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

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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 B_1. 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

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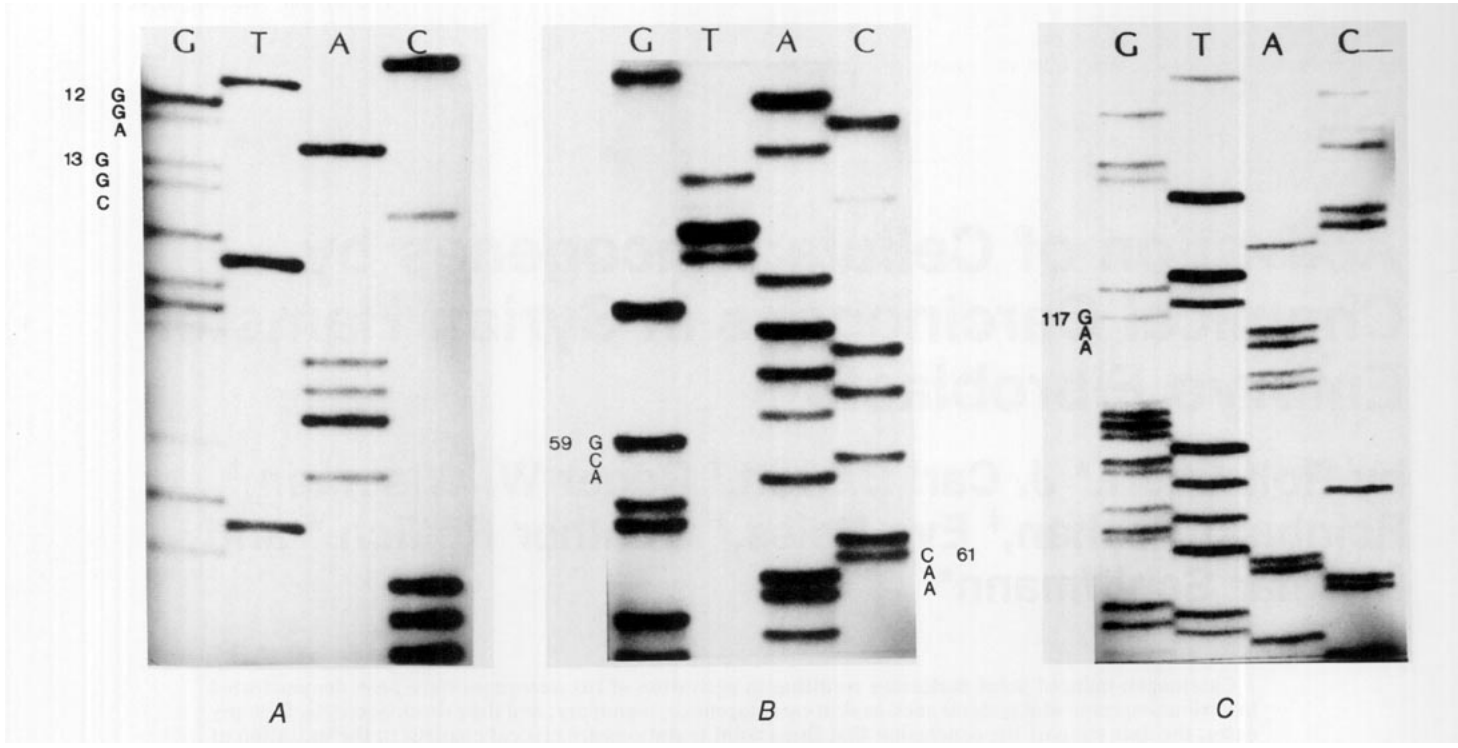

FIGURE 1. SHE c-Ha-*ras* wild-type sequences around codons 12/13 (*A*), 59/61 (*B*), and 116/117 (*C*).

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 ampliTaq (Perkin Elmer Cetus). The appropriate sequencing primers were end-labeled with ^{32}P-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 B_1(AFB_1). 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-

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

	Codon				
Cell line	12	13	59	61	117
SHE cells (wt)	GGA	GGC	GCA	CAA	AAG
DES T1	wt[a]	wt	wt	wt	ND[b]
DES T2	wt	wt	wt	wt	wt
DES T3	wt	wt	wt	wt	wt
DES T4	wt	wt	wt	wt	wt
DES T5	wt	wt	wt	wt	wt
DES T6	wt	wt	wt	wt	wt
BaP T1	wt	wt	wt	wt	wt
BaP T2	wt	wt	wt	wt	ND[b]
BaqP TPA T	wt	wt	wt	wt	wt
Trenbolon T	wt	wt	wt	wt	wt
AFB_1T	wt	wt	wt	wt	wt
NMU T	wt	wt	wt	wt	wt
IQ T	wt	wt	wt	wt	wt
Asbestos T	wt	wt	wt	wt	wt

[a] wt, Wild type.
[b] ND, not determined.

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

	Passage number		
Cell line	10/25	60	Tumor
Levels of c-Ha-*ras* expression			
Trenbolon	6	ND[a]	6
Diethylstilbestrol	1	1–2	6.5
Diethylstilbestrol treated with ConA	—	—	1–2
Levels of c-*myc* expression			
Trenbolon	6	ND[a]	6
Diethylstilbestrol	1	1	1

[a] ND, not determined.

vealed 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, non-agglutinating 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).

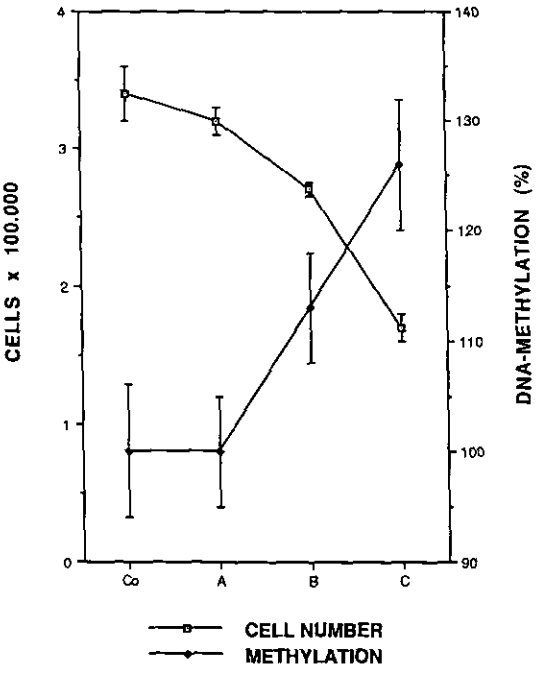

FIGURE 2. Growth inhibition and simultaneous DNA methylation increase of DES-transformed SHE cells 5 days after treatment with Concanavalin A. Concentrations of Concanavalin A used were 0.1 μg/mL (*A*), 0.5 μg/mL (*B*), 1.0 μg/mL (*C*). Co indicates control.

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, AFB_1). 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.

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