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Over expression of proto-oncogenes: ki-ras, fos and myc in rat liver cells treated in vitro by two liver tumor promoters: phenobarbital and biliverdin

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Summary

Among the liver cell strains established in the laboratory from the liver of 10-day-old rats, some of them (FV) underwent spontaneous neoplastic transformation after a number of subcultures. However one (Cl_3) had maintained a non-transformed phenotype (persistence of contact inhibition, lack of growth in soft agar and of gamma glutamyltranspeptidase activity). These cells were grown either for a short time (48 h) or a longer time (3 weeks) in the presence of two liver tumor promoters: phenobarbital (0.2×10^{-3} M) or biliverdin (10^{-6} M). Total RNA was analysed by dot blot and Northern blot, then hybridized with ki-ras, fos and myc probes, previously labelled with ^{32}P by nick translation. The three oncogenes were well expressed by the two strains but while an over-expression was observed for the Cl_3 non-transformed cells when they were grown in presence of phenobarbital or biliverdin, the FV-transformed cells were not sensitive to the two promoters.

Keywords: cell culture; liver tumor promotion; phenobarbital; biliverdin; oncogenes expression.

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Introduction

One of the most exciting developments in the discovery of proto-oncogenes as cellular homologues of the viral genes responsible for neoplastic transformation of cells was the demonstration that these genes could exist in activated forms in non virus infected cells transformed by chemicals [1,2]. Chemical carcinogenesis is classically considered as a multistep process, and two steps at least have been defined: initiation, which is a latent irreversible genetic change caused by a chemical agent and promotion induced by repetitive treatment with a non or a weakly carcinogenic substance. Initiators (which can be complete carcinogens including a promoting effect) are thus considered as mutagenic substances while promoters are regarded as agents which perturb genes expression, or pattern of differentiation, permitting the genetic changes acquired during initiation to be expressed.

In the field of hepatocancerogenesis, many authors have demonstrated the role of initiators as proto-oncogenes activators [3—6], however the promotional event has been studied essentially with the skin model using phorbol esters and related substances as promoters [7,8] or hormones which often act as promoters in the target tissues [9]. Our purpose was to study the effects of two liver promoters, pheno-

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barbital [10—12] and biliverdin (a bile pigment) [13], on the expression of three proto-oncogenes c-ki-ras, c-myc and c-fos in two strains of epithelial cells derived from rat liver.

Materials and methods

Cell lines and medium

Epithelial cell lines, fibroblast free, were prepared from the liver of 10-day-old Fischer rats according to Williams et al. [14]. They were grown in William's medium (Eurobio, France) supplemented with 10% foetal calf serum (Flow, England), glutamine and antibiotics. The cells were usually plated at 5×10^4 cells/ml medium in plastic flasks, incubated at 37 °C in a humidified incubator with a 5% CO_2 atmosphere and subcultured weekly. They were screened periodically for mycoplasma contamination and kept frozen for use at sequential passages.

Chemicals

Phenobarbital was purchased from Rhone-Poulenc. Biliverdin was provided by Sigma Chemical Company.

Experimental procedure

The two cell lines, FV and Cl_3, were respectively subcultured in three groups.

One group of cells were cultivated in presence of phenobarbital : (PB) : 50 μg/ml of culture (0.2×10^{-3} M) in solution in dimethylsulfoxide (DMSO) for either a short time (48 h) or a longer period of time (3 weeks).

One group of cells was grown in presence of biliverdin (BV): 500 ng/ml of culture (10^{-6} M) in the same solvent and conditions as for the first group.

The cells of the third group were used as controls and were treated with DMSO alone. The concentration of DMSO in the medium must be always below 1% in order to avoid toxicity.

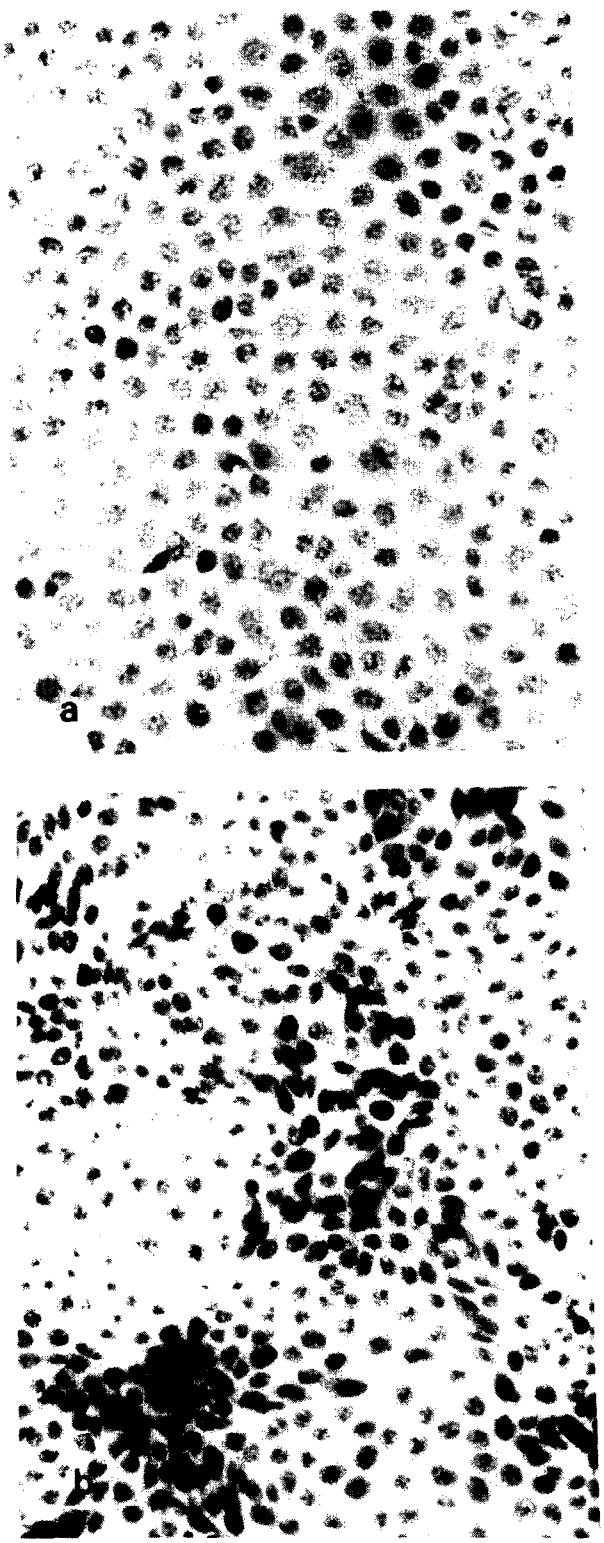
Dot blot and Northern analysis

Total RNA from fresh or frozen (−80 °C) cells was isolated using the guanidine thiocyanate method of Chirgwin et al. [15]. The total RNA was spotted directly on biodyne nylon membrane or analysed by Northern blotting. In this case, 20 μg of total RNA were dissolved in MOPS buffer containing 50% formamide and 6% formaldehyde then incubated at 60 °C for 5 min and electrophoresed in a 1.2% agarose gel in MOPS buffer. Samples were transferred to biodyne nylon paper and baked at 80 °C in a vacuum oven for 2 h. Dot blot and Northern transferred membranes were hybridized to the following oncogene clones: *Myc*, the 1.5 kb sac 1 fragment containing the second exon from a human c-myc, provided kindly by Dr. D. Stehelin (Lille); *Ki-ras*, the 1 kb Eco RI fragment from the clone Hi Hi 3 of Kirsten murine sarcoma virus provided kindly by Dr. E. Scloelnick (USA); *Fos*, the 3.1 kb Xhol-Ucol DNA fragment of the human fos gene (Amersham). The probes were labelled by nick translation with ^{32}P dCTP (Amersham) to a specific activity of approximately 1×10^8 dpm μg^{-1}. Prehybridation was performed at 42 °C for 12 h in a solution containing 50% deionised formamide, 5 × SSC, 0.1% SDS, 5 × Dehnardt, 50 mM sodium phosphate pH 6.5 and 250 μg/ml of sonicated salmon testes DNA. Hybridization was performed for 48 h at 42 °C with the same mixture containing the labelled probes. The membranes were then washed three times for 5 min at room temperature with a solution containing 2 × SSC and 0.1% SDS and once at 50 °C for 15 min with a solution containing 0.1 × SSC and 0.1% SDS. Membranes were then dried and exposed to Amersham Hyperfilm MP with Dupont Cronex intensifying screens at −80 °C for 1 week.

Results

Morphological features of the cells

The FV cells during the first transfer has a flattened epithelial shape and grew in a mosaic-like pattern. These cells divided slowly with high adhesiveness to the substrate and neighboring cells (Fig. 1a). At this stage they had a non-transformed phenotype with no ability to



grow in soft agar and they did not express gamma-glutamyltranspeptidase (γGT). After a number of transfers (between 30 and 40) some evidence of spontaneous neoplastic transformation was observed [16]. Gradual overlapping appeared in the cultures (Fig. 1b), the cells were able to form colonies in soft agar, and expressed γGT.

The Cl_3 cells were cloned from FV cells at an early transfer. This clone conserved, even after a great number of subcultures (more than 100), a non-transformed phenotype (Fig. 1c) with maintenance of high adhesiveness to the substrate and the neighboring cells, lack of growth in soft agar and no γGT activity.

Expression of the oncogenes

In these experiments the Cl_3 non-transformed cells were used at the 127th transfer and compared to their transformed counterpart FV cells at the 112th transfer both after

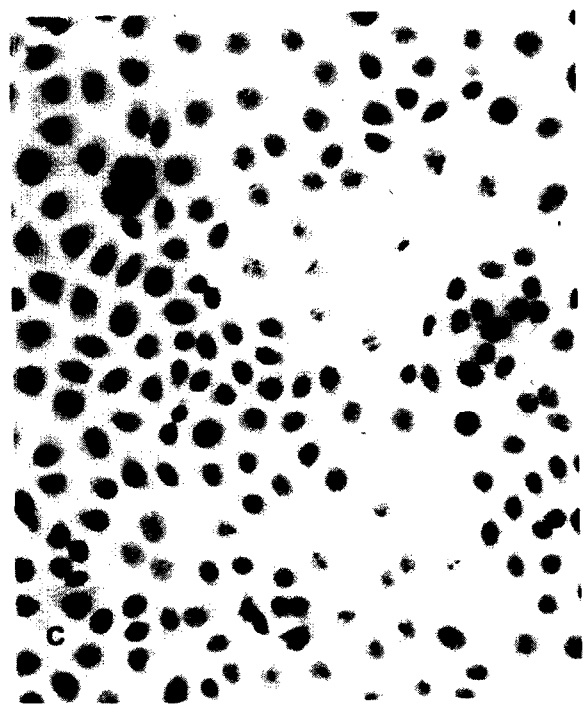


Fig. 1. Morphological aspect of the cells. a: FV cells 17th transfer × 160; b: FV cells 126th transfer × 160; c: Cl_3 cells 112th transfer × 160.

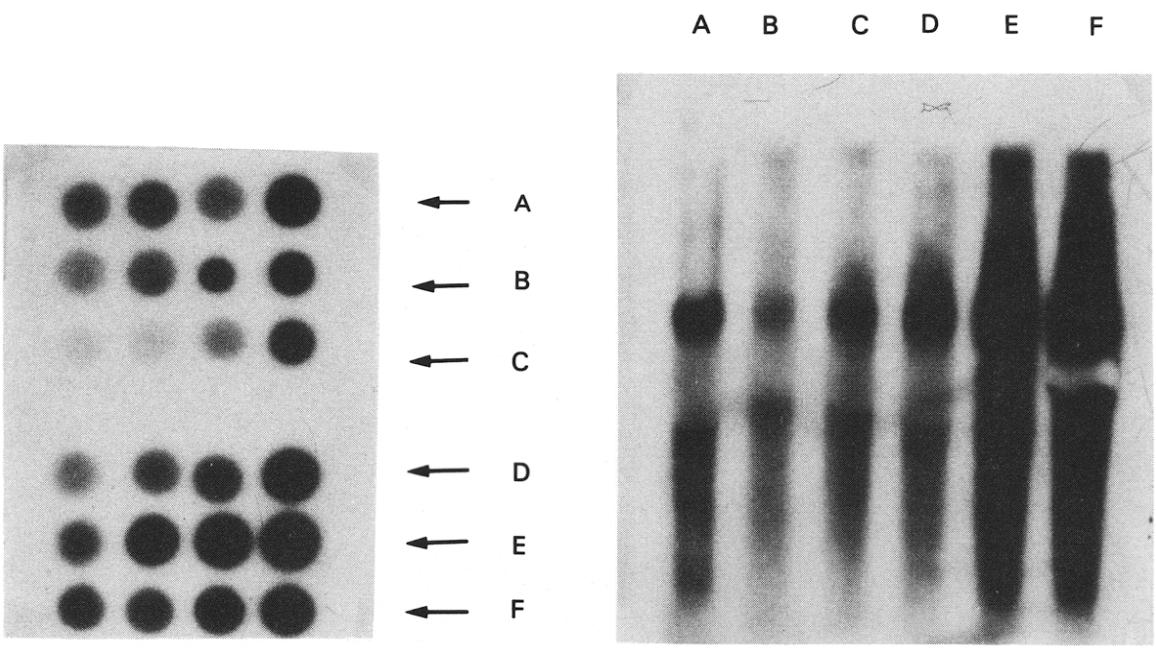


Fig. 2. Stimulation of ki-ras proto-oncogenes expression in liver cells in culture after long term exposure to tumor promoters. A: FV control; B: FV biliverdin; C: FV phenobarbital; D: Cl_3 control; E: Cl_3 biliverdin; F: Cl_3 phenobarbital. 4 μg, 2 μg, 1 μg, 0.5 μg of total RNA were spotted for the dot blot; 20 μg of total RNA for each lane of the Northern.

exposure to phenobarbital (PB) or biliverdin (BV).

FV transformed cells expressed strongly Ki-ras mRNA (Fig. 2) by dot blot as well as by Northern analysis. Neither PB nor BV increased this expression. By contrast, though Cl_3 non-transformed cells expressed Ki-ras mRNA at approximately the same level as for FV cells they were very sensitive to the action of both PB and BV. The expression of the oncogene Ki-ras was strong as observed in Northern analysis, after 3 weeks exposure to PB and BV.

The fos oncogene was also well expressed by both FV and Cl_3 cells, but as for Ki-ras the two promoters were without affect on FV cells fos expression after 48 h (Fig. 3) as well as following long time exposure (Fig. 4). For Cl_3 cells the stimulation of fos expression by PB and BV was perceptible after 48 h of culture (Fig. 3) and an increased expression was evident after 3 weeks exposure (Fig. 4).

As for the two other oncogenes c-myc was well expressed by the two cell strains but again, FV cells were not sensitive to PB and BV, but an over expression was registered for Cl_3 when the cells were grown for 3 weeks in presence of the PB or BV (Fig. 5).

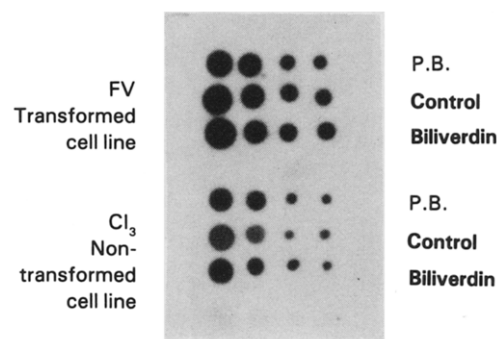


Fig. 3. Stimulation of fos proto-oncogenes expression in liver cells in culture after 48 h exposure to tumor promoters. 4 μg, 2 μg, 1 μg, 0.5 μg of total RNA were spotted for each sample.

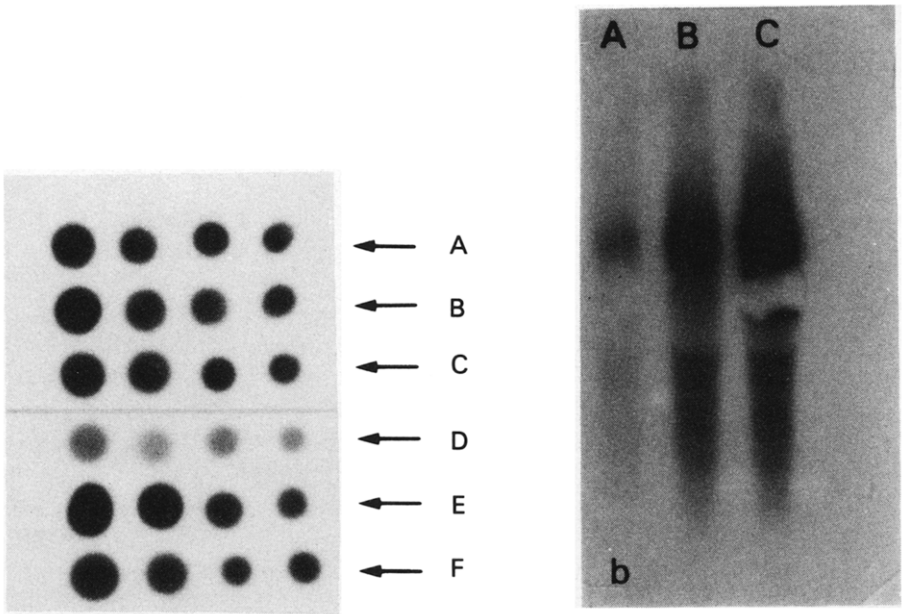


Fig. 4. Stimulation of fos proto-oncogenes expression in liver cells in culture after long term exposure to tumor promoters. (a) A: FV control; B: FV phenobarbital; C: FV biliverdin; D: Cl_3 control; E: Cl_3 phenobarbital; F: Cl_3 biliverdin. (b) A: Cl_3 control; B: Cl_3 biliverdin; C: Cl_3 phenobarbital. 4 μg, 2 μg, 1 μg, 0.5 μg of total RNA were spotted for each sample on the dot blot; 20 μg of total RNA for each lane of the Northern.

Discussion

A great variety of agents with different chemical structures and biological functions have been identified as promoters in hepatocarcinogenesis. Among them is phenobarbital, a drug used widely in human therapy and biliverdin, an endogenous substance which accumulates in pathological conditions following liver damage. Tumor promoters may be defined as compounds either not having or with weak carcinogenic activity when tested alone, but that enhance markedly tumor formation when they are applied following a low or suboptimal dose of an initiating carcinogen.

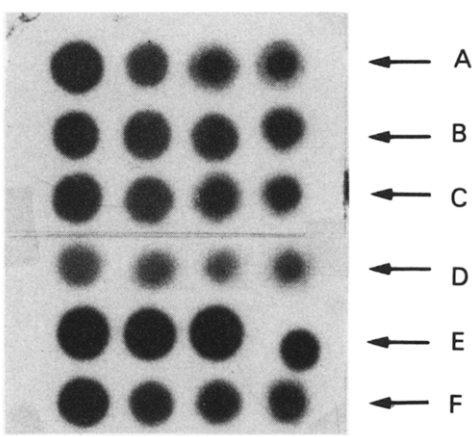


Fig. 5. Stimulation of myc proto-oncogenes expression in liver cells in culture after long term exposure to tumor promoters. A: FV control; B: FV phenobarbital; C: FV biliverdin; D: Cl_3 control; E: Cl_3 phenobarbital; F: Cl_3 biliverdin. 4 μg, 2 μg, 1 μg, 0.5 μg of total RNA spotted for each sample.

The exact mechanisms of tumor promotion are not known, but a wide spectrum of biological and morphological changes have been described for the most intensively studied tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), in the skin model as well as for phenobarbital in the liver model. TPA acts at the membrane level [17] induces ornithine decarboxylase activity [18] and modifies the cytoskeleton of the cells [19]. Phenobarbital plays a similar role for the liver [20,21] and we have demonstrated recently that biliverdin, like phenobarbital, induces important changes in the cytoskeleton features of the two liver strains studied (Decloître et al. unpublished data).

However, the activation of cellular oncogenes is considered to play an important role in carcinogenesis. The present study was undertaken to obtain information at the genomic level on the mechanism of liver tumor promotion by phenobarbital and biliverdin.

We have shown that the two strains studied expressed the three oncogenes at approximately the same level, but the part of cell proliferation and malignancy is not well delineated, as one strain is transformed and the other not, though both having a comparable growth rate. The two promoters exert similar effects on the oncogenes studied : c-ki-ras, c-fos and c-myc are over expressed in Cl_3 cells but the promoters are without effect on FV-transformed cells. An increased expression of c-myc oncogenes and of two endogenous retrovirus-like DNA sequences have also been observed in regenerating rat liver by Hsieh et al. [22]. At first sight, it seems paradoxical that after exposure to phenobarbital or biliverdin the non-transformed Cl_3 cells expressed much more the three oncogenes than the transformed FV cells. In fact, the transformed cells have lost their ability to respond to promoters. For example *P*-450 cytochromes were no more inducible by phenobarbital in transformed cells (Decloître et al. unpublished data). Furthermore we have demonstrated some years ago [23] that biliverdin enhanced [^{3}H]thymidine incorporation in the DNA of rat epithelial liver cells but that this property was lost after neoplastic transformation of the cells. One explanation might be the membranes and cytoskeleton changes, that we observed in these cells after their transformation (Decloître et al. unpublished data).

Oncogenes can be activated by point mutation, enhanced gene expression, gene amplification or chromosomal rearrangements. Ras genes are known to acquire transforming properties by single point mutations [24] whereas most examples for activation of myc genes involve chromosomal translocation or gene amplification and over expression [25,26]. The activation of fos gene is more ambiguous and requires the addition of viral LTR sequences and removal of sequences from 3′ non-coding regions of the gene [27]. In most cases, the co-operation of two oncogenes, belonging to complementary classes of genes, is needed to obtain the transformation of the cells. This is the case for ki-ras oncogenes which encode proteins acting at the membrane level, while the proteins encoded by myc and fos genes are located in the nucleus of cells.

In our experimental model we have observed an over expression of the three oncogenes studied. This result is in agreement with the concept of multistage carcinogenesis. The initiation step is a genetic event which may correspond to a point mutation of an oncogene, but in many cases this DNA alteration alone is insufficient. Balmain and co-workers [28,29] have shown the presence of an activated and transforming ha-ras in many chemically induced skin carcinomas and papillomas. The presence of an active oncogene in both benign and malignant lesions may indicate that the activation of the ha-ras alone is not sufficient for complete malignant development. A process such as the action of promoters by which normal cellular proliferation might be disrupted, may lead to increase expression and/or amplification of the transformed genes and hence to malignant conversion. Furthermore the idea of negative modulation of cancer may be introduced at the promotional step, for example, the indole-3-carbinol may inhibit or enhance aflatoxin B_1 induced hepatocellular carcinoma in the rainbow trout according to the experimental protocol used [30].

Initiation-promotion experiments with the Cl_3 cells using aflatoxin B_1 as an initiator and biliverdin or phenobarbital as promoters are in progress in our laboratory. The detection at the different initiation-promotion steps of the transforming potency of the genomic DNA of the cells, in DNA transfection assays using NIH/3T3 cells should give us some more information in the future.

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References

1 Reddy, E.P., Reynolds, R.K., Santos, E. and Barbacid, M. (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature, 300, 149—152.

2 Barbacid, M. (1985) Oncogenes in human cancers and in chemically induced animals tumors. Prog. Med. Virol., 32, 86—100.

3 Raymondjean, M., Defer, N., Tichonicky, L., Kruh, J., Kneip, B. and Guguen Guillouzo, C. (1985) Changes in poly (A)$^+$ translational pattern during chemically induced hepatocarcinogenesis. Carcinogenesis, 6, 1797—1803.

4 Wiseman, R.W., Strowers, S.J., Miller, E.C., Anderson, M.W. and Miller, J.A. (1986) Activating mutations of the C-Ha-ras protooncogene in chemically induced hepatomas of the male B6C3F1 mouse. Proc. Natl. Acad. Sci., 83, 5825—5829.

5 Tashiro, F., Morimura, S., Hayashi, K., Makino, R., Kawamura, H., Horikoshi, N., Nemoto, K., Ohtsubo, K., Sugimura, T. and Ueno, Y. (1986) Expression of the c-Ha-Ras and c-myc genes in aflatoxin B_1 induced hepatocellular carcinomas. Biochem. Biophys. Res. Commun., 138, 858—864.

6 McMahon, G., Hanson, L., Lee, J.J. and Wogan, G.N. (1986) Identification of an activated c-Ki-ras oncogene in rat liver tumors induced by aflatoxin B_1. Proc. Natl. Acad. Sci., USA, 83, 9418—9422.

7 Tofgard, R., Roop, D.R. and Yuspa, S.H. (1985) Protooncogenes expression during two stages carcinogenesis in mouse skin. Carcinogenesis, 6, 655—657.

8 Peling, J.C., Neades, R. and Strawhecker, J. (1988) Epidermal papillomas induced in uninitiated mouse skin by tumor promoters alone contain a point mutation in the blot codon of the Ha-ras oncogene. Carcinogenesis, 9, 665—667.

9 Guernsey, D.L. and Leuthauser, S.W.C. (1987) Correlation of thyroid hormone dose dependent regulation of Ki-ras protooncogene expression with oncogene activation by 3-methylcholanthrene: Loss of thyroidal regulation of the transformed mouse cell. Cancer Res., 47, 3052—3056.

10 Peraino, C., Fry, R.J.M., Staffeldt, E. and Christopher, J.P. (1975) Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin and dichlorodiphenyltrichloroethane on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res., 35, 2884—2890.

11 Goldsworthy, T., Campbell, H.A. and Pitot, H.C. (1984) The natural history and dose response characteristics of enzyme-altered foci in rat following phenobarbital and diethylnitrosamine administration. Carcinogenesis, 5, 67—71.

12 Lans, M., de Gerlache, J., Taper, H., Preat, V. and Roberfroid, M.D. (1983) Phenobarbital as a promoter in the initiation/selection process of experimental rat liver hepatocarcinogenesis. Carcinogenesis, 4, 141—144.

13 Lafarge-Frayssinet, C., Morel-Chany, E., Trincal, G. and Frayssinet, C. (1983) Promoting effect of biliverdin for liver cells initiated by aflatoxin B_1. Cancer Lett., 18, 277—282.

14 Williams, G.M., Weisburger, E.K. and Weisburger, J.H. (1971) Isolation and long-term culture of epithelial-like cells from rat liver. Exp. Cell Res., 69, 106—112.

15 Chirgwin, J.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, 18, 5294—5299.

16 Morel-Chany, E., Lafarge-Frayssinet, C. and Trincal, G. (1985) Progression of spontaneous malignant transformation of epithelial rat liver cell lines. Cell Biol. Toxicol., 1, 11—22.

17 Horowitz, A.D., Greenbaum, E. and Weinstein, I.B. (1981) Identification of receptors for phorbol ester tumour promoters in intact mammalian cells and of an inhibitor of receptor binding in biological fluids. Proc. Natl. Acad. Sci., USA, 78, 2315—2319.

18 O'Brien, T.G., Simsiman, R.C. and Boutwell, R.K. (1975) Induction of polyamine biosynthetic enzymes in mouse epidermis by tumour promoting agents. Cancer Res., 35, 1662—1670.

19 Takahashi, K., Heine, U.I., Junker, J.L., Colburn, N.H. and Rice, J.M. (1986) Role of cytoskeleton changes and expression of H-ras oncogene during promotion of neoplastic transformation in mouse epidermal JB6 cells. Cancer Res., 46, 5923—5932.

20 Schulte-Hermann, R. (1985) Tumor promotion in the liver. Arch. Toxicol., 57, 147—158.

21 Eckl, P.M., Meyer, S.A., Whitcombe, W.R. and Jintle, R.L. (1988) Phenobarbital reduces EGF receptors and the ability of physiological concentrations of calcium to suppress hepatocyte proliferation. Carcinogenesis, 9, 479—483.

22 Hsieh, L.L., Peraino, C. and Weinstein, I.B. (1988) Expression of endogenous retrovirus-like sequences and cellular oncogenes during phenobarbital treatment and regeneration in rat liver. Cancer Res., 48, 265—269.

23 Lafarge-Frayssinet, C., Morel-Chany, E., Trincal, G. and Frayssinet, C. (1981) Enhancement of DNA synthesis by biliverdin in a non-transformed liver cell strain. Cell Mol. Biol., 27, 77—82.

24 Barbacid, M. (1987) Mutagens, oncogenes and cancer. In: Oncogenes and Growth Factors, pp. 90—99. Editor: R.A. Bradshaw and S. Prentis. Elsevier Science Publishers, Amsterdam.

25 Dalla-Favera, R., Martinotti, S., Gallo, R.C., Erikson, J. and Croce, C.M. (1983). Translocation and rearrangements of the c-myc oncogene locus in human undifferentiated B-cell lymphoma. Science, 219, 963—967.

26 Marcu, K.B., Harris, L.J., Stanton, L.W., Eriskson, J.,

Watt, R. and Croce, C.M. (1983). Transcriptionally active c-myc oncogene is contained within NIARD, a DNA sequence associated with chromosome translocation in B cell neoplasma. Proc. Natl. Acad. Sci., USA, 80, 519—523.

27 Verma, I.M. (1987) Proto-oncogene fos: a multifaceted gene. In: Oncogenes and Growth Factors, pp. 67—73. Editors: R.A. Bradshaw and S. Prentis. Elsevier Science Publishers, Amsterdam.

28 Balmain, A. and Pragnell, I.B. (1983) Mouse skin carcinomas induced in vivo by chemical carcinogens have a transforming Harvey-ras oncogene. Nature, 303, 72—74.

29 Balmain, A., Ramsden, M., Bowden, G.T. and Smith, J. (1984) Activation of the mouse cellular Harvey-ras gene in chemically induced benign skin papillomas. Nature, 307, 658—660.

30 Bailey, G.S., Hendricks, J.D., Shelton, D.W., Nixon, J.E. and Pawlovski, N.E. (1987) Enhancement of carcinogenesis by the natural anticarcinogen indole-3-carbinol. J. Natl. Canc. Inst., 78, 931—934.