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CORRELATED INCREASE OF THE EXPRESSION OF THE C-RAS GENES IN CHEMICALLY INDUCED HEPATOCARCINOMAS

D. Corcos, N. Defer, M. Raymondjean, B. Paris, M. Corral, L. Tichonicky and J. Kruh

Institut de Pathologie et Biologie Cellulaires et Moléculaires
INSERM U137, 24 rue du Fg Saint-Jacques 75014 Paris, France

D. Glaise, A. Saulnier and C. Guguen-Guillouzo

INSERM U49, Hôpital Pontchaillou, 35011 Rennes Cedex, France

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SUMMARY : The expression of the c-Ha-ras, the c-Ki-ras and the N-ras genes was measured by the dot blot technique in rat liver tumors induced by a short diethylnitrosamine (DENA) treatment and in the surrounding liver cells. A 2 to 25 times higher level of transcript was found as well in the surrounding cells, as in the tumor cells, as compared to the level in hepatocytes. In addition the increase of expression was parallel for the three ras genes.

We conclude that this enhanced expression can be attributed to an epigenetic mechanism and it can, in certain cases, be dissociated from cell proliferation.

Cellular genes analogous to retroviral onc genes have been shown to be transcribed in tumor cells as well as in normal cells (1,2). It is assumed that the involvement of these genes in malignancy may result either from a change in their coding sequence or from an alteration of their expression. Enhanced expression of these genes is in some cases related to insertion of retroviral sequences (3), translocation (4) or gene amplification (5,6). Alternatively it can be caused by an epigenetic mechanism (7). c-myc and c-ras genes are the most ubiquitous oncogenes (8). We have found that unlike c-myb, c-sis, c-mos, and c-abl (data not shown) they are expressed in normal rat hepatocytes and overexpressed in a rat hepatoma cell line (HTC cells). In order to know whether that enhanced expression was a frequent feature during hepatocarcinogenesis we turned ourselves to chemically induced liver cancer. We have modified the regimen developed by Solt and Farber (9) for inducing hepatocarcinogenesis by subjecting female rats to a short DENA treatment after a two-third partial hepatectomy. This treatment induces hyperplastic foci of hepatocytes which exhibit a number of characteristic morphological and biochemical alterations (10,11). Then, the majority of the hyperplastic foci undergoes regression whereas some of them progress to form carcinoma nodules (10,12). We report here that the level of RNA specified by the three ras genes is increased not only in neoplastic but also in perineoplastic cells. Moreover, as

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great variations of gene expression, possibly related to tumor heterogeneity, are detected, it clearly appears that the amounts of transcripts of the three ras genes vary in a correlated way.

MATERIALS AND METHODS

Cells and tumors : HTC cells have been established from a Morris Hepatoma (13). Hepatocytes from normal and regenerating liver were obtained by collagenase perfusion of rat liver as previously described (14). Four female rats (Sprague-Dawley) were given three doses of DENA (25mg/kg) by stomach tube 24h, 48h and 72h after a two-third partial hepatectomy. As previously shown (15) the early and late alterations induced by this regimen resemble those described by others (11,12). Rats were sacrified 70 weeks after the DENA treatment. Each liver was perfused via the portal vein with a collagenase solution. Undissociated nodules were easily collected from the cellular suspension of the surrounding hepatocytes. Isolated hepatocytes were purified from the non parenchymal cells by three differential centrifugations ; the hepatocytic nature of these cells was confirmed by their ability to form hepatic molecular colonies and by their incapacity to grow **in vitro.**

Preparation of poly (A)$^+$ RNAs, dot blots and Northern blots hybridization. Frozen tumors and hepatocytes were lysed in 5M guanidium thiocyanate, o.1M sodium acetate (pH 5.5) 1mM EDTA, 5% (v/v) 2-mercaptoethanol and 2% (v/v) N-laurylsarcosine. Total cellular RNAs were prepared as previously described (16,17). Poly (A)$^+$ RNAs were isolated by oligo dT cellulose chromatography.

RNA dotting was performed as described by Thomas (18) using either 1µg or 0.2 µg of poly (A)$^+$ RNA. The blots were hybridized with 50% formamide, 5xSSC, 2xDenhardt (19), 0.1% SDS at 42°C. Washings were performed with 0.1SSC at 52°C.

In each case, various DNA concentrations of the corresponding clones were treated in the same conditions.

In order to ascertain that the same amounts of RNAs were fixed, filters were then rehybridized with the cDNA of one of the samples, since these RNA populations share at least 90% common sequences (unpublished results).

For Northern blotting, poly (A)$^+$ RNAs were denatured for 10 min at 60°C in 10mM phosphate buffer (pH 7.0), 50% formamide, 2.2M formaldehyde, 1mM EDTA, 5mM sodium acetate and electrophoresed on 0.8% agarose gels. Ribosomal RNAs were used as size markers. RNA was transferred to nitrocellulose papers in 20xSSC.

Hybridizations were performed as described above.

Probes : The c-Ha-ras probe was either the clone BRI, a Bam-H_1-Eco R_1 subclone of the clone BS9 (20) or the Sac I-PstI fragment of the BRI clone which corresponds to $p21^{ras}$. The c-Ki-ras probe was either clone KBE-2, or the sac II-Hinc II fragment of the clone KBE-2. The N-ras probe was the human genomic 9.2 Kb probe (21). The c-myc probe was the sac I genomic fragment which corresponds to the first exon (22). The albumin probe was the clone pRSA 8 isolated by T.D. Sargent **et al** (23). The actin probe was the clone 91 isolated by A.J. Minty **et al** (24).

Quantitation of the dots was done by densitometric scanning of the radioautograms.

RESULTS AND DISCUSSION

Relative to normal rat hepatocytes, the levels of the ras gene transcripts were increased from 2 to around 25 times as well on tumor as in perineoplastic cells (fig. 1 and 2). In two cases, the levels were found even higher in perineoplastic cells (samples 4 and 6) than in tumor cells (samples 3 and 5) of the same rat. The same pattern was observed with either whole BRI

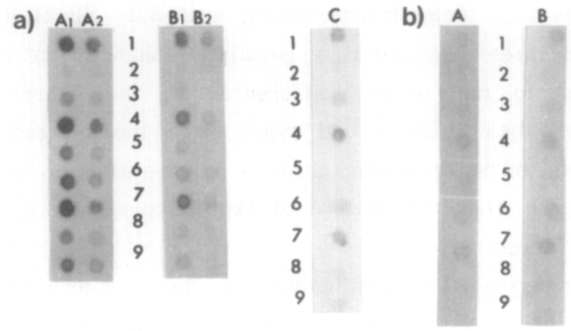

Figure 1 : Expression of the c-ras sequences in rat hepatocarcinomas.
Poly $(A)^+$ RNAs were isolated from the following sources : 1) hepatoma tissue cultured cells (HTC cells) ; 2) adult rat isolated hepatocytes ; 3) nodules from adult rat 1 treated with Diethylnitrosamine after partial hepatectomy and sacrified 70 weeks later ; 4) perinodular hepatocytes from the same adult rat 1; 5) nodules from adult rat 2 treated as rat 1 ; 6) perinodular hepatocytes from rat 2 ; 7) liver from rat 3 which contains microscopic collections of tumour foci ; 8) perinodular hepatocytes from rat 4 ; 9) nodules from adult rat 4 treated as rat 1.
a) Dot blot hybridization using A) v Ha-ras probe (clone BRI) ; B) v-Ki-ras probe (clone KBE-2) (14) ; and C) human N-ras probe (15). b) Dot blot hybridization using A) the Sac I-Pst I fragment of the v-Ha-ras clone BRI ; B) the Sac II-Hinc II fragment of the v-Ki-ras clone KBE-2.

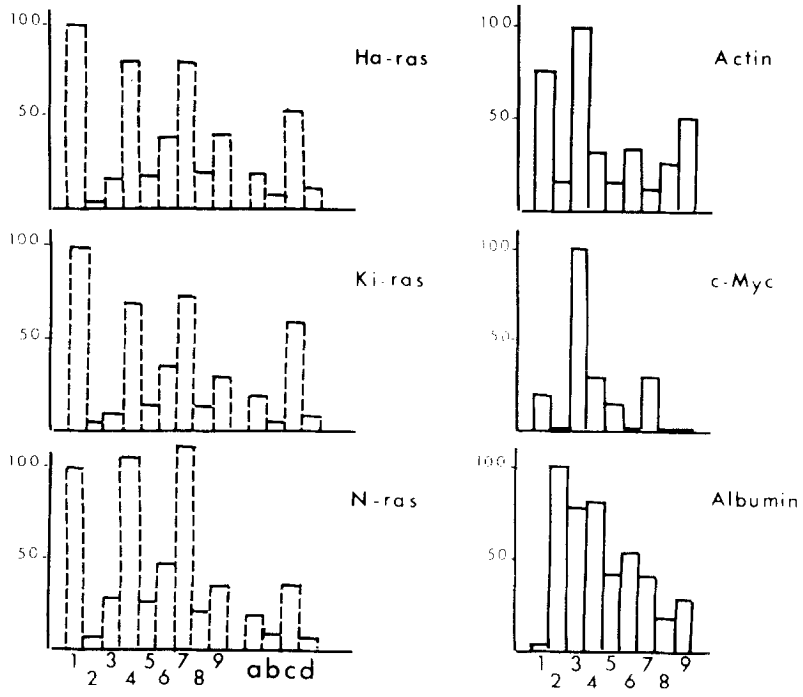

Figure 2 : Gene expression in hepatocarcinomas.
Samples 1 to 9 are described in figure 1 legend. a) Nodules from animals treated chronically by DENA, without partial hepatectomy ; b) perinodular hepatocytes from the same animal ; c) hepatocytes from regenerating liver 30h after partial hepatectomy ; d) hepatocytes from regenerating liver 7 days after partial hepatectomy.

and KBE-2 clones or their insert coding for p21 (fig.1). In addition, the expression of the three ras genes is parallely enhanced in each case. These parallel variations do not reflect differences in the general level of gene expression between the different cell types since the expression of c-myc, of serum albumin and of non-muscular actin is not parallel to the expression of the ras genes (fig.2). Thus the pattern of expression of the c-ras genes appears to be specific for these genes.

Before considering the significance of these observations, we have to discard the possibility that the parallel increase might be apparent and due to cross-hybridization between each of the ras transcript and the labelled probes. This possibility may be ruled out for two reasons : 1/ under our experimental conditions, there was very weak cross-hybridization between v-Ha-ras and v-Ki-ras specific inserts (data not shown) ; Northern blot hybridizations performed in the stringency conditions used for the dot blot showed different transcripts for the three ras genes (fig.3). One transcript of approximately 1.2 kb was detected with the v-Ha-ras probe, whereas the Ki-ras probe detected two major transcripts around 5.6 and 2.1 kb and the N-ras probe showed a transcript around 5kb. To our knowledge this is the first observation of a parallel expression of the 3 ras genes.

Goyette **et al** (25) observed an increase in the expression of c-Ha-ras gene during liver regeneration. We confirm here these data and show that a similar pattern of expression occurs for the two other ras genes.

While this manuscript was in preparation, Makino **et al** (26) have reported an overexpression of the c-myc in tumors but not in non-tumorous parts of the liver. We found an enhanced expression of the myc gene in one sample of perineoplastic hepatocytes but cannot rule out the possibility that microscopic

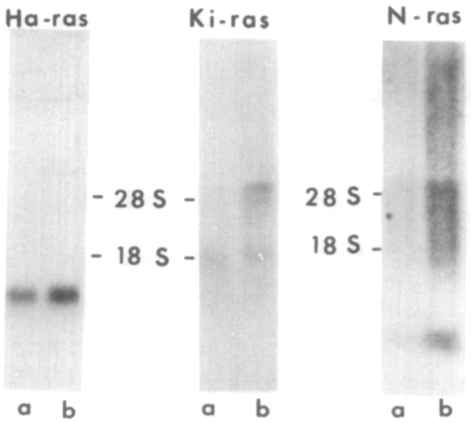

Figure 3 : Northern blot of the ras gene transcripts in rat hepatoma.
a) perinodular hepatocytes from adult rat 4 ; b) nodules from adult rat 4.

foci could be present. On the other hand, high expression of the ras genes in perinodular hepatocytes cannot be attributed to the presence of small populations of microscopic nodules since in two cases the ras expression was higher in the perinodular tissue than in the tumour itself. C-Ha-ras and c-myc gene expression have been shown to be increased during liver carcinogenesis induced in rat by a choline deficient diet containing 0.1% ethionine (27). Data from this model lend support to the view that oval cells, a new cell population, may constitute the cell group responsible for the metabolic changes including enzyme modifications as well as for the enhanced oncogene expression (27-28). However such an explanation cannot be applied to the DENA induced hepatocarcinomas studied here, since only foci of altered hepatocytes have been described with this regimen (10).

So we are left with two hypotheses : i-perinodular hepatocytes may correspond to the numerous focal areas of altered hepatocytes (hyperplastic nodules which have disappeared, undergoing remodelling and regression, whereas some of their oncodevelopmental markers (29,30) remain expressed. In this case the situation may be analogous to that of the chemically induced skin papilloma model. Papillomas, which are premalignant lesions, have an activated c-Ha-ras oncogene, which is overexpressed in these benign tumours (31). It will be of interest to know if one of the three ras genes, the expression of which is increased in perinodular hepatocytes, has a transforming capacity, as assayed on NIH/3T3 cells ; ii- alternatively, perinodular hepatocytes may exhibit changes in gene expression as a secondary effect of neoplasm on the surrounding tissular areas and on other host tissues (32).

In any case it is difficult to assume that the coordinated expression of these three genes could result from a DNA rearrangement like translocation or amplification, as shown with c-myc (5) and c-Ki-ras (6). It is more likely that it results from some epigenetic mechanism. An increase of the c-Ha-ras gene expression in tumors and non tumorous part of the liver or rats treated by 3' Me DAB has been reported (26). This enhanced expression has been attributed to a compensatory cell proliferation caused by the toxic effect of 3' Me DAB. This cannot be the case in our experiments since the increased expression was observed long after DENA treatment. It may be suggested that overexpression of the ras genes is necessary for cell proliferation but not sufficient and some other event has to be involved. Increase of c-myc expression could be one of these events.

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REFERENCES

1. Eva, A., Robbins, K.C., Andersen, P.R., Srinivasan, A., Tronick, S.R., Reddy, E.P., Ellmore, N.W., Galen, A.T., Lautenberger, J.A., Papas, T.S., Westin, E.H., Wong-Staal, F., Gallo, R.C. and Aaronson, S.A. (1982) Nature **265**, 116-119.
2. Westin, E.H., Wong-Staal, F., Gelmann, E.P., Dalla Favera, R., Papas, T.S., Lautenberger, J.A., Eva, A., Reddy, E.P., Tronick, S.R., Aaronson, S.A. and Gallo, R.C. (1982) Proc. Natl. Acad. Sci. USA **79**, 2490-2494.
3. Hayward, W.S., Neel, B.G. and Astrin, S.M. (1981) Nature **290**, 475-480.
4. Erikson, J., Ar-Rushdi, A., Drwinga, H.L., Nowell, P.C. and Crole, C..M. (1983) Proc. Natl. Acad. Sci. USA **80**, 820-824.
5. Collins, S. and Groudine, M. (1982) Nature **298**, 679-681.
6. Schwab, M., Alitalo, K., Varmus, H.E., Bishop, J.M. and George, D. (1983) Nature **303**, 497-501.
7. Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983) Cell **35**, 603-610.
8. Slamon, D.J., de Kernion, J.B., Verma, I.M. and Cline, M.J. (1984) Science **224**, 256-262.
9. Solt, D.B. and Farber, E. (1976) Nature **263**, 701-706.
10. Ogawa, K., Medline, A. and Farber, E. (1979) Lab. Invest. **41**, 22-34.
11. Farber, E., Cameron, R.G., Laisnes, R.A., Lin, J.C., Medline, A., Ogawa, R., Solt, D.B. (1979) In Carcinogens : identification and mechanisms of action (eds Clark, A.C., Shan, C.R.), New York Raven Press.
12. Scherer, E. and Emmelot, P. (1975) Europ. J. Cancer **11**, 145-154.
13. Thompson, E.B., Thomkins, G.M. and Curran, J.F. (1966) Proc. Natl. Acad. Sci. USA **56**, 296-302.
14. Seglen, P.O. (1973) Exp. Cell. Res. **76**, 25-30.
15. Beaumont, C., Lerumeur, E., Guillouzo, C., Latinier, M.F., Bourel, M. and Guillouzo, A. (1981) Biochem. Biophys. Res. Commun. 99, 879-885.
16. Raymondjean, M., Kneip, B. and Kruh, J. (1983) Biochim. Biophys. Acta **741**, 30-37.
17. Chirgwin, J., Przybyla, A., Mac Donald, R. and Rutter, W. (1980) Biochemistry **19**, 857-864.
18. Thomas, P. (1980) Proc. Natl. Acad. Sci. USA **77**, 5201-5205.
19. Denhardt, D.T. (1966) Biochem. Biophys. Res. Commun. **23**, 641-648.
20. Chang, E.H., Furth, M.E., Scolnick, E.M. and Lowy, D.R. (1982) Nature **297**, 479-484.
21. Taparowsky, E., Shimizu, K., Goldfarb, M. and Wigler, M. (1983) Cell **34**, 581-586.
22. Dalla Favera, R., Gelmann, E.P., Martinotti, S., Franchini, G., Papas, T.S., Gallo, R.C. and Wong-Staal, F. (1982) Proc. Natl. Acad. Sci. USA **79**, 6497-6501.
23. Sargent, T.D., Salat-Trepat, J.M., Bruce-Wallace, R., Reyes, A.A. and Bowner, J. (1979) Proc. Natl. Acad. Sci. USA **76**, 3256-3260.
24. Minty, A.J., Caravatti, M., Robert, B., Cohen, Daubas, P., Weydert, A. Gros, F. and Buckingham, M.E. (1981) J. Biol. Chem. **256**, 1008-1014.
25. Goyette, M., Petropoulos, C.J., Shank, P.B. and Fausto, N. (1983) Science **219**, 510-512.
26. Makino, R., Hayashi, K., Sato, S. and Sugimura, T. (1984) Biochem. Biophys. Res. Commun. **119**, 1096-1102.
27. Fausto, N. and Shank, P.R. (1983) Hepatology **3**, 1016-1023.
28. Schapira, F. (1981) in "Isozymes : current topics in biological and medical research" **5**, 27-75 (Alan R. Liss, Inc.).
29. Guguen-Guillouzo, C., Szajnert, M.F., Glaise, D., Gregori, C. and Schapira, F. (1981) In Vitro **17**, 369-377.
30. Rabes, N.M., Schulze, P. and Jantsch, B. (1972) Cancer Res. **32**, 2577-2586.
31. Balmain, A., Ramsden, M., Bowden, G.T. and Smith, J. (1984) Nature **307**, 658-660.
32. Koss, B. and Greengard, O. (1982) Cancer Res. **42**, 2146-2157.