

UNDERMETHYLATION OF THE *C-MYC* GENE IN A RAT HEPATOMA CORRELATES WITH INCREASED EXPRESSION OF THE GENE

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ABSTRACT

The extent of DNA methylation of the *c-myc* gene and the levels of *c-myc* mRNA transcripts have been determined in a rat ascitic hepatoma and in normal adult and fetal liver tissues. The *myc* gene is undermethylated to a higher extent in the hepatoma and moderately undermethylated in fetal liver compared to adult liver. Higher levels of *myc* transcripts are present in the hepatoma and fetal liver than in adult liver. Our observation suggests differential activation of *myc* gene at the transcriptional level in the tumour and in fetal liver.

INTRODUCTION

THERE is considerable evidence that an increased expression of certain cellular homologues of viral oncogenes (or proto-oncogenes) can directly be linked to tumourigenesis in several animal species. The enhanced expression of the *c-myc* gene, which is the cellular homologue of the oncogene of the avian myelocytomatosis virus (MC29) has been demonstrated in several murine plasmacytomas, Burkitt's lymphomas and certain rat hepatomas¹⁻³. The expression of the *c-myc* gene has also been recently shown to be regulated during the cell cycle in normal fibroblasts and lymphocytes^{4,5}. The available evidence suggests two kinds of *c-myc* regulation. In Burkitt's lymphoma cell lines where there is evidence for chromosomal translocation of the *myc* gene, expression of the translocated genes has been observed, but not of the normal alleles. This observation has been interpreted in terms of regulation by a *trans*-acting element which represses the normal alleles^{6,7}. On the other hand, in lung fibroblasts and embryonal carcinoma cells, *myc* regulation appears to be primarily at the level of mRNA stability, since in these cells *c-myc* is transcribed constitutively, but its mRNA is rapidly degraded^{8,9}. In regenerating rat liver and certain hepatomas, *c-myc* mRNA levels are considerably higher than in adult liver tissues³ but it is not clear whether *c-myc* regulation is transcriptional or post-transcriptional.

Recent advances in understanding eukaryotic gene regulation have indicated several potential mechanisms for altering the transcriptional activity of the DNA template. These include chemical and conformational modifications of DNA, chemical

modifications of histones and changes in the binding of non-histone DNA-binding proteins¹⁰⁻¹². For many higher eukaryotes, an inverse correlation exists between the transcriptional activity of a gene and the level of DNA methylation at cytosine residues in the dinucleotide CpG at specific sites in the gene. Actively transcribed genes are thus relatively undermethylated at certain sequences containing the dinucleotide CpG as compared to inactive genes¹³⁻¹⁵.

In the present study, we have compared the levels of *c-myc* mRNA expression in normal adult and fetal liver tissues and in a rat hepatoma, the Zajdela ascitic hepatoma (ZAH), and determined methylation levels of the *c-myc* gene in these tissues, specifically at C^mCGG and G^mCGC sequences by restriction analysis of the DNAs.

METHODS

The maintenance and characteristics of ZAH have been described earlier¹⁶. Adult and fetal liver [15-day-old fetuses] were obtained from Wistar rats. For dot hybridizations, total RNA was extracted by the guanidinium thiocyanate procedure¹⁷ and poly A⁺ mRNA was isolated by oligo dT-cellulose chromatography¹⁸. Poly A⁺ mRNA samples were applied to a nitrocellulose filter (Schleicher and Schuell BA85) and hybridized to a nick-translated *myc* probe (specific activity 2×10^8 cpm/ μ g), corresponding to a 1.6 kb *Pst* I fragment containing the entire *v-myc* gene¹⁹ as described elsewhere^{20,21}. The filter was washed in $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) at 50°C for 2×45 min and exposed to Agfa Curix RPI film for 2-5 days at -70°C (with intensifying screens). To obtain relative intensities of spots, autoradiograms were scanned on a soft laser scan-

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ning densitometer (model SL2 DUV, Biomed). For Southern hybridizations, high molecular weight DNA (25 μg), isolated as described elsewhere²² was digested to completion with different restriction enzymes as recommended by the manufacturers (Bethesda Research Laboratories), subjected to electrophoresis in 1% agarose gels and transferred to nitrocellulose filters²³. In figure 2, transfer of fragments < 1 kb in length was carried out for a shorter time (18 hr) compared to that for the rest of the gel (36 hr) for optimal transfer. Hybridization was carried out with the nick-translated *myc* probe as described elsewhere^{20,24}. Filters were washed in $0.5 \times \text{SSC}$ at 65°C for 2×30 min and exposed to Agfa Curix RPI film for 2–5 days at -70°C (with intensifying screens).

RESULTS AND DISCUSSION

The results of dot hybridization of poly A⁺ mRNA from different sources with a radiolabelled *myc* probe are shown in figure 1. The data clearly indicate that the expression of the *myc* gene is about 2-fold higher in ZAH cells as compared with that in adult liver. It is interesting to note that the *myc* gene is expressed at considerably higher levels (3–4 fold) in 15-day-old rat fetal liver. This observation is consistent with a recent report that the *myc* gene is expressed during fetal development in the rat²⁵.

The presence of methylation in the *myc* gene at CCGG sequences was analysed by the use of the restriction endonucleases *Msp I* and *Hpa II*. *Msp I* cleaves this sequence whether the second cytosine is methylated or not but not if the first cytosine is methylated. *Hpa II* cleaves the sequence only if the second cytosine is unmethylated regardless of the methylation of the first cytosine. The gel profiles of *Msp I* and *Hpa II* digests after hybridization with the *myc* probe are shown in figure 2A. DNA samples were also digested with *Hha I*, which cleaves the sequence GCGC only if the first cytosine is unmethylated, and the *Hha I* digests after hybridization with the *myc* probe are presented in figure 2B.

The presence of a number of fragments (< 1.2 kb size) hybridizing with the *myc* probe in *Hpa II* digests of the tumour DNA, in comparison with corresponding digests of normal adult DNA, suggests the presence of several C^mCGG sites in the normal DNA which are unmethylated in the tumour DNA. Likewise a comparison of the *Msp I* digests of ZAH DNA and normal adult liver DNA would argue for the presence of fragments containing sequences such as GGC^mCGG or C^mCGGCC in the

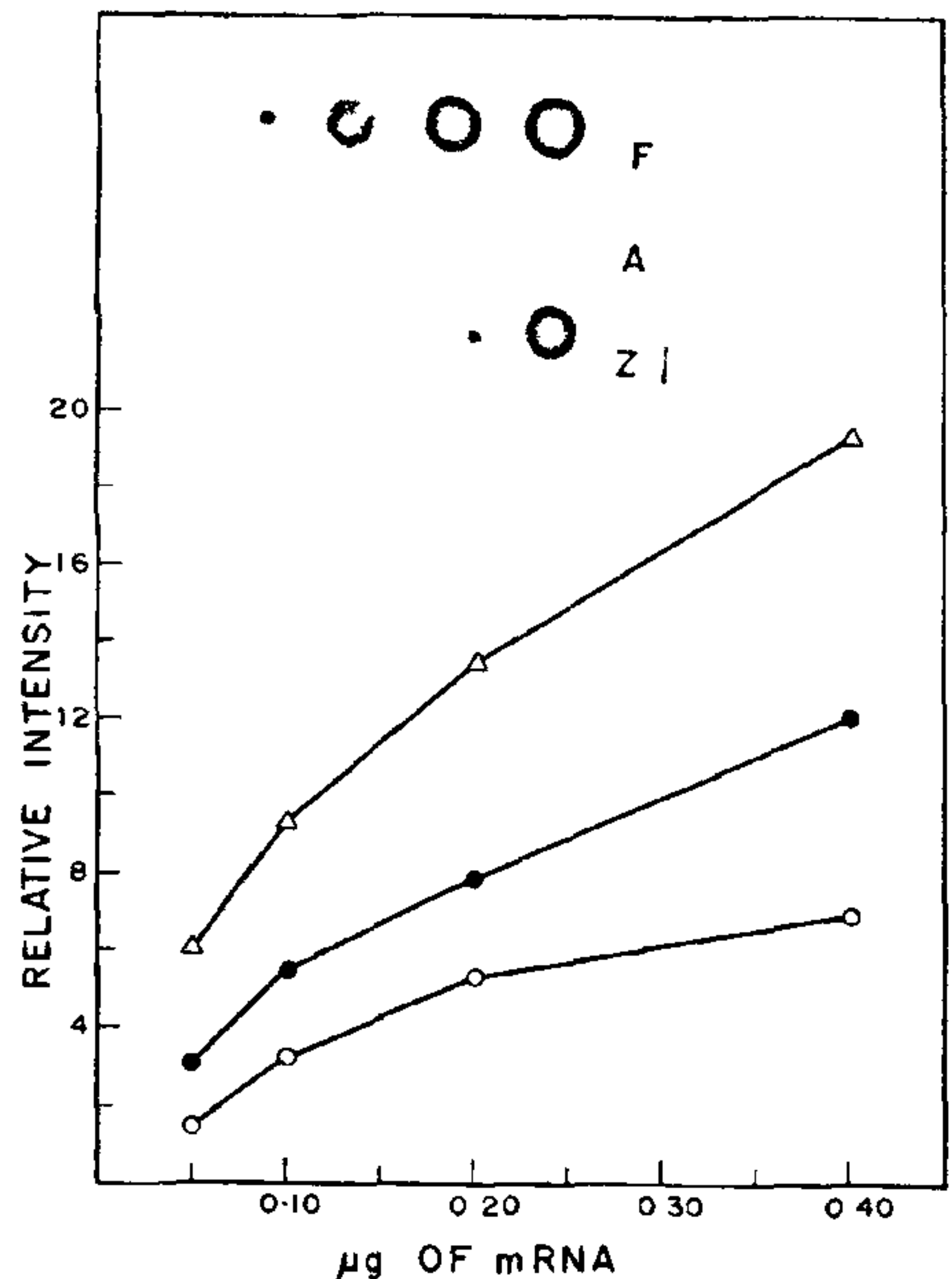


Figure 1. Quantitation of *myc* mRNA levels by dot hybridization of total poly A⁺ mRNA from ZAH (Z), normal adult (A) and fetal liver (F) with radiolabelled *myc* probe (see Methods) (●-●) ZAH; (Δ-Δ) fetal liver; (○-○) adult liver. The most intense spot for normal adult liver corresponds to approximately 20 μg of *myc*-specific transcripts or 0.005% of the total poly A⁺ mRNA.

adult DNA which are unmethylated in the tumour DNA^{26,27}. Another interesting feature that may be noticed is that both *Msp I* and *Hpa II*, digests of fetal DNA show at least two almost identical sized fragments (1.2 and 0.74 kb) which are absent in the corresponding digests of adult DNA. If fragments of the type GGC^mCGG, which are not restricted either by *Msp I* or *Hpa II* remain unmethylated in the fetal DNA, such sites become available for restriction by both *Msp I* and *Hpa II*. This type of situation is also obtained for the tumour DNA, in comparison with adult liver DNA. The *Hha I* digests (figure 2B) suggest that a GCGC site which is methylated in a 7 kb fragment of adult and fetal liver, is undermethylated in ZAH cells, as the disappearance of this band from the ZAH lane coincides with the appearance of a 2 kb fragment and several fragments of size ~ 0.5 kb. Since our *v-myc* probe is

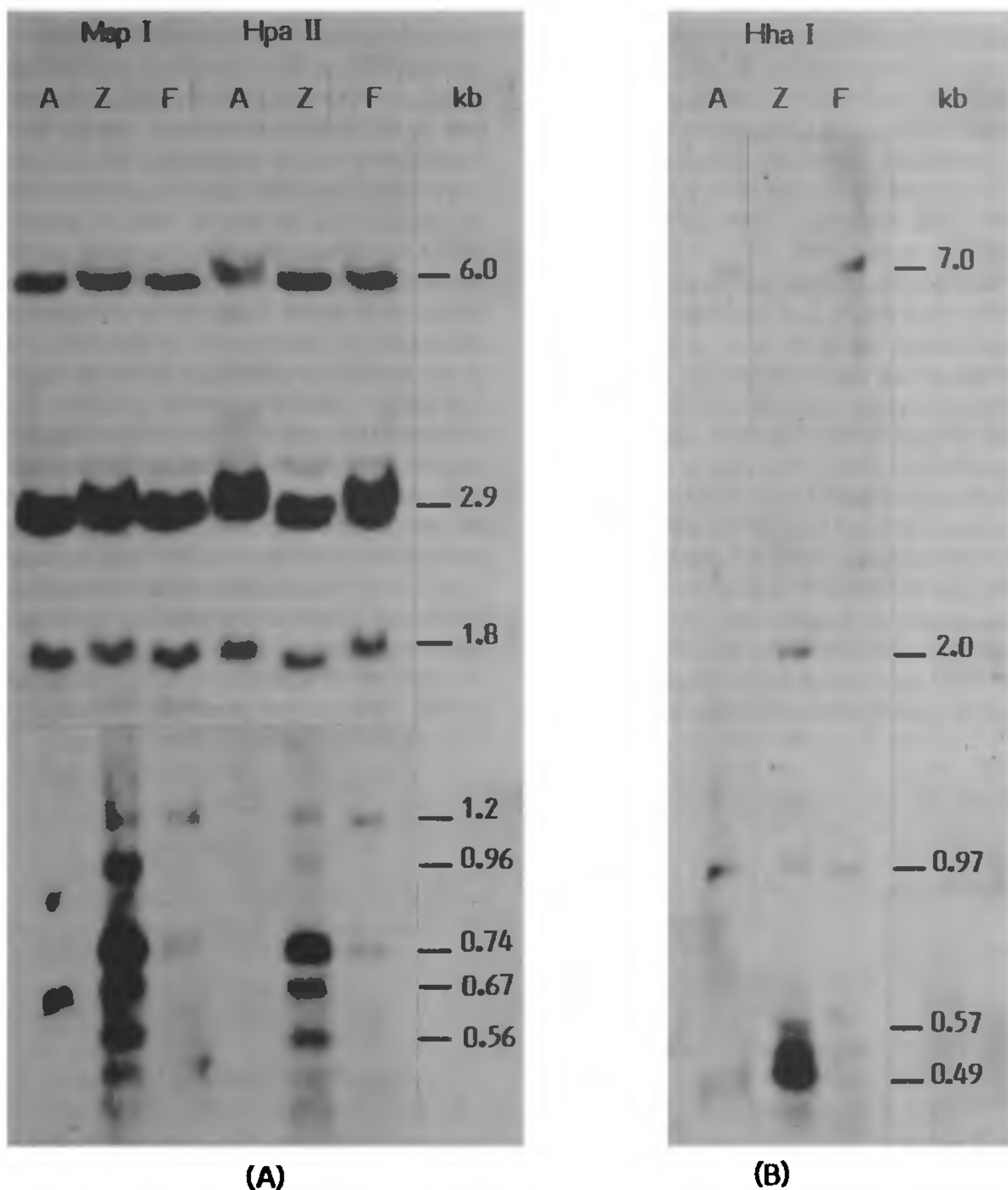


Figure 2A and B. DNA methylation analysis of the *myc* gene by Southern hybridization of total DNA from ZAH (Z), normal adult (A) and fetal liver (F) with radiolabelled *myc* probe (see Methods).

known to hybridize to exons 2 and 3 of the *c-myc* gene in several species (mouse, rat, human)²⁸, the small size of the fragments obtained on digestion of ZAH DNA with *Hpa II* or *Hha I* suggests that the sites of undermethylation are in exons 2 and 3. In a recent study on human tumour cell lines, a specific undermethylated site was shown to be present exclusively in exon 3 of the human *myc* gene²⁹. However, levels of expression of *myc* mRNA had not been determined in these tumour lines.

Although correlations of increased gene expression with undermethylation in the 5' region of a gene have been reported in a number of instances, there have also been instances where increased expression was found to correlate with undermethylation within the structural gene as in the case of globin, albumin and immunoglobulin genes³⁰⁻³². However, the precise mechanism by which the latter type of correlation is brought about remains unknown. It is also important to look for undermethylation at the 5'

region of *c-myc*, but probes specific to this region are not presently available. We have been unable to map the exact sites of undermethylation as a detailed restriction map or sequence of the rat *c-myc* is not available. Double digests with *Eco RI* or *Hind III*/*Msp I* and *Eco RI* or *Hind III*/*Hpa II* give rise to very small fragments (~300 bp) which do not hybridize to the probe.

Our data demonstrate that increased expression of the *c-myc* gene in a rat hepatoma correlates with undermethylation of the gene at specific sites as compared with that of normal liver. At least two of these sites (giving fragments of 1.2 and 0.74 kb) are also present in fetal liver. The absence of other demethylated sites in fetal liver as compared with ZAH suggests that the additional sites in ZAH may be sites of aberrant hypomethylation in the tumour not necessarily connected with increased transcription (also see below). It is possible that only certain specific sites of demethylation seen both in fetal liver and in ZAH may be the ones required for increased expression of the *myc* gene. The correlation of transcriptional activity with some but not all

demethylated sites has been observed also for the globin, ovalbumin, lens γ -crystallin and metallothionein genes³³. The occurrence of modifications at the level of the structure of the *myc* gene by DNA demethylation strongly suggests that the expression of *myc* is regulated at the transcriptional level in this rat hepatoma and in fetal rat liver. At present, several mechanisms have been suggested for the transcriptional activation of the *myc* gene³⁴ such as changes in 5'-control sequences by chromosomal rearrangements, amplification of the number of copies of the gene or changes in the binding of *trans*-acting regulatory factors. It is unlikely that activation of the gene in the ZAH cells is caused by a rearrangement in flanking DNA sequences since our restriction mapping of the gene in the hepatoma and normal liver with several restriction enzymes shows identical maps for the gene in both the tissues (figure 3). Our Southern hybridization data also do not give any indication of amplification of the gene in this hepatoma.

It may be noted that ZAH is originally derived from a rat hepatoma induced by

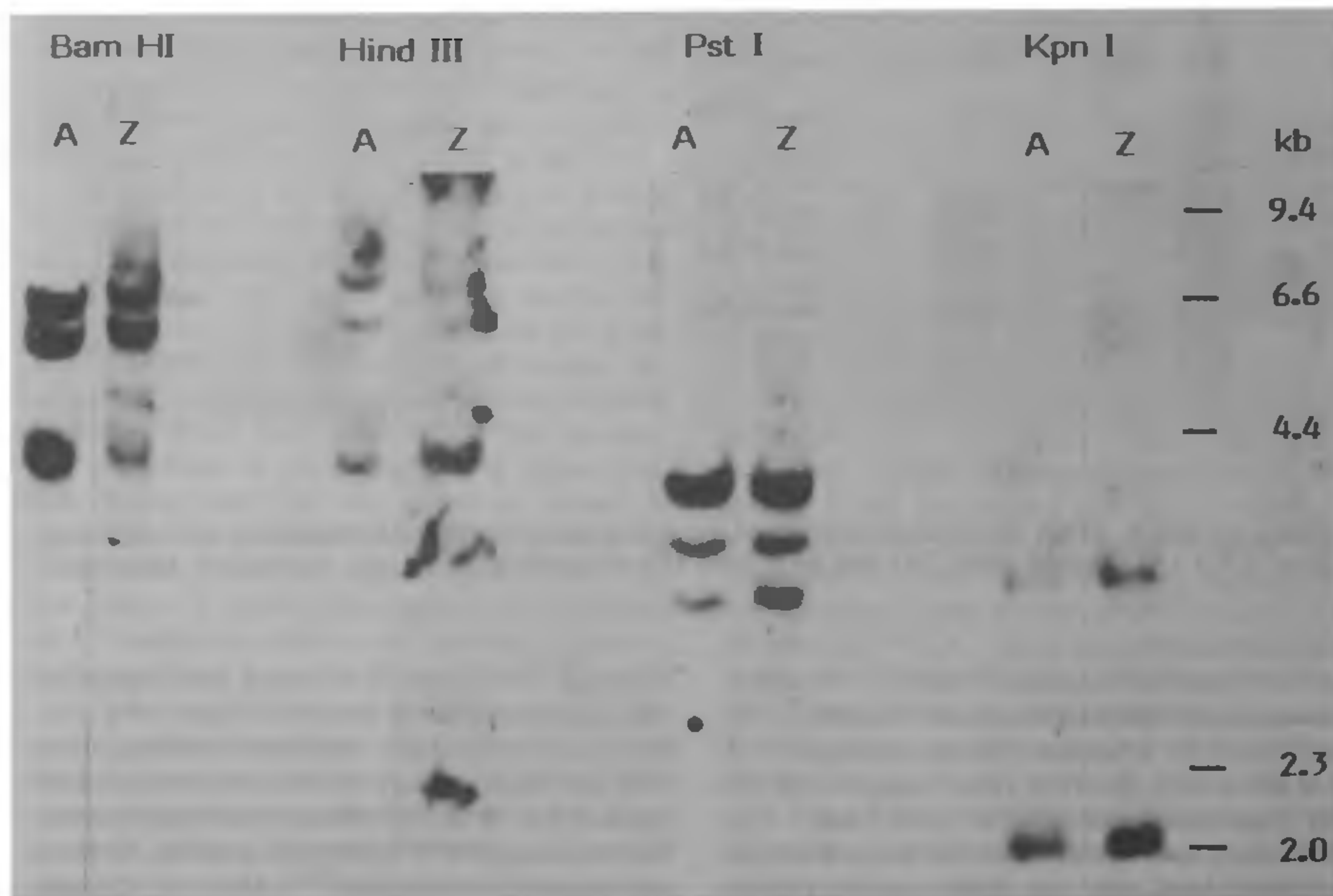


Figure 3. Restriction mapping of the *myc* gene by Southern hybridization of total DNA from ZAH (Z) and normal adult liver (A) with radiolabelled *myc* probe (see Methods).

dimethylaminoazobenzene^{35,36}. It has also been reported that the DNA of primary hepatocarcinomas induced by this carcinogen contains less 5-methylcytosine than does the DNA of normal liver tissue^{37,38}. Our experiments further narrow down at least part of the difference in the methylation status of cytosine to the *c-myc* regions of the DNA. It has been hypothesized that there exists a cellular mechanism that ensures the maintenance of methylation patterns which would mirror earlier carcinogen-induced aberrant methylations and that a carcinogen-induced damage to DNA can thus be fixed in an altered methylation pattern³⁸. Hypomethylation is expected to occur during the DNA synthetic phase of the cell cycle and our observations on the relative, but prominent, difference in the levels of methylation between fetal liver DNA and tumour DNA, and the distinct nature of the fragments obtained in the *Msp I* and *Hpa II* digests of fetal DNA and tumour DNA would argue for the presence of different kinds of hypomethylation patterns in the two systems. Hypomethylation found in the DNA during development is likely to be reversible whilst those induced by chemical carcinogens are probably inheritable.

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ANNOUNCEMENTS

NINTH NATIONAL SYMPOSIUM ON CATALYSIS

The ninth National Symposium on Catalysis under the theme: "Catalysis—concepts and applications" will be held at Madras Refineries Ltd., R&D Centre, Manali, Madras from December 15 to 17, 1988.

The Symposium will provide a forum for exchange of ideas and views on new and emerging concepts in the field of catalysis with special reference to: a) New developments in catalysis, b) Catalysis for petroleum refining and petrochemical industries, c) Catalysis for fertilizer, organic chemi-

als and other industries, d) Catalysis for synthetic fuels, e) Catalysis for environmental protection.

Abstracts of papers in triplicate not exceeding two typed/printed sheets (A4 size) in double line spacing should reach the Secretary, Ninth National Symposium on Catalysis before March 1st, 1988.

Further particulars may be had from: Dr R. Sitaraman, Secretary, Ninth National Symposium on Catalysis, Madras Refineries Ltd., R&D centre, Manali, Madras 600 068.

INTERNATIONAL CONFERENCE ON COMPOSITE MATERIALS AND STRUCTURES

The FRP Research Centre, Indian Institute of Technology, Madras is organising the above Conference during January 6–9, 1988 to be held at the Indian Institute of Technology, Madras.

The programme contains seven invited lectures and 47 contributed papers. The contributors are from Bulgaria, China, France, India, Italy, Japan, U.K., USA, USSR, West Germany etc. There will be around 20 technical sessions on the following topics: 1. Processing of raw materials and composites; 2. Micro and macro mechanics; 3. Failure analysis; 4. Fatigue and fracture; 5. Static analysis; 6. Dynamic analysis and buckling, 7. FEM applied

to composites, and 8. Experimental techniques.

Sponsors of the Conference are: a) Indian Institute of Technology, Madras, b) Aeronautical Society of India, c) Indian Space Research Organisation, d) Aeronautical Research and Development Board, e) Indian Academy of Sciences, Bangalore, f) Chinese Society of Theoretical and Applied Mechanics, and g) Indian Rocket Society.

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