Activation of Cellular Oncogenes by Chemical Carcinogens in Syrian Hamster Embryo Fibroblasts

by Rolf Ebert,* J. Carl Barrett,† Roger W. Wiseman,† Reinhard Pechan,‡ Eva Reiss,* Günther Röllich,* and Dietmar Schiffmann*

Carcinogen-induced point mutations resulting in activation of ras oncogenes have been demonstrated in various experimental systems such as skin carcinogenesis, mammary, and liver carcinogenesis. In many cases, the data support the conclusion that these point mutations are critical changes in the initiation of these tumors. The Syrian hamster embryo (SHE) cell transformation model system has been widely used to study the multistep process of chemically induced neoplastic transformation. Recent data suggest that activation of the Ha-ras gene via point mutation is one of the crucial events in the transformation of these cells. We have now cloned the c-Ha-ras proto-oncogene from SHE cDNA-libraries, and we have performed polymerase chain reaction and direct sequencing to analyze tumor cell lines induced by different chemical carcinogens for the presence of point mutations. No changes were detectable at codons 12, 13, 59, 61, and 117 or adjacent regions in tumor cell lines induced by diethylstilbestrol, asbestos, benzo(a)pyrene, trenbolone, or aflatoxin B1. Thus, it is not known whether point mutations in the Ha-ras proto-oncogene are essential for the acquisition of the neoplastic phenotype of SHE cells. Activation of other oncogenes or inactivation of tumor suppressor genes may be responsible for the neoplastic progression of these cells. However, in SHE cells neoplastically transformed by diethylstilbestrol or trenbolone, a significant elevation of the c-Ha-ras expression was observed. Enhanced expression of c-myc was detected in SHE cells transformed by benzo(a)pyrene or trenbolone.

Introduction

Mechanisms of carcinogen-induced activation of oncogenes are being widely studied, and the implications of the results are highly important for understanding the genotoxicity of chemicals. The importance of the results is illustrated by the fact that carcinogen-induced point mutations resulting in activation of ras oncogenes have been demonstrated in skin carcinogenesis (1), mammary (2), and liver carcinogenesis (3). In many cases, the data indicate that these point mutations are critical changes in the initiation of tumors. These findings provide strong experimental evidence for relating the most commonly studied end point with genotoxic activity of chemicals in carcinogenesis. Furthermore, the identification of specific base pair substitutions in oncogenes allows toxicologists to evaluate the relevance of specific carcinogen metabolites and carcinogen-DNA adducts.

The Syrian hamster embryo (SHE) cell transformation model system has been widely used to study the multistep process of chemically induced neoplastic transformation. DNAs from 5 of 12 carcinogen-induced SHE cell lines were shown to cause transformed foci in NIH 3T3 cells. The focus cells contained hamster repetitive sequences and novel Ha-ras restriction patterns (as revealed by Southern hybridization experiments). Electrophoresis of p21 ras proteins from transformed foci showed an altered protein with slower mobility, indicating a possible point mutation in codon 12 (4). These data suggest that activation of the Ha-ras gene via point mutation is one of the key events in the transformation of SHE cells. As we have cloned the Syrian hamster c-Ha-ras gene, we are now able to analyze SHE DNA directly for the presence of point mutations in this gene.

Changes in gene expression have been discussed as a further mode of activation of cellular oncogenes, e.g., for c-myc and c-Ha-ras. Gene rearrangements (5–7) and

*Institute of Toxicology, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany.
†Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.
‡Present address: Klimg Pharma GmbH, Klinische Forschung, D-8000 Munich, Federal Republic of Germany.
Address reprint requests to D. Schiffmann, Institute of Toxicology, University of Würzburg, D-8700 Würzburg, Federal Republic of Germany.
changes in DNA methylation (8) have been identified as possible reasons for these alterations. Therefore we have investigated the transcriptional frequency of c-myc and c-Ha-ras in various cell lines transformed by different chemical carcinogens.

Materials and Methods

Normal, nontransformed SHE fibroblasts were isolated from 12-day-old embryos as previously described (9). After treatment with chemicals, the cells were repeatedly subcultured before reaching confluence. In general, senescence occurred between passages 8 and 15. Immortalized cells escaping senescence were subcultured until passage 70 to 80 and subsequently injected into thymus aplastic mice, giving rise to fibrosarcomas at the injection site. The tumors developed within 4 to 6 weeks. Subsequently, different tumor cell lines were derived from the obtained tissue.  

To clone a Syrian hamster c-Ha-ras cDNA, two cDNA libraries were screened with a 460 bp fragment of v-Ha-ras (BS9) (10). Positive clones were sequenced using direct sequencing of double strand miniprep DNA (11,12).

c-Ha-ras sequences were amplified by polymerase chain reaction (PCR) as described (13). Briefly, 1 to 2 μg genomic DNA was subjected to 35 cycles of amplification using ampiTaq (Perkin Elmer Cetus). The appropriate sequencing primers were end-labeled with 32P-ATP using T4 polynucleotide kinase, annealed to PCR-generated DNA, and elongated with cloned T7 DNA polymerase and a ddNTP/dNTP mix. Samples were analyzed on 8% acrylamide/urea gels. Southern and Northern blotting and hybridization experiments were performed according to Maniatis et al. (14).

Results

After screening two Syrian hamster cDNA libraries, we isolated and sequenced 12 Ha-ras positive clones. A comparison to known c-Ha-ras coding sequences (15,16) revealed a homology of 95% to rat and 89% to human. The deduced amino acid sequence of the Syrian hamster p21 Ha-ras protein is identical to that of rat and human.  

Using the sequence data, we analyzed 14 tumor cell lines for activating point mutations in the c-Ha-ras gene by means of PCR and direct sequencing (Fig. 1). The cell lines investigated had been induced with benzo(a)pyrene (BaP), N-methylnitrosourea (NMU), diethylstilbestrol (DES), asbestos, or aflatoxin B1 (AFB1). No point mutations were detected in codons 12, 13, 59, 61, 117, or surrounding regions (Table 1). Northern analysis revealed enhanced expression of c-Ha-ras in cell lines transformed or immortalized by DES or trenbolone. Under the influence of DES, levels of c-Ha-ras mRNA were elevated only at a late stage, i.e., in tumor cells, whereas trenbolone caused an elevated transcription of this gene already in preneoplastic cells of early passage. In addition, transcriptional levels of c-myc were enhanced in cell lines induced by trenbolone and BaP. Again, trenbolone-treated cells already re-
revealed this elevation in the preneoplastic state (Table 2).

The degree of DNA methylation is inversely correlated with gene expression (17), and in vitro methylation of a number of genes causes inhibition of their expression after transfer into cells. It is known that Concanavalin A (ConA) is able to activate the de novo methylating activity of cytosine-5-methyltransferase without affecting the maintenance methylation activity of this enzyme (18). Moreover, ConA inhibits tumor cell growth in vivo and in vitro (19). ConA (1 μg/mL, nonagglutinating dose) induces growth inhibition after 5 days in a SHE cell line neoplastically transformed by DES (DES T2), while DNA methylation is increased simultaneously (Fig. 2). No change in growth and de novo methylation of normal SHE cells was found under the same conditions. Since it is known that expression of c-Ha-ras is influenced by its methylation status (8), we have investigated whether ConA-mediated de novo methylation reduces c-Ha-ras overexpression in a DES tumor cell line in which this gene showed a 6-fold overexpression compared to normal SHE cells. In fact, we found a reduction to 1- to 2-fold expression after 48 hr ConA treatment (Table 2).

**Table 1. Results of direct sequencing of DNA from SHE cells and tumor cell lines generated by various carcinogens.**

| Cell line          | Codon | 12 | 13 | 59 | 61 | 117 |
|--------------------|-------|----|----|----|----|-----|
| SHE cells (wt)     | GGA   | GGC | GGA | AAA | AAG |
| DES T1             | wt*   | wt | wt | wt | wt | NDa |
| DES T2             | wt | wt | wt | wt | wt | wt |
| DES T3             | wt | wt | wt | wt | wt | wt |
| DES T4             | wt | wt | wt | wt | wt | wt |
| DES T5             | wt | wt | wt | wt | wt | wt |
| DES T6             | wt | wt | wt | wt | wt | wt |
| BaP T1             | wt | wt | wt | wt | wt | wt |
| BaP T2             | wt | wt | wt | wt | wt | NDa |
| BaP TPA T          | wt | wt | wt | wt | wt | wt |
| Trenbolon T        | wt | wt | wt | wt | wt | wt |
| AFB1, T            | wt | wt | wt | wt | wt | wt |
| NMU T              | wt | wt | wt | wt | wt | wt |
| IQ T               | wt | wt | wt | wt | wt | wt |
| Asbestos T         | wt | wt | wt | wt | wt | wt |

*a* wt, Wild type.

*a b* ND, not determined.

**Table 2. mRNA levels of c-Ha-ras and c-myc in chemically transformed SHE cell lines.**

| Cell line          | Passage number | 10/25 | 60 | Tumor |
|--------------------|----------------|-------|----|-------|
| Levels of c-Ha-ras expression | 6 | NDa | 6 |
| Trenbolon         | 1 | 1-2 | 6.5 |
| Diethylstilbestrol treated with ConA | — | — | 1-2 |
| Levels of c-myc expression | 6 | NDa | 6 |
| Trenbolon         | 1 | 1 | 1 |
| Diethylstilbestrol | 1 | 1 |

*a* ND, not determined.

**Figure 2.** Growth inhibition and simultaneous DNA methylation increase of DES-transformed SHE cells 5 days after treatment with Concanavalin A. Concentrations of Concanavalin A used were 0.1 μg/mL (A), 0.5 μg/mL (B), 1.0 μg/mL (C). Co indicates control.

**Discussion**

The absence of point mutations in all chemically transformed Syrian hamster cell lines examined may indicate that c-Ha-ras activation via point mutation is not involved in the transformation of these cells, although this might have been expected from the kind of primary DNA interaction of some of the chemicals used (e.g., NMU, BaP, AFB1). At the present, we cannot exclude the involvement of other ras genes (Ki-ras, N-ras), which might also be a target for these chemicals. The possible existence of small subpopulations carrying c-Ha-ras mutations also cannot be ruled out. Therefore, we are currently screening morphologically transformed foci using PCR and direct sequencing to have access to the very early stage of chemical carcinogenesis after 10 to 12 cell divisions. In DES- and trenbolone-transformed tumor cell lines, enhanced expression of the c-Ha-ras gene may contribute to their malignant state. In case of DES-induced cells, a correlation of c-Ha-ras transcription with their proliferation rate was detected. It is known that enhanced c-Ha-ras expression of the normal or the mutated gene is sufficient to induce cell transformation (20).

Furthermore, our results suggest that stimulation of de novo DNA methylation reduces oncogene expression and most likely, as a consequence, tumor cell growth. Thus our findings provide a possible new approach to influence the malignant phenotype of transformed cells.
This work was supported by the Deutsche Forschungsgemeinschaft, SFB 172, the Stifterverband für die Deutsche Wissenschaft, and the National Cancer Institute.

REFERENCES

1. Quintanilla, M., Brown, K., Ramsden, M., and Caputo, A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. Nature 322: 78–80 (1986).
2. Zarbl, H., Sukumar, S., Arthur, A. V., Martin-Zanca, D., and Barbacid, M. Direct mutagenesis of Ha-ras-1 oncogenes by N-nitroso-N-methyl-urea during initiation of mammary carcinogenesis in rats. Nature 315: 382–385 (1985).
3. Wiseman, R. W., Stowers, S. J., Miller, E. C., Anderson, M. W., and Miller, J. A. Activating mutations of the C-Ha-ras protooncogene in chemically induced hepatomas of the male B6C3F1 mouse. Proc. Natl. Acad. Sci. USA 83: 5825–5829 (1986).
4. Gilmer, T. M., Annab, L. A., and Barrett, J. C. Characterization of activated protooncogenes in chemically transformed Syrian hamster embryo cells. Mol. Carcinog. 1: 189–188 (1988).
5. Battey, J., Moulding, C., Taub, R., Murphy, W., Stewart, T., Potter, H., Lenoir, G., and Leder, P. The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell 34: 779–787 (1983).
6. Hayward, W. S., Neel, B. G., and Astrin, S. M. Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukemia. Nature 296: 475–480 (1981).
7. Dalla-Favera, R., Wong-Staal, F., and Gallo, R. C. Onc gene amplification in promyelocytic leukemia cell line HL-60 and primary leukemia cells of the same patient. Nature 299: 61–63 (1982).
8. Feinberg, A. P., and Vogelstein, B. Hypomethylation of ras oncogenes in primary human cancers. Biochem. Biophys. Res. Commun. 111: 47–54 (1983).
9. Schiffmann, D., Reichert, D., and Henschler, D. Induction of morphological transformation and unscheduled DNA synthesis in Syrian hamster embryo fibroblasts by hexachlorobutadiene and its putative metabolite pentachlorobutenoic acid. Cancer Lett. 23: 297–305 (1984).
10. Ellis, R. W., DeFeo, D., Maryak, J. M., Young, H. A., Shih, T. Y., Chang, E. H., Lowy, D. R., and Scolnick, E. M. Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. J. Virol. 36 (2): 406–420 (1980).
11. Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463–5467 (1977).
12. Toneguzzo, F., Glynn, S., Levi, E., Mjosness, S., and Hayday, A. Use of a chemically modified T7 DNA polymerase for manual and automated sequencing of supercoiled DNA. Biotechniques 6(5): 460–469 (1988).
13. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491 (1988).
14. Maniatis, T., Fritsch, E. F., and Sambrook, J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
15. Ruta, M., Wolford, R., Dhar, R., Defeo-Jones, D., Ellis, R. W., and Scolnick, E. M. Nucleotide sequence of the two rat cellular ras genes. Mol. Cell. Biol. 6(6): 1706–1710 (1986).
16. Capon, J. D., Chen, E. Y., Levinson, A. D., Seeburg, P. H., and Goeddel, D. V. Complete nucleotide sequences of the T24 human bladder carcinoma oncogene and its normal homologue. Nature 302: 33–37 (1983).
17. Doerfler, W. DNA methylation—a regulatory signal in eukaryotic gene expression. J. Gen. Virol. 57: 1–20 (1981).
18. Szhydlo, M., Kaplan, F., Mann, V., Giloh, H., Kedar, E., and Razin, A. Cell cycle-dependent regulation of eukaryotic DNA methylase level. J. Biol. Chem. 260: 8653–8656 (1985).
19. Shoham, J., Inbar, M., and Sachs, L. Differential toxicity of normal and transformed cells in vitro and inhibition of tumor development in vivo by Concanavalin A. Nature 227: 1244–1246 (1970).
20. Lacal, J. C., Srivastava, S. K., Anderson, P. S., and Aaronson, S. A. Ras p21 proteins with high or low GTPase activity can efficiently transform NIH/3T3 cells. Cell 44: 609–617 (1986).