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SITE-SPECIFIC HYPOMETHYLATION OF c-*myc* PROTOONCOGENE IN LIVER NODULES AND INHIBITION OF DNA METHYLATION BY *N*-NITROSOMORPHOLINE

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Abstract—The protooncogene c-*myc* was investigated in *N*-nitrosomorpholine-induced rat liver nodules to elucidate the role of altered DNA methylation in chemical carcinogenesis. Furthermore, *Micrococcus luteus* DNA and chicken erythrocyte DNA were modified *in vitro* by reactive metabolites of *N*-nitrosomorpholine, generated by P450-dependent monooxygenases. The modified DNAs were less methylated *in vitro* than control DNAs by DNA-(cytosine-5)-methyltransferase (DNA methylase). The DNA methylase assay and ^{32}P-postlabeling analysis revealed lowered levels of DNA methylation in nodular DNA. In nodular tissue, c-*myc* messenger RNA levels were found to be increased compared to normal liver. DNA methylation analysis using the restriction endonucleases HpaII/MspI indicated hypomethylation in the first intron of c-*myc* DNA in liver nodules. The results suggest that genotoxic lesions may cause stably inherited, aberrant DNA methylation patterns which may be responsible for site-specific hypomethylation of the c-*myc* protooncogene in liver nodules.

To investigate possible relations between genotoxic lesions, hypomethylation of DNA and persistently increased expression of critical genes at cancer prestages, we studied effects of *N*-nitrosomorpholine-induced DNA alterations on DNA-(cytosine-5)-methyltransferase (DNA methylase§) function *in vitro* and on hypomethylation of c-*myc* protooncogene in hepatocyte nodules. The cyclic nitrosamine *N*-nitrosomorpholine represents a strong carcinogen in experimental hepatocarcinogenesis [1, 2]. It is found in ambient air at working places [3] and in human urine [4]. It has also been reported as a common contaminant of toiletries and cosmetic products [5] and is found in snuff tobacco [6]. After treatment with *N*-nitrosomorpholine *in vivo* alkylated DNA bases have been found in rat liver DNA [7]. *N*-Nitrosomorpholine is extensively metabolized in rats. Recent studies support the hypothesis that cyclic nitrosamines are metabolically activated by α-hydroxylation [8–10].

In eukaryotic DNA cytosine methylation provides a signal function with plurivalent potential, leading, for example, to alterations of chromatin structure [11] and to decreased gene expression [12, 13].

Alterations of the DNA methylation pattern such as hypomethylation have been frequently observed at liver cancer prestages [14–18]. It is conceivable that genotoxic lesions may impair maintenance methylation of DNA by DNA methylase [19]. Low fidelity repair of these lesions may cause an aberrant methylation pattern which is inherited in subsequent cell cycles [20]. Hypomethylation of genomic DNA at regulatory sites may in part be responsible for increased expression of many proteins in liver nodules, such as drug metabolizing enzymes [21–23]. For example, it has been demonstrated recently that the gene of NAD(P)H:quinone reductase is hypomethylated in liver nodules [24]. However, the relevance of altered methylation patterns for carcinogenicity still has to be elucidated.

The protooncogene c-*myc* was studied since it codes for a nuclear protein which plays an important role in the control of proliferation/differentiation of hepatocytes [25]. Expression of c-*myc* which is low in resting liver, is temporarily increased in regenerating liver and is persistently increased in liver nodules, hepatomas and hepatocellular carcinomas [26–28]. However, the mechanisms responsible for the loss of controlled c-*myc* expression are still unknown.

The present report demonstrates site-specific hypomethylation of c-*myc* in *N*-nitrosomorpholine-induced liver nodules. Moreover, DNA methylase function was found to be inhibited using DNAs as the substrate which were modified by treatment with P450-generated *N*-nitrosomorpholine metabolites. A causal relationship between genotoxic lesions leading to stably inherited, aberrant methylation patterns and hypomethylation of oncogenes such as c-*myc* is suggested.

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§ Abbreviations used: DNA methylase, DNA-(cytosine-5-)-methyltransferase (EC 2.1.1.37); m^{5}C, 5-methylcytosine.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: Chicken erythrocyte DNA and T4 polynucleotide kinase from PL Biochemicals

(Milwaukee, WI, U.S.A.); *Micrococcus luteus* DNA and dithiothreitol from the Sigma Chemical Co. (St Louis, MO, U.S.A.); *S*-Adenosyl-L-(methyl)-methionine and nuclease P1 from Boehringer (Mannheim, F.R.G.); *S*-adenosyl-L-[*methyl*-^{3}H]-methionine (15 Ci/mmol) and [γ-^{32}P]ATP from Amersham (Amersham, U.K.); proteinase K from Appligène (Strasbourg, France); micrococcal nuclease and spleen phosphodiesterase from Worthington Biochemicals (Freehold, NJ, U.S.A.). DNA methylase was prepared from the rat spleen as described [29]. A partially purified enzyme with a specific activity of 3 units/mg protein was used. Units are expressed in pmol CH_3 incorporated into 10μg chicken erythrocyte DNA in 1 hr by 1 mg protein.

Measurement of 5-methyldeoxycytidine (m^{5}C) in DNA samples. m^{5}C was determined by the method of Wilson *et al.* [30], modified as follows: DNA (10 μg) was digested at 37° for 3 hr with micrococcal nuclease (10 μg) and spleen phosphodiesterase (10 μg) in a reaction mixture (total volume: 25 μL) containing 20 mM sodium succinate and 10 mM $CaCl_2$, pH 6.0. The digested DNA was diluted 30-fold. Aliquots (2.5 μL) of the mixture of nucleoside monophosphates were converted to ^{32}P-labeled nucleoside 3',5'-bisphosphates by incubation for 2 hr at 37° with 2 units of T4 polynucleotide kinase and 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol) in 50 mM Tris–HCl, pH 9.0, containing 100 μM ATP, 10 mM $MgCl_2$, 1 mM spermidine and 15 mM β-mercaptoethanol in a volume of 10 μL. Labeled nucleoside 3',5'-bisphosphates were then converted to nucleoside monophosphates by incubation for 2 hr at 37° with nuclease P1 (0.175 μg) in 50 mM ammonium acetate, pH 5.3. Labeled nucleoside 5'-monophosphates were separated by two-dimensional TLC using 20 × 20 cm glass-cellulose plates. Unlabeled nucleotides were visualized under UV light (254 mm). Chromatography in the first dimension was carried out overnight with isobutyric acid: H_2O: NH_4OH (66:20:1, by vol.) using a 3 cm wick (Whatmann 3 MM), and chromatography in the second dimension with saturated $(NH_4)_2SO_4$:isopropanol:1 M sodium acetate (80:2:18, by vol.). Quantification of individual nucleotides was carried out by scraping off the UV quenching spots and counting their radioactivity. The m^{5}C content of DNA was calculated from the radioactivity found in m^{5}dCMP and dCMP by the following equation:

$$\% \text{ m}^5\text{dCMP} = \text{m}^5\text{dCMP} \times 100/\text{m}^5\text{dCMP} + \text{dCMP}.$$

Generation of liver nodules. Male Wistar rats (150–180 g) were fed a standard diet (Altromin, Lage, F.R.G.) and treated for 7 weeks with *N*-nitrosomorpholine (Sigma; 160 mg/L, in drinking water). Four months after withdrawal of *N*-nitrosomorpholine (at weeks 20–25 of the experimental model) the animals were killed and the liver excised, washed with 0.9% NaCl and placed on ice. Hepatic nodules of about 0.5 cm diameter were dissected from surrounding liver and used for analysis. At weeks 30–40 most rats had died of metastasizing liver cancer. Some of the hepatocellular carcinomas were also dissected out and investigated.

Extraction of RNA and Northern blot analysis. RNA was isolated from liver nodules by the method of Chirgwin *et al.* [31]. RNA was denatured for electrophoresis in a 1.5% agarose gel containing 2.2 M formaldehyde. RNA was released from agarose gels and transferred to Hybond N (Amersham Buchler) by capillary blotting and was covalently bound to the membrane by UV irradiation. Nylon membranes containing the samples were prehybridized and hybridized for 24 hr at 44° in a solution containing 50% deionized formamide, 5 × Denhardt's solution, 5 × SSC (0.75 M NaCl/0.075 M sodium citrate, pH 7.0), 0.5% SDS (sodium dodecyl sulfate) and 400 μg/mL herring sperm DNA [32]. The Nylon membranes were hybridized with the 1.1 kb BamHI/SalI 3'-fragment of v-*myc* MC29 in pBR 322 [33], which was used as a probe corresponding to c-*myc* exon III. The probe was labeled with [α-^{32}P]dCTP (sp. act. 3000 Ci/mmol) using the multiprime DNA labeling system (Amersham Buchler, UK). After hybridization, membranes containing RNA were washed twice in 2 × SSC, 0.1% SDS for 20 min at 44°. Blots were exposed to Kodak XAR-5 film at −70° with intensifying screens for 7 days. The relative amounts of mRNA were estimated by densitometric scanning of autoradiograms of Northern blots. Northern blots were reprobed with albumin cDNA to confirm that all lanes contained equal amounts of RNA.

Extraction of DNA and Southern blot analysis. DNA was prepared as described by Blin and Stafford [34] and used for digestion with restriction enzymes or as a substrate for DNA methylase. Restriction enzymes were obtained from Bethesda Research Laboratories (Bethesda, MD, U.S.A.) and used under conditions supplied by the manufacturer. Digestions of DNA samples with MspI and HpaII and Southern blot analysis were carried out as follows: DNA (20 μg) was digested to completion with MspI and HpaII (20 units/μg DNA). The digested DNA was electrophoresed in a 1% agarose gel for 18 hr at 16 V/cm in buffer containing 40 mM Tris–HCl, pH 8.0, 20 mM acetic acid and 20 mM EDTA. Phage Lamda DNA, digested with HindIII, was used as a size marker during electrophoresis. The electrophoresed DNA fragments were transferred to nitrocellulose. Electrophoresis and transfer to nitrocellulose was followed by ethidium bromide staining, in order to ensure that each sample contained approximately equal amounts of DNA. Nitrocellulose filters containing the samples were hybridized with the same ^{32}P-labeled v-*myc* probe used for Northern blot analysis. Prehybridization and hybridization were carried out at 42° for 20 hr in a solution containing 50% formamide, 5 × SSC, 10 × Denhardt's solution, 0.1% SDS and 100 μg/mL herring sperm DNA. This procedure was followed by two washes with 2 × SSC, 0.1% SDS for 20 min at 65°. Blots were exposed to Kodak XAR-5 film at −70° with intensifying screens for 10 days. The relative amounts of DNA fragments were estimated by densitometric scanning of autoradiograms.

DNA modification by treatment with reactive metabolites of N*-nitrosomorpholine generated by P450-dependent monooxygenases.* DNAs (100 μg) from *Micrococcus luteus* (low methylation level) or

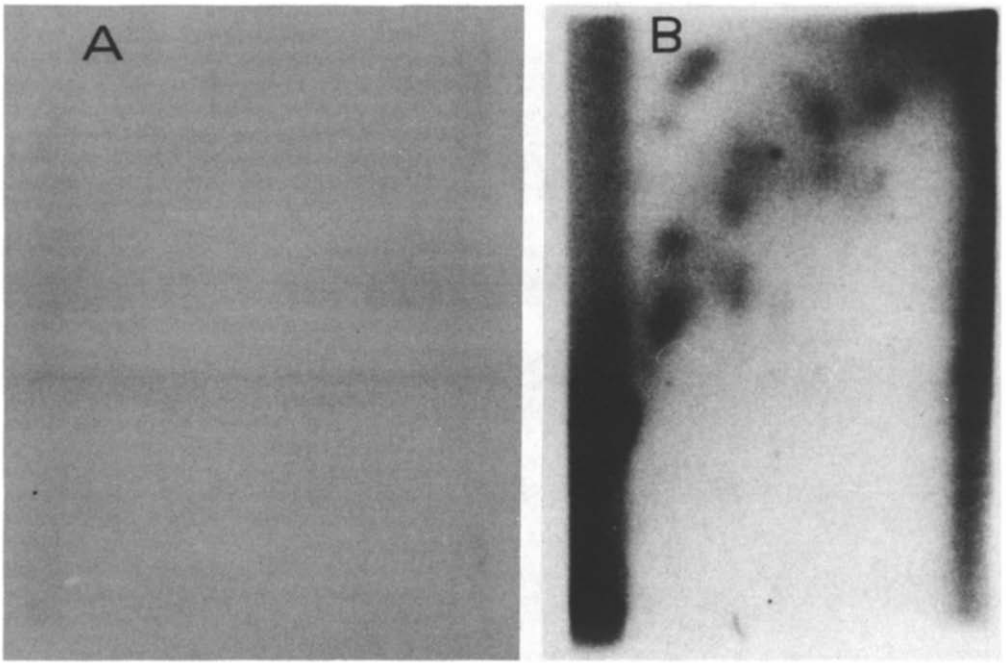


Fig. 1. Autoradiograms of TLC maps of ^{32}P-labeled hydrophobic DNA adducts obtained by treatment of *Micrococcus luteus* DNA with *N*-nitrosomorpholine metabolites. (A) Control *Micrococcus luteus* DNA, (B) *Micrococcus luteus* DNA modified by *N*-nitrosomorpholine metabolites. DNA modification, ^{32}P-postlabeling of DNA adducts, their separation by TLC and autoradiography were carried out as described in Materials and Methods.

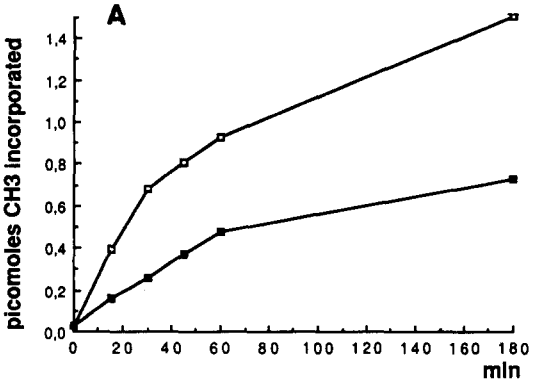
from chicken erythrocytes (high methylation level) were incubated at 37° in the presence of 50 mM *N*-nitrosomorpholine, liver microsomes from phenobarbital-treated rats (1 mg protein), 0.1 M Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 0.5 mM NAD, 5 mM sodium isocitrate, 10 μL isocitrate dehydrogenase (Boehringer, Mannheim, F.R.G.) in a total volume of 5 mL. As a second control, DNA (100 μg) was incubated in the absence of *N*-nitrosomorpholine for 30 min. DNA was then treated for 1 hr at 37° with proteinase K (1 mg/mL) in 25 mM Tris–HCl, pH 8.0, 100 mM NaCl, 2.5 mM EDTA and 1% SDS. DNA was precipitated by addition of ethanol (2.5 volumes in 100 mM NaCl). After two additional washes with ethanol the precipitated DNA was lyophilized.

^{32}P-Postlabeling of DNA adducts. The method used was that of Randerath and co-workers [35, 36]. The DNAs were digested with micrococcal nuclease and spleen phosphodiesterase (see above), and adducts were enriched (versus normal nucleotides) by further nuclease P1 digestion. The remaining nucleotides were ^{32}P-labeled and the labeling mixture was directly applied to PEI-cellulose thin layers. *Dimension 1* was on a one dimension plate: the solvent was 2.3 M sodium phosphate, pH 5.7. After detection of the hydrophobic nucleotides at the lower part of the migration track, the corresponding region was cut out and transferred onto the origin point of a new plate. Further separation of the labeled nucleotides was performed using the following solvents, *dimension 2*: 8.5 M urea, 4.25 M lithium formiate, pH 3.5; *dimension 3*: 7 M urea, 0.7 M sodium phosphate, pH 6.0; *dimension 4*: 1.7 M sodium phosphate pH 6.0. Areas of radioactivity were visualized by autoradiography [36].

RESULTS AND DISCUSSION

To investigate the causal relationship between genotoxic lesions caused by the cyclic nitrosamine *N*-nitrosomorpholine and altered methlyation patterns, DNA was treated in an *in vitro* P450-dependent monooxygenase system in the presence of *N*-nitrosomorpholine. The latter is known to be converted to DNA reactive metabolites by P450-dependent monooxygenases [8–10] which lead to alkylated guanines in rat liver DNA [7]. These are not the only modified bases obtained by this treatment. Using ^{32}P-postlabeling of the nucleotides obtained by exhaustive digestion of *N*-nitrosomorpholine-treated *Micrococcus luteus* DNA, we could also detect several hydrophobic adducts (Fig. 1). Similar but less abundant DNA adducts could be detected after a similar treatment of chicken erythrocyte DNA (not shown), possibly reflecting the different GC contents of these two DNAs. The exact nature of these adducts could not be determined.

The findings about DNA modifications by metabolites of *N*-nitrosomorpholine (alkylation and production of hydrophobic adducts) prompted us to determine whether these modifications affect the overall *in vitro* methylation pattern of DNAs. Using this assay, three types of carcinogens could be distinguished previously: (a) carcinogens inhibiting DNA methylation (2-acetylaminofluorene and



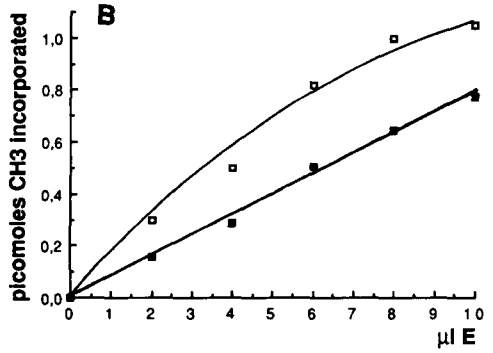
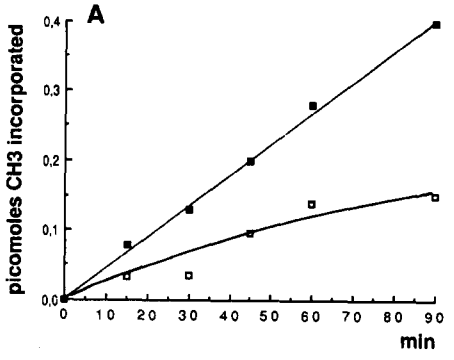


Fig. 2. *In vitro* enzymatic DNA methylation of *Micrococcus luteus* DNA, modified by *N*-nitrosomorpholine metabolites. Results represent means of triplicates and are expressed as picomoles of CH_3 incorporated into 1 µg DNA. (□) Control DNA; (■) modified DNA. (A) Time course of DNA methylation: DNA (1 µg) was incubated at 37° in the presence of *S*-adenosyl-[*methyl*-^{3}H]methionine (2 µCi) and DNA methylase (10 µL = 25 µg protein) in 50 mM Tris–HCl, pH 7.6 and 1 mM dithiothreitol (80 µL final volume). The reaction was stopped at the time points indicated. (B) Protein dependency of DNA methylation: DNA (1 µg) was incubated for 90 min at 37° in the presence of increasing amounts of DNA methylase. E, enzyme (described in A and in Materials and Methods).



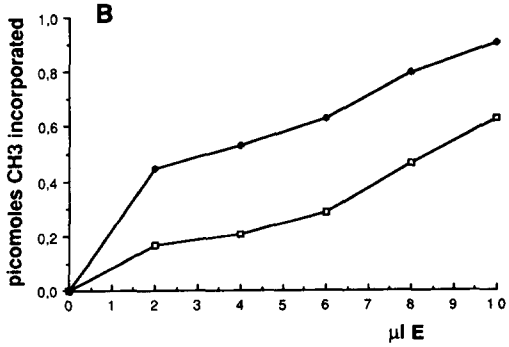


Fig. 3. *In vitro* enzymatic DNA methylation of liver and nodular DNA by rat spleen DNA methylase. Conditions are the same as those given in Fig. 2. (□) Liver DNA; (■) nodular DNA. E, enzyme (described in Materials and Methods).

methylnitrosourea [9, 37–39]); (b) carcinogens stimulating DNA methylation (aminofluorene and 4-nitroquinoline-1-oxide [40, 41]); (c) carcinogens without effect on DNA methylation (dimethylsulfate [37]). To study methylation of *N*-nitrosomorpholine-modified DNA, we used an *in vitro* enzymatic system with rat spleen DNA methylase. DNA methylation in eukaryotic cells is a post-replicative process involving the transfer of methyl groups from *S*-adenosyl-L-methionine to the carbon in position 5 of cytosine residues through the action of DNA methylase. The major function of this enzyme is 'maintenance methylation' of hemimethylated sites after replication, in order to preserve the pattern from one generation to the next. The enzyme is also involved in '*de novo* methylation'.

To obtain information on both maintenance and *de novo* methylation the following DNAs were studied: (a) *Micrococcus luteus* DNA, which contains no m^5C to measure *de novo* methylation; and (b) chicken erythrocyte DNA, which contains a large number of hemimethylated sites [42] to measure maintenance methylation. The time course and protein dependency showed a marked decrease of DNA methylation with modified *Micrococcus luteus* DNA (Fig. 2). Similar results were obtained using modified erythrocyte DNA (not shown).

Since inhibition of methylation is tissue specific and clonally inherited it was of interest whether the resulting hypomethylation could be detected *in vivo*. Therefore, preneoplastic liver nodules were produced by treatment with *N*-nitrosomorpholine. Using ^{32}P-postlabeling analysis the levels of m^5C were found to be reduced by 43% in nodular DNA (1.7 ± 0.1% of m^5C) compared to normal liver DNA (3.0 ± 0.1% of m^5C). This result was further confirmed by *in vivo* enzymatic methylation of DNA isolated from nodules. As shown in Fig. 3, nodular DNA appeared to be a much better substrate for DNA methylase

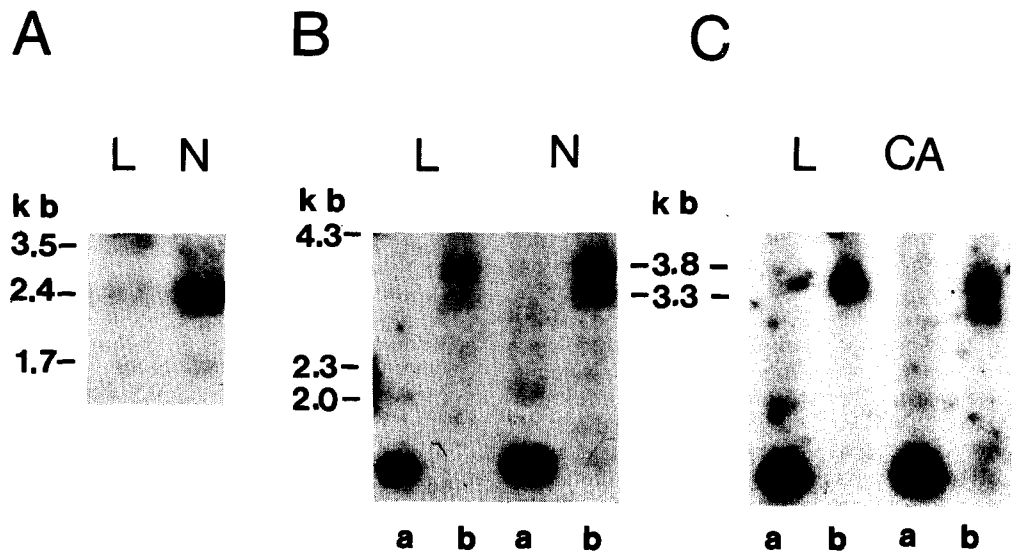


Fig. 4. (A) Northern blot analysis of c-*myc* in normal liver (L) and in liver nodules (N). Total RNA (20 μg) from the tissues was subjected to Northern blot analysis. (B) DNA methylation analysis of c-*myc* from rat liver (L) and liver nodules (N). DNA (20 μg) was digested with MspI (a) and HpaII (b). (C) DNA methylation analysis of c-*myc* from rat liver (L) and a liver carcinoma (CA). Conditions were the same as in (B). In A to C v-*myc* MC29 was used as a probe corresponding to c-*myc* exon III.

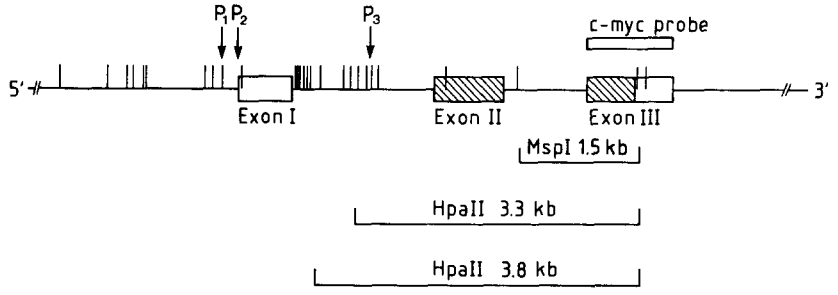


Fig. 5. Pattern of MspI/HpaII restriction sites of c-*myc* (vertical lines). The pattern is based on the c-*myc* sequence published by Hayashi *et al.* [47]. Restriction sites are deduced by the computer program DNASIS. Open boxes of exons represent non-coding regions. P_1, P_2 and P_3 indicate promoter regions.

in vitro than control liver DNA, confirming the overall hypomethylation of DNA.

It is known that changes in DNA methylation may be important events in carcinogenesis [14, 43]. Many studies have shown that hypomethylation of the transcriptional control regions of genes is correlated with enhanced gene expression [12, 44]. In the case of the c-*myc* protooncogene, gene expression is low in normal liver, temporarily increased in regenerating liver [25] and persistently increased (3-fold) in liver nodules (Fig. 4A). In addition, *in situ* hybridization using ^{35}S-labeled mouse c-*myc* exon II RNA revealed increased c-*myc* expression in hepatocytes of nodular tissues (not shown).

Multiple mechanisms are known to be responsible for persistently increased c-*myc* expression [45], for example, amplification of the c-*myc* gene as previously found in rat hepatoma H4IIE cells [46]. However, in human hepatoma HepG2 cells, amplification or rearrangements of the c-*myc* gene were not responsible for the persistently increased c-*myc* expression [45]. In liver nodules produced by treatment with *N*-nitrosomorpholine no amplification and rearrangements of the c-*myc* gene could be detected (not shown). Hypomethylation has been suggested to be responsible for c-*myc* overexpression during early stages of chemical carcinogenesis [15] and in human hepatocellular carcinomas [16–18]. Therefore hypomethylation of the c-*myc* gene was analysed in *N*-nitrosomorpholine-induced liver nodules. DNA methylation analysis was carried out using the isoschizomeric restriction endonucleases HpaII and MspI. Digestion with MspI (cleaving at both Cm^{5}CGG and CCGG sequences) led to a major fragment of about 1.5 kb. However, digestion with HpaII (cleaving only at CCGG sequences) led to

two major fragments of 3.8 and 3.3 kb (Fig. 4B). Densitometric scanning of autoradiograms indicated that in the control liver the relative amount of the 3.3 kb fragment compared to the 3.8 kb fragment was low (up to 30%), while in liver nodules almost equal amounts of the two fragments were detected. Similar results were obtained with DNA extracted from a hepatocellular carcinoma (Fig. 4C). Using the c-*myc* sequence data published by Hayashi *et al.* [47], our results suggest site-specific hypomethylation of the c-*myc* gene in nodular DNA. The DNA fragments obtained by MspI/HpaII digestion (Fig. 4B) can be assigned to defined regions of the c-*myc* gene on the following grounds (Fig. 5): assuming complete digestion the MspI fragment (1.5 kb) starts from the cleavage site close to the translated part of exon III. Assuming hypomethylated regions in exon III the HpaII fragments may start from the same cleavage site. Hence the 3.3 and 3.8 kb fragments may include exons II and III as well as a part of the first intron and the promoter P_3. In the rat, the promoters P_1 and P_2 are normally operating whereas promoter P_3 appears to be activated when P_1 and P_2 are damaged [47]. Therefore, our results suggest that hypomethylation of the P_3 promoter region could be responsible for persistently increased c-*myc* expression in liver nodules.

Hypomethylation of genes in preneoplastic liver nodules may also explain persistently increased expression of other proteins, such as drug metabolizing enzymes [21–23]. This suggestion is substantiated by studies with 5-azacytidine, an inhibitor of DNA methylation. These studies demonstrated increased expression of NAD(P)H quinone reductase [48]. In addition, treatment with 5-azacytidine during the phase of carcinogen-induced repair synthesis potentiated initiation of the carcinogenic process [49].

In conclusion, it is shown that *in vitro* treatment of DNA with *N*-nitrosomorpholine metabolites leads to the formation of hydrophobic DNA adducts (in addition to the known alkylated adducts). The modified DNAs were poor substrates for spleen DNA methylase. These *in vitro* findings suggest inherited DNA hypomethylation as a result of genotoxic lesions which may affect the regulation of critical genes *in vivo*. In fact, site-specific hypomethylation of the first intron (containing a cryptic promoter) of the c-*myc* gene could be demonstrated in DNAs from liver nodules and hepatocellular carcinomas.

However, more work is needed to understand the complex regulation of this protooncogene and the consequences of its hypomethylation at critical sites.

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