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Expressions of *c-fos* and *c-myc* Genes during 3'-methyl-4-dimethylaminoazobenzene (3'-MeDAB)-induced Rat Hepatocarcinoma

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We investigated the expression of the growth-related nuclear proto-oncogenes, c-fos and c-myc, in early preneoplastic regions and tumor nodules of 3'-MeDAB induced rat hepatocarcinoma. To amplify the levels of these transcripts, we gave cycloheximide (100 mg/kg B.W. i.p.) to each group of rats. The elevated levels of the 2.2 kb c-fos and 2.4 kb c-myc transcripts appeared as early as the 2nd week after feeding on the 3'-MeDAB diet and lasted through the 4th, 6th weeks and tumor. Southern blot analysis indicated that gross amplification or rearrangements were not observed in DNA of the preneoplastic livers and hepatoma nodules. We also measured the rate of the incorporation of [^{3}H] thymidine into hepatic DNA in order to monitor the rate of cell proliferation occurring at the early preneoplastic periods. We have found that the rate of [^{3}H] thymidine incorporation corresponds to the elevated levels of c-fos and c-myc transcripts in the precancerous stages. This finding suggests that the elevated expressions of c-fos and c- myc may result from the continuous cell proliferative stimuli generated in the carcinogen altered cells, which is essential to the initiation and promotion of chemical hepatocarcinogenesis.

Key Words: 3'-methyl-4-dimethylaminoazobenzene(3'-MeDAB), c-*fos*, c-*myc*, preneoplastic livers, cell proliferation

Cell proliferation has often been implicated in the development of cancer caused by chemicals (Craddock, 1976; Cayama *et al.* 1978). Supportive evidence for this is observation that several carcinogens that normally do not induce liver cancer in adult animals, especially with only a single dose, become hepatocarcinogenic when given in a single dose coupled with a liver cell proliferative stimulus, such as partial hepatectomy (PH) (Craddock, 1976). It is now generally accepted that the compensatory type of cell proliferation, which occurs following PH or treatment with chemicals having the property of cell necrosis, has a significant role in the initiation and promotion of liver carcinogenesis, unlike the mitogen-induced hyperplasia which results in a controlled type of cell death, namely apoptosis (Ledda-Columbano *et al.* 1989; Columbano *et al.* 1990). Recently, it was shown that the enhanced expressions of growth-related proto-oncogenes, c-*fos* and c-*myc* (Muller *et al.* 1984; Bravo *et al.* 1985) were observed only when compensatory cell proliferation was occurring and not when mitogen-induced hyperplasia was occurring in the liver cells (Coni *et al.* 1990), although it is not known what are the exact roles of c-*fos* and c-*myc* in the carcinogenic process. 3'-Methyl-4-dimethylaminoazobenzene (3'-MeDAB), which is a potent hepatocellular carcinogen without requiring additional cell stimulators to induce rat hepatoma, is known to cause widespread necrosis of hepatocyte and cell division, especially at an early phase of hepatoma formation (Goldfarb, 1973; Becker *et al.* 1975; Sell *et al.* 1976). We therefore examined the expressions of c-*fos* and c-*myc* during the period of 3'-

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MeDAB-induced hepatocarcinogenesis of the rats, particularly in the early preneoplastic regions and tumor nodules. We also measured the rate of the incorporation of [^{3}H] thymidine into hepatic DNA in order to monitor the cell proliferation rate occurring at the early preneoplastic periods.

MATERIALS AND METHODS

Animals and tumors

Preneoplastic livers and hepatomas were produced by feeding Sprague-Dawley female rats (150-160 g, initial weight) with a semipurified diet (Table 1) containing 0.06% 3'-MeDAB which was dissolved in corn oil (Miller, 1948). Control rats were fed the same diet without 3'-MeDAB. For the preneoplastic livers, rats were sacrificed after taking the 3'-MeDAB diet for 1, 2, 4 and 6 weeks. Hepatoma nodules were obtained after 12 weeks of 3'-MeDAB diet feeding followed by a normal diet for an additional week.

Tissue samples for RNA and DNA extractions

For the extraction of RNA, three rats from each group were injected with cycloheximide intraperitoneally (100 mg/kg, body weight) one hour before sacrifice to amplify the levels of c-*fos* and c-*myc* transcripts. Three preneoplastic livers, hepatoma nodules and normal livers of cycloheximide-treated rats from each group were pooled, quickly frozen in liquid nitrogen, causing the frozen tissues to be disrupted into powder and stored at −70°C until the RNA extraction. DNA was extracted from the rats not treated with cycloheximide.

Table 1. The composition of diets (per kg)

Constituent/Diet group	Control	3'-MeDAB
Casein, g	180	180
Corn oil, g	50	50
Glucose monohydrate, g	770	770
Salt mixture, g	40	40
Riboflavin, g	0.001	0.001
Vitamin mixture*, ml	5	5
3'-MeDAB, g	–	0.6

*Contains vitamin A 20,000 units, vitamin D 2,000 units, choline chloride 1.5 g, pteroylglutamic acid 0.6 mg, biotin 1.5 mg, thiamine-HCl 20 mg, pyridoxine-HCl 20 mg, menadione 50 mg, nicotinamide 50 mg, potassium parabenzoic acid 50 mg, calcium pantothenate 60 mg, inositol 100 mg, and cyanocobalamine 40 μg.

Northern blot analysis

Total cellular RNA was isolated by homogenation of the tissue in 4M guanidinium thiocyanate (Sambrook *et al.* 1989). Samples containing 20 μg of total RNA were fractionated on formaldehyde-1% agarose gels and the integrity of the extracted RNA was monitored by ethidium bromide staining of the gel and transferred to the nitrocellulose membrane. Membranes were then prehybridized and hybridized to heat-denatured ^{32}P-labeled probes at 42° C. Prehybridization and hybridization conditions for c-*fos* and c-*myc* RNA were 50% (v/v) formamide, 0.2% (w/v) sodium dodecyl sulfate, 5×SSPE, 5× Denhardt's solution, 200 μg/mL denatured herring sperm DNA, 10 mg/mL glycine and 50% (v/v) formamide, 0.2% sodium dodecyl sulfate, 5×SSC, 5 ×Denhardt's solution, and 250 μg/mL denatured herring sperm DNA, respectively. After hybridization, the filters were washed to a final stringency of 1×SSC at 50°C for the c-*fos* probe and 0.1×SSC/0.1% SDS at 55°C for the c-*myc* probe.

Southern blot analysis

High mol. wt. genomic DNA was extracted as described (Sambrook, 1989). Ten micrograms of DNA were digested with EcoRI and fractionated in a 0.8 % agarose gel, transferred to a nitrocellulose membrane and hybridized to ^{32}P-labeled probes (Sambrook *et al.* 1989). Hybridization and washing conditions were the same as those used in the Northern blot analysis.

Probes

The c-*fos* and c-*myc* probes were generated respectively from p*fos*-1 (Verma I, Salk Institute, USA) and pMC-*myc* 54 (Marcu K, Dept of Biochem, Sunny-Stony Brook, NY, USA) by PstI restriction digestion. 1.0kb-v-*fos* (Fig. 1) and 0.75kb (Exon I) and 1.0kb (Exon III)-murine c-*myc* (Fig. 2) specific fragments were purified by the electroelution method (Sambrook, 1989) and labeled with [α-^{32}P] dCTP (3000 Ci/mmol, Amersham) using the random priming method (Feinberg and Vogelstein, 1983) to a specific activity of 1×10^9 cpm/μg.

Determination of the rate of [^{3}H] thymidine incorporation into DNA

In order to determine the extent of cell proliferation occurring at the preneoplastic stages and in the

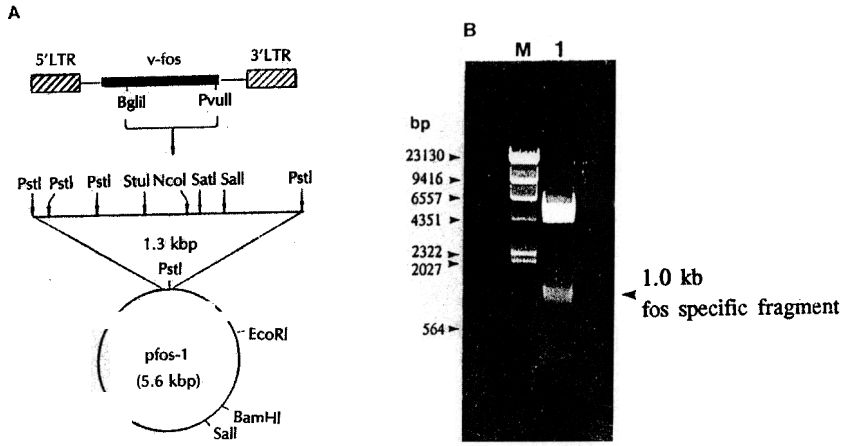

Fig. 1. Agarose gel electrophoresis (1%) of PstI-digested pfos-1. A. Physical map of pfos-1 showing the v-fos specific region. B. PstI-digested pfos-1 was run in 1% agarose gel electrophoresis. Lane M shows the molecular weight marker, lane 1 shows the pfos-1 digested with PstI; 4.4 kb of vector (pBR 322) and 1.0 kb, 0.26 kb, 0.06 kb of three fos-specific fragments.

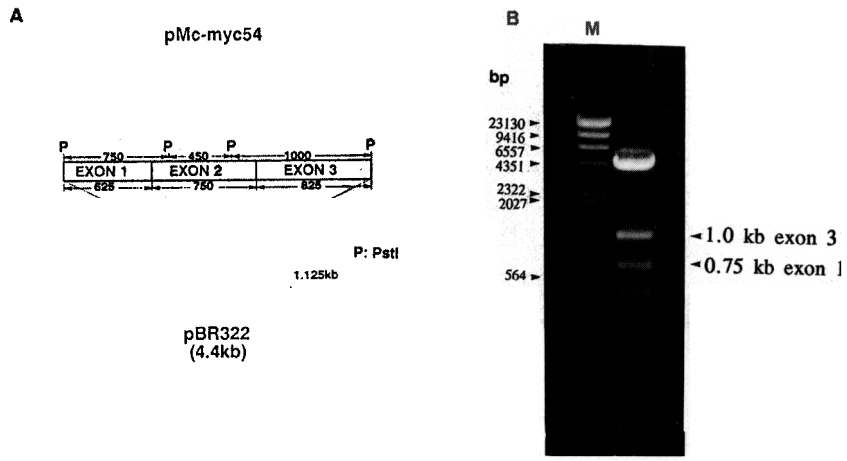

Fig. 2. Agarose gel electrophorsis (1%) of PstI-digested pMc-myc 54. A. Physical map of pMc-myc 54. B. PstI-digested pMc-myc 54 was run in 1% agarose gel electrophoresis. Lane M shows the molecular weight marker, lane 1 shows the pMc-myc 54 digested with PstI; 4.4 kb vector (pBR 322) and 1.0 kb, 0.75 kb, 0.45 kb of three c-myc specific fragments.

normal adult rat liver, a single injection of [^{3}H] thymidine (New England Nuclear, sp.act. 25 Ci/mmol) was administrated intraperitoneally at a dose of 100 Ci/100 g body weight. Two hours after the injection of thymidine, rats were sacrificed, and the livers of three to five rats per group were pooled and minced well and stored at −70° C until the assay. For the assay, one gram of tissue sample was homogenized in 6 volumes of 0.075 M NaCl/0.025 M EDTA (pH 7.6) and was then precipitated in ice-cold 1N perchloric acid (PCA). The pellet was washed three times with ice-cold 0.5N PCA and ex-

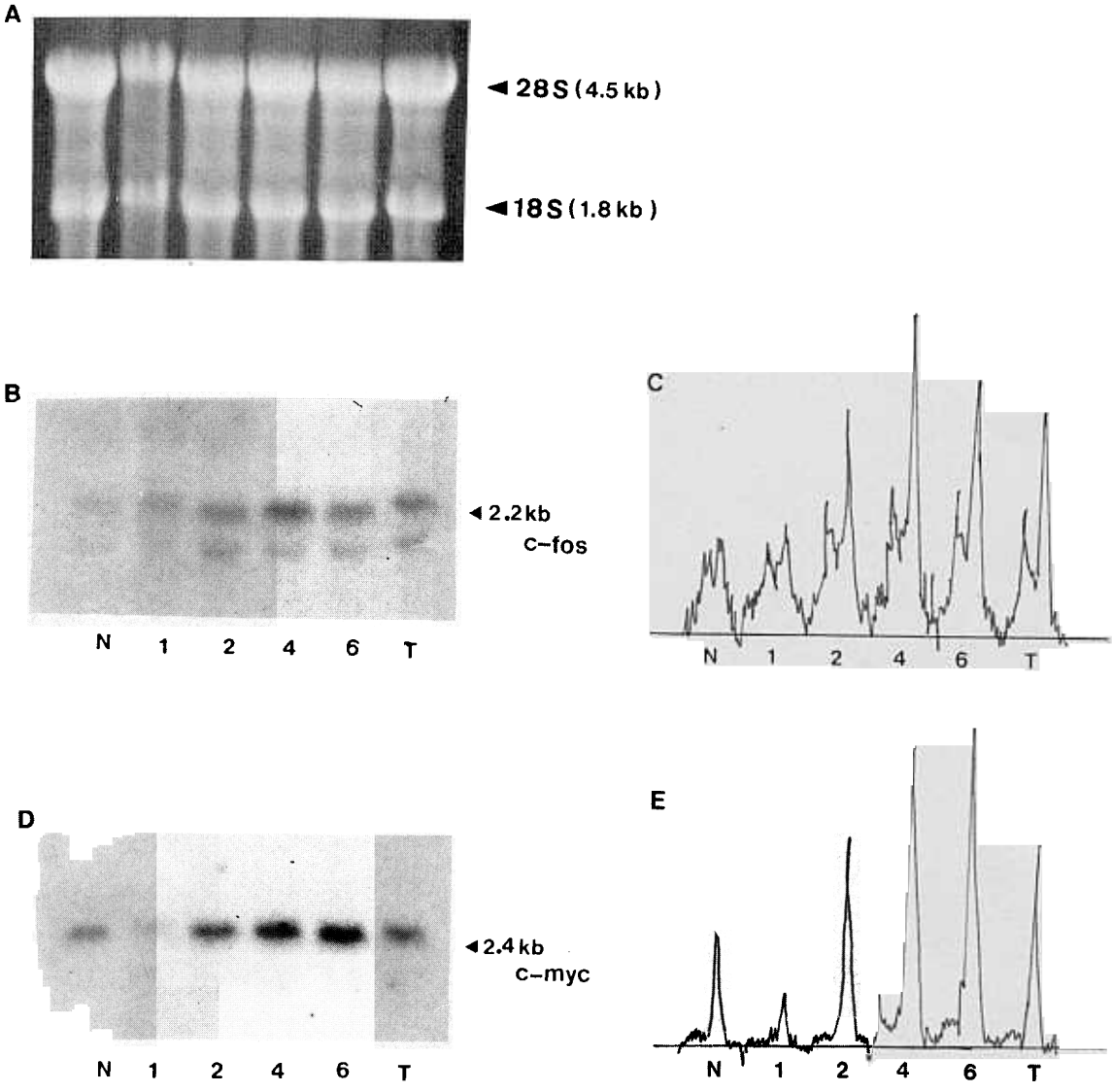

Fig. 3. Northern blot analysis of c-fos and c-myc mRNA during 3'-MeDAB-induced rat liver carcinogenesis. Total RNA (30 μg) was extracted from the normal adult rat livers (N), the preneoplastic livers of rats maintained on 3'-MeDAB diet (1, 2, 4, 6, weeks) and tumor group (T) rats and fractionated in 1% agarose formaldehyde gels, blotted to a nitrocellulose membrane and hybridized with ^{32}P-labeled probes described in the text. Each group was consisted of three cycloheximide (100 mg/kg, intraperitoneally)-treated rats. A. Major ribosomal RNAs (28S, 18S) B. Hybridization with the v-fos (1.0 kb). c-fos transcripts migrated in a 2.2 kb band. C. Densitometric analysis of panel B (c-fos mRNA). D. Hybridization with the murine c-myc probe (exon III). c-myc transcripts migrated in a 2.4 kb band. E. Densitometric scanning analysis of panel D (c-myc mRNA).

tracted with 0.5N PCA at 70°C for 1 hour. Suitable aliquots of the supernatant were used for measurement of radioactivity and of DNA content by using Burton's diphenylamine method (Burton, 1956).

RESULTS

Elevated expressions of c-*fos* and c-*myc* genes in early preneoplastic livers and tumor nodules of 3'-MeDAB-induced rat hepatocarcinoma

Total cellular RNA from the preneoplastic livers of the 1, 2, 4 and 6 week rats and nodules of rat hepatoma were examined for expressions of c-*fos* and c-*myc* genes (Fig. 3). The elevated levels of the 2.2kb c-*fos* transcripts (van Straaten et *al.* 1983) and 2.4kb c-*myc* transcripts (Watt et *al.* 1983) and 2.4kb c-*myc* transcripts (Watt et *al.* 1983) appeared in preneoplastic livers as early as 2 weeks after the feeding of the 3'-MeDAB diet and were maintained through 4, 6 weeks and tumor. The densitometric scanning (Laser densitometer, LKB) analysis showed that the fold induction of c-*fos* mRNA compared to that of control liver was 2.1, 3.1, 2.4 and 2.1 at 2, 4, 6 weeks and tumor nodules respectively (Fig. 3, C). The fold increases of c-*myc* mRNA were very similar to those of the c-*fos* gene, but the signal at 6 weeks was slightly higher than that at 4 weeks (Fig. 3, E).

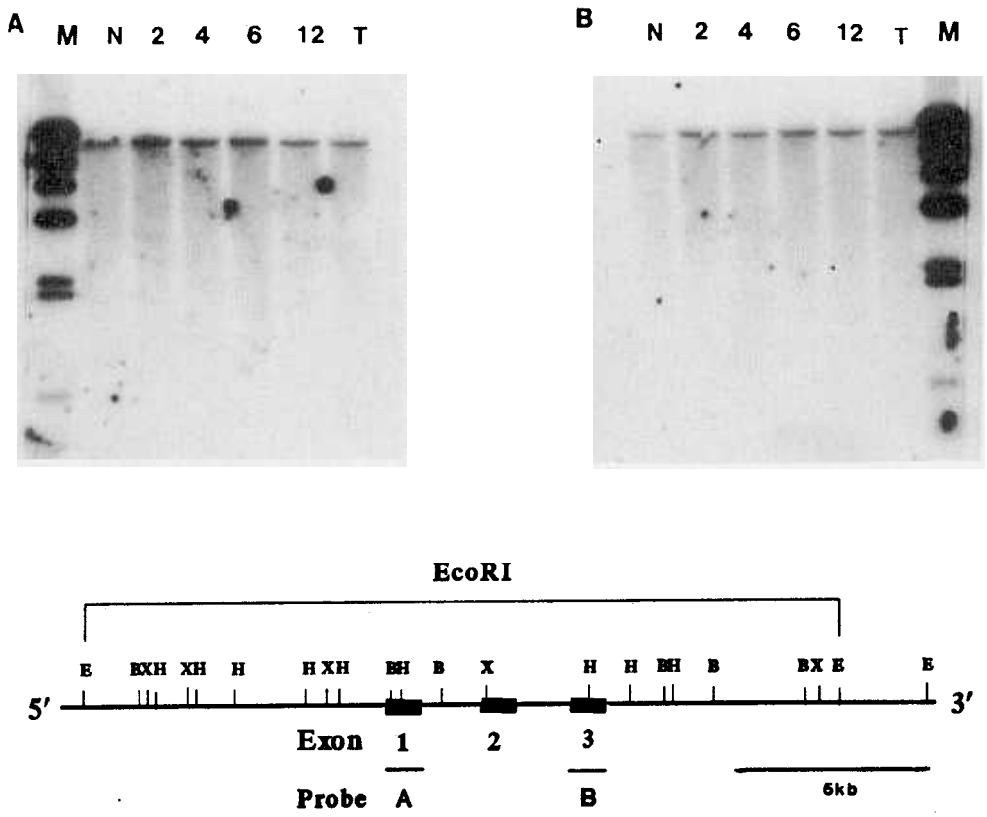

Fig. 4. Southern blot analysis of the c-myc locus in DNA during 3'-MeDAB-induced rat liver carcinogenesis. Genomic DNA (10 μg/lane), isolated from nomal adult rat liver (N) and the preneoplastic liver of rats maintained on 3'-MeDAB diet (2, 4, 6, 12 weeks) and tumor group (T) rats were fully digeset to completion with EcoRI. The digests were fractionated on 0.8% agarose gel transferred to a nitrocellulose membrane, and the digests in panel A and B were hybridized to ^{32}P-labeled exon I (probe A) and exon III (probe B) of murine c-myc respectively. Molecular weight marker was a ^{32}P-labeled λ/Hind III fragments. Abbreviations for the restriction enzymes are as follows: E, EcoRI: B, BamHI; X, XbaI; H, Hind III. Restriction sites and exon I, II, III of murine c-myc on the genomic map are based on the published sequence of the gene (Corcoran, 1984).

Southern blot analysis

Southern blot analysis with v-*fos* or c-*myc* probes revealed that c-*fos* and c-*myc* loci were in approx. 5.5kb (Fig. 5) and 20.0kb (Fig. 4) DNA bands respectively in the preneoplastic livers and hepatoma nodules as well as in the normal adult livers. Gross amplification or rearrangements were not observed in the DNA of the preneoplastic livers and hepatoma nodules. Whether point mutations or small deletions or insertions which may affect c-*fos* and c-*myc* expressions occurred cannot be determined by this analysis.

Determination of the rate of [^{3}H] thymidine incorporation into DNA

We measured the rate of [^{3}H] thymidine incorporation into hepatic DNA in order to monitor the rate of cell proliferation occurring at the early preneoplastic periods. Table 2 indicates that the rate of thymidine incorporation was significantly increased over the control value beginning at 2 weeks of 3'-MeDAB diet feeding and continuing through 6

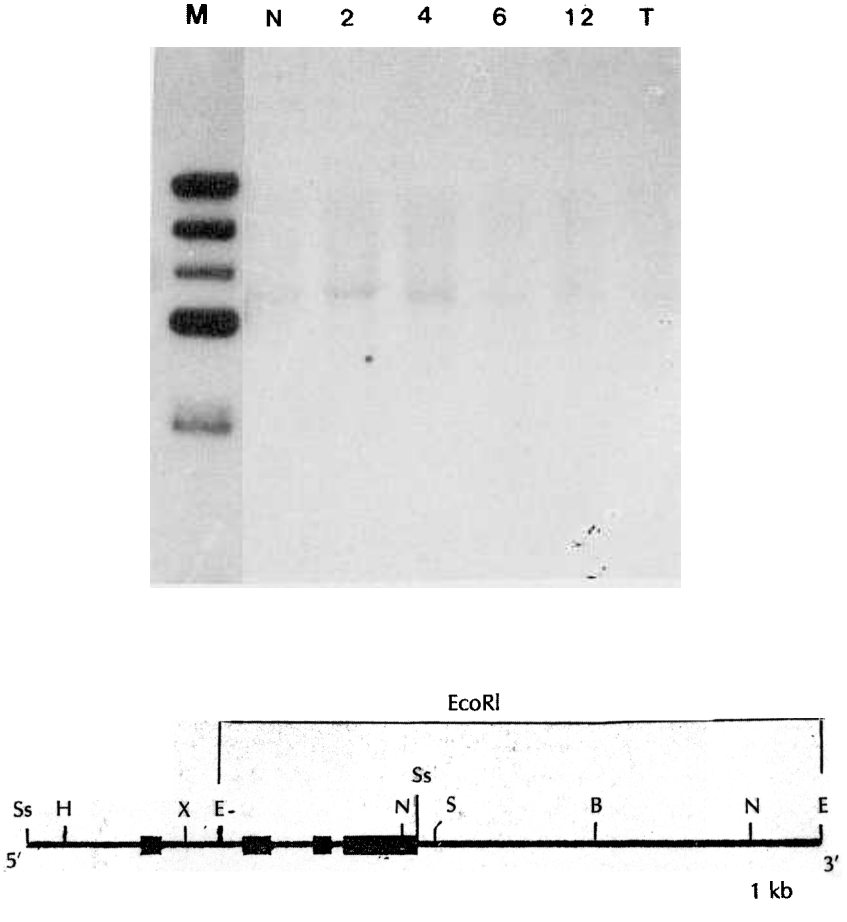

Fig. 5. Southern blot analysis of the c-fos locus in DNA during 3'-MeDAB-induced rat liver carcinogenesis. Same genomic DNA (10 μg/lane) in Fig. 2. was fully digested with EcoRI. The digests were fractionated on an 0.8% agarose gels, transferred to a nitrocellulose membrane, and hybridized to ^{32}P-labeled v-fos. Molecular weight marker was a ^{32}P-labeled λ/Hind III fragment. Abbreviations for the restriction enzymes are as follow: Ss, SstI; H, Hind III; X, XhoI; E, EcoRI: N, NcoI, S, SalI; B, BamHI. Murine c-fos sequences on the genomic map are based on the published sequence of the gene (Miller et al, 1984).

Table 2. [^{3}H] Thymidine incorporation into hepatic DNA in preneoplastic period of 3'-MeDAB-induced rat liver carcinogenesis

	Total rats	DNA/g liver (mg)	[^{3}H] thymidine incorporation (cpm/mg DNA)	p-value
Control	3	1.80±0.05	14700±8681	
1 week	5	1.95±0.21	19160±2550	NS
2 week	3	2.50±0.08	25300±2022	0.05
4 week	4	2.91±0.25	56750±6130	0.05
6 week	4	3.25±0.33	28160± 763	0.05

Rats were fed 3'-MeDAB diet for 1-6 weeks *ad libitum* and control rats were fed normal diet. [^{3}H] thymidine (100 μCi/100g B.W.) was injected intraperitoneally to each group of rats 2 hours before sacrifice. Their livers were homogenized in 6 vol. of 0.075 M NaCl-0.025 M EDTA, pH 7.6. The measurement of radioactivity and quantitation of DNA were done as described in materials and methods. Values represent the mean±SD of 3-5 rats per group. $P<0.05$ compared to control group, by ANOVA and multiple-comparison tests.

weeks. There was a gradual increase in DNA content from the 1st week through the 6th week over control value. Since the amount of thymidine incorporation represents the rate of incorporation per unit time at that specific period, it does not have to be increased in parallel with the absolute amount of DNA at the corresponding periods in order to indicate the increase in ongoing cell proliferation over the control value. In our study the peak response of thymidine incorporation induced by 3'-MeDAB diet appeared at 4 weeks, notably somewhat later than in the previous study (Becker *et al.* 1975).

DISCUSSION

3'-MeDAB causes widespread necrosis of rat hepatocytes and rapid and intense compensatory cell division at an early stage of tumor development (Sell *et al.* 1976). Early changes also include extensive proliferation of, especially, oval cells and bile duct cells (Price *et al.* 1952; Goldfarb 1973; Sell *et al.* 1976). Cirrhosis with hyperplastic nodules and cholangiofibrosis also develops during the preneoplastic periods, and later these develop into multiple and mixed-type hepatomas, cholangiocarcinomas and undifferentiated carcinoma (Price *et al.* 1952). In this study, we obtained clear evidence that c-*fos* and c-*myc* transcripts were elevated as early as the 2nd week after feeding with the 3'-MeDAB diet, continuing through the 4th, 6th weeks and in tumor nodules over control livers (Fig. 3). Since these proto-oncogenes are superinduced by cycloheximide, an inhibitor of protein synthesis (Kelly *et al.* 1983; Leder *et al.* 1983; Sassone-Corsi *et al.* 1988), we gave cycloheximide for 1 hour before sacrifice in order to amplify the levels of these transcripts, thereby allowing easy comparisons of the low amounts of mRNA accumulated in the samples. The increases that we observed are likely to be the result of enhanced synthesis at the transcriptional level rather than the stabilization of mRNA in general because the mechanism of superinduction of these genes by cycloheximide is well known to be working at the transcritional level (Leder *et al.* 1983; Sassone-Corsi *et al.* 1988). These growth-related nuclear proto-oncogenes (Muller *et al.* 1984) are expressed at elevated levels in a wide range of experimental animals and human cancerous tissues (Adams *et al.* 1983; Ren *et al.* 1986; Boultwood *et al.* 1988; Terrier *et al.* 1988; Klimpfinger *et al.* 1988; Goto *et al.* 1990).

The overexpression of c-*myc* is usually a consequence of a specific translocation (Klein, 1983), promoter insertion (Hayward *et al.* 1981) or amplification (Riou *et al.* 1987). However, there are several reports that high levels of expression of the c-*fos* and c-*myc* genes occur without concomitant amplification or rearrangement of the loci in tumor tissues (Erisman *et al.* 1985; Rothberg *et al.* 1984; Terrier *et al.* 1988). We also found that elevated expressions of c-*fos* and c-*myc* at the precancerous stages and tumor nodules of 3'-MeDAB induced rat hepatoma occurred without any apparent rearrangement or amplification of the loci (Fig. 4, 5). The results of our examination and others (Erisman *et al.* 1985; Rothberg *et al.* 1984; Terrier *et al.* 1988) suggest that neither rearrangement nor amplification of c-*fos* and c-*myc* is a necessary primary

genetic change linked to enhanced expression of these transcripts, and the overexpression without concomitant amplification or rearrangement of the loci also appears to be an important consideration in oncogenesis. However, in our study, it is interesting to note that the elevated levels of c-*fos* and c-*myc* transcripts in the precancerous stages correspond to the rate of [^{3}H] thymidine incorporation into hepatic DNA during this period (Table 2). The highest signals of c-*fos* mRNAs were found at 4 weeks-feeding of 3'-MeDAB, particularly, parallel with the highest rate of [^{3}H] thymidine incorporation at this period. This may suggest that the high-level expressions of c-*fos* and c-*myc* observed here may result from the continuous cell proliferative stimuli generated in the carcinogen altered cells, which are essential to the initiation and promotion of chemical hepatocarcinogenesis (Columbano *et al.* 1990). Since the precancer and tumor tissues we have analyzed are mixed populations of cells, we are not certain in what cells the c-*fos* and c-*myc* transcripts are expressed. However, Yaswen *et al.* (1985) found a proliferation of oval cells in the precancerous stages after the feeding of ethionine in a choline-deficient (CDE) diet with concomitant increase of c-*myc* and c-Ki-*ras* mRNA. Sell and Salmon (1984) also reported a proliferation of mixed cells including oval cells after the feeding of an acetylaminofluorene-containing CDE diet. Therefore, the elevated levels of c-*fos* and c-*myc* transcripts in our study may be partially, if not all, related to the proliferations of the oval cells and bile duct cells which occur in the early stages of 3'-MeDAB-induced hepatocarcinogenesis. Important questions, however, remain. First, what are the exact roles of c-*fos* and c-*myc* expressions in the precancerous stages of 3'-MeDAB-induced hepatocarcinoma? To answer this, we only have a general idea that elevated expressions of these proto-oncogenes, whose translated products are related to nuclear transcription regulation (Bradshaw and Prentis, 1987), may be necessary for cell cycle progression and the growth transformation aspect of the precancersous cells which may contribute to tumor progression. Secondly, if the elevated expressions of c-*fos and c-myc* transcripts indicate an increase in cell proliferation, what causes the continuous growth signal in the early preneoplastic regions as well as in the tumor nodules? The present experiment does not provide any definite clues to answer this question. We can only predict one possibility that preexisting mutations of other oncogenes (e.g. *ras*) caused by 3'-MeDAB could initiate this signal. The loss of tumor suppressor genes (e.g. *p53*), of which a key role is in the inhibiton of cell proliferation (Sanger, 1989), can also occur along this path. This hypothesis fits well into the multi-stage theory of cancer development (Klein and Klein, 1985). Apparently cooperating lesions in cellular oncogenes accumulate during tumor progression and clonal selection and increase the malignant potential of the tumor cells. This experimental model, therefore, is attractive for further analysis of the signal transduction mechanisms responsible for the overexpressions of c-*fos* and c-*myc* in the carcinogen altered cells *in vivo*. We are now investigating these questions.

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