequences of rat liver following carcinogen treatment is described. After restriction, the genomic DNA is heated to 65° at pH 8.0 to cause depurination of 7-alkylguanine and 3-alkyladenine residues. Reaction with spermidine then generates strand breaks. After gel electrophoresis and Southern transfer, sequences of interest are visualized using specific probes. The presence of strand breaks in restriction fragments reduces their intensity compared to unmethylated fragments. The method was applied to show that the transcriptionally active albumin gene is damaged by the hepatocarcinogen N-nitrosodimethylamine to a much greater extent than the untranscribed IgE gene in rat liver.

Most measurements of DNA damage produced by chemical carcinogens have utilized techniques that average in their effects over entire genome (1). Attempts to quantify damage in subsets of the genome have usually relied on the isolation of DNA having characteristic physical properties which differ from the bulk of genomic DNA such as repetitive sequences, satellite DNA, ribosomal DNA and eu- and hetero- chromatin (2,3). These methods still suffer from the disadvantage of averaging the effects over maybe a few percent of the total cellular DNA. Elucidation of the molecular mechanisms of tumour induction by carcinogens, however, will require detailed knowledge of DNA modification within defined gene sequences in target tissues that can be related to alterations in gene function.

Using techniques pioneered by Bohr et al. (4) for quantifying pyrimidine dimers, Thomas et al. (5) have recently described a method for measuring base adducts in specific mammalian genes. This technique, however, which utilizes Escherichia coli UvrABC excision nuclease, is limited to carcinogen adducts which are substrates for this enzyme. Damage caused by methylating agents is not recognized by the nuclease system. Here we describe a technique for measuring alkylation of individual genes, and we show that the

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hepatocarcinogen N-nitrosodimethylamine (NDMA) preferentially methylates a gene that is actively transcribed in rat liver (albumin) compared to an untranscribed gene (immunoglobulin ϵ-heavy chain constant domain, IgE).

MATERIALS AND METHODS

Male Sprague-Dawley rats (90-100g) were injected i.p. with various doses (shown in Fig. 1) of NDMA in normal saline. Six h later, the rats were sacrificed by cervical dislocation, and hepatic DNA was prepared by standard methods (6). Ten μg samples of DNA were restricted with either PvuII or Hind III. The buffer was replaced with TE (10 mM Tris, pH 8.0/1 mM EDTA) by dialysis, one tenth volume of a solution containing 250 mM Tris (pH 8.0) and 5 mM spermidine was added, and the resulting solution was heated at 65° for 16h. The DNA samples were then subject to electrophoresis using 0.7% agarose at 1 Vcm^{-1} for ca 20 h. The DNA was finally transferred to a Gene Screen Plus nylon membrane (NEN Research Products, Boston MA) under conditions recommended by the manufacturer.

The plasmids pRSA13 containing rat albumin cDNA (7), and pSD11-ϵ, containing a genomic clone of IgE (8), were kindly supplied by Drs. Tom Sargent of the N.I.H., Bethesda, MD, U.S.A. and Ulf Pettersson of the Biomedical Center, University of Uppsala, Sweden, respectively. A 600 bp Hinf I fragment from pRSA13 and a 1500 bp BamHI-BglII fragment from pSD11-ϵ were used as probes. The restriction fragments were electroeluted from 1% agarose gels and further purified on a hydroxyapatite column eluted with 400 mM phosphate, pH 7.0. The fractions containing the restriction fragments were finally dialyzed extensively against TE. One hundred ng aliquots were radio-labelled with ^{32}P using second strand synthesis (9) to a specific activity of ca 10^8cpm μg^{-1}. The labelled probes were hybridized under stringent conditions to the rat liver DNA bound to the nylon membrane as described by the supplier. Bands were visualized by autoradiography at -70° using XAR-5 film with an intensifying screen.

RESULTS AND DISCUSSION

The method we have developed for quantifying alkylated bases in specific genes in rat liver following carcinogen treatment is similar to that used by Bohr et al. for measurement of pyrimidine dimers (4) and Thomas et al. for measurement of bulky lesions (5). However, instead of using phage T4 endonuclease V or UvrABC excision nuclease (2), after digesting the genomic DNA with a restriction endonuclease, the DNA is heated in the presence of spermidine. Under these conditions, 7-alkylguanine and 3-alkyladenine are unstable and spontaneously depurinate to form apurinic (AP) sites. Spermidine then reacts at AP sites to generate strand breaks (10,11). Single strand breaks may then be analyzed by denaturing gels or double strand breaks by non-denaturing gels. After Southern transfer, sequences of interest are visualized using specific probes. A restriction fragment in which there are alkylated purines will show diminished intensity compared to the unalkylated fragment and a smear of lower molecular weight fragments may be visible. Any

hot spots for alkylation would lead to the formation of a sharp band of lower molecular weight than the parent fragment.

The method is suitable for monitoring any lesion at the 7-position of guanine or the 3-position of adenine which leads to spontaneous depurination. Spermidine treatment does not cause breaks at phosphotriesters (although use of an alkaline denaturing gel would cause breaks at these sites). Furthermore, adducts at the O^6-position of guanine are not detected by this method since they are refractory to depurination. At least for simple alkylating agents, however, 7-alkylguanine and 3-alkyladenine are likely to give a good indication of the distribution of total alkylation damage in DNA (12).

The results of an experiment to assess the damage caused by NDMA in the transcriptionally active albumin gene and the untranscribed IgE gene in rat liver are shown in Figure 1. When the hepatic DNA is cleaved with PvuII, the probe we selected for the albumin gene hybridizes to a 7.5 kb fragment. Similarly, the probe we selected for the IgE gene hybridizes to a 7.2 kb HindIII fragment. The intensity of the 7.2 kb HindIII fragment in the IgE gene remains unchanged as the dose of NDMA increases from zero to 3 mg/kg. This implies that most of these fragments have not been interrupted by strand breaks. In contrast, the intensity of the 7.5 kb PvuII fragment in the

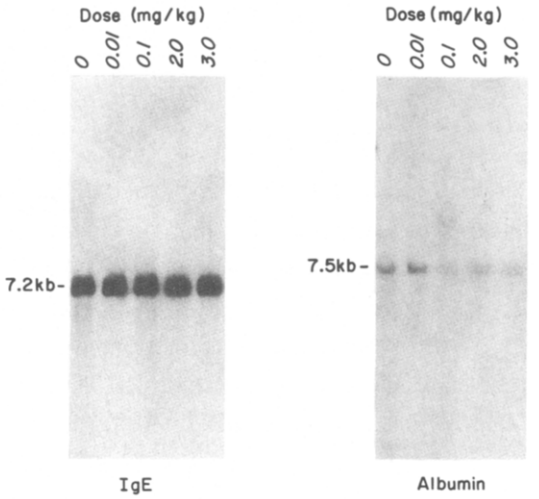

Figure 1. Assay for damage caused by NDMA in the IgE and albumin genes of rat liver. Genomic DNA was isolated from rats treated with 0-3 mg/kg NDMA as described in Methods. Ten μg of DNA from each rat were digested with a restriction endonuclease (PvuII or HindIII) then treated with spermidine. The resulting fragments were fractionated by gel electrophoresis, transferred to a nylon membrane, and hybridized to a probe for IgE or albumin. The 5 lanes on the left contain DNA digested with PvuII and probed with an IgE genomic clone. The 5 lanes on the right contain DNA digested with HindIII and hybridized to an albumin cDNA probe.

albumin gene decreases as the NDMA dose increases. This trend suggests that an increasing fraction of these fragments contain breaks (double stranded in this case, since the gel was non-denaturing) caused by NDMA.

The data in Figure 1 indicate that the albumin locus is damaged by NDMA to a much greater extent than the IgE locus at which we could detect no damage. This effect is not due to different sizes of the target restriction fragments in the two genes, since we chose these to be essentially equal. Our observations are in agreement with data which suggest that transcriptionally active DNA is more susceptible to NDMA-induced damage than untranscribed DNA (13-15). It appears that in rat liver, the albumin gene is considerably more accessible than the IgE gene to damage by reactive intermediates derived from NDMA. This suggests that any gene involved in the initiation of hepatocarcinogenesis by NDMA is likely to be one that is being actively transcribed.

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