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Oncogene activation: c-*raf*-1 gene mutations in experimental and naturally occurring tumors

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SUMMARY

We demonstrate here consistent point mutations of the c-*raf*-1 proto-oncogene, within a small region of the kinase domain, in a mouse model for chemical tumor induction. This is the first demonstration of point mutated *raf* genes in vivo, and the first isolation of activating in vivo point mutations in the kinase domain of a proto-oncogene. The specific region where these mutations are clustered also has biological significance. This is precisely the region where 5/5 independently generated monoclonal antibodies raised against *Raf*-1 map to [29], and predictions based upon the crystal structure of A kinase identify this as the substrate pocket. The tumors examined show a selective specificity for *Raf*-1 mutations in that another family of genes, the *ras* proto-oncogenes which are frequently activated by point mutation in both animal and human tumors [15–21,26], is not involved. Our consistent finding of *Raf*-1 mutations in a mouse tumor model also has consequences for further evaluation of the role of *Raf*-1 in human tumor development, as it emphasizes the need to examine c-*raf*-1 at the sequence level. In fact preliminary screening of human lung tumors indicates point mutations at amino acid 533 (John Lyons, personal communication). Finally, the cumulative data on the critical role of *Raf*-1 in signal transduction and the occurrence of oncogenic *Raf*-1 in tumors [32–41] highlight this enzyme as an attractive target for development of novel anticancer regimens.

INTRODUCTION

A critical set of genes that become altered in the course of carcinogenesis specify components of homeostatic growth control, and this is the arena in which cancer genes function. Homeostatic growth control is achieved through positive and negative signalling pathways that impinge on the cell cycle (Fig. 1). Elements in negative

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pathways are also known as tumor suppressors (Fig. 2A), whereas positive pathways link oncogenes in signalling cascades (Fig. 2B). The current dilemma in molecular oncology stems from the finding of a very large number of positive and negative growth regulating genes, which may be activated or inactivated by a multiplicity of genetic changes resulting in tumorigenesis [1,2]. Moreover, a tumor cell, once emerged, appears to display Protean qualities in its ability to utilize newly acquired genetic alterations for growth promotion. In order to reduce interference from the multiplicity of growth-stimulating gene products, mapping of mitogenic signal transduction pathways is now of particular importance because it holds the promise of identifying critical gatekeeper positions through which peripheral signals are channeled to central targets. *Raf* family oncogenes (the properties of which are summarized in Table I) have been identified as holding such a position. We have, therefore, undertaken a search for oncogenic alterations in the *Raf* genes. Based on findings with other growth control genes a variety of potential genetic lesions had to be considered including deletion, truncation, amplification, translocation, and point mutation. As the history of isolation of the original *Raf* oncogene-carrying virus (3611 MSV [3,4]) pointed to a connection with lung adenocarcinoma, and the human *Raf*-1 gene mapped to a position on the short arm of chromosome 3 (3p25 [5]) which is frequently altered in lung carcinoma, we have developed a mouse model for studying the potential role of *Raf* in lung cancer.

This system involves mating NFS female mice with AKR males and giving pregnant females a transplacental injection of 1-ethyl-1-nitrosourea (ENU) on day 16 of gestation [6]. ENU was chosen for tumor induction since it is a very potent direct-acting carcinogen capable of modifying any base in vivo [7]. ENU alkylates all tissues with roughly the same efficiency and has a very short half-life in vivo [8,9] allowing specific mutagenesis of tissues which are mitotically active at a particular time. NFS and AKR were chosen as parental strains based on earlier studies which showed them to be particularly susceptible to lung tumors following ENU exposure [10,11]. With this procedure, nearly 100% of the offspring develop lung adenocarcinomas and approx. 70% develop, in addition, T-cell lymphomas with a mean latency of approx. 20 weeks. In order to achieve more rapid tumor development we treated weanling mice with weekly injections of a tumor promoter, the antioxidant butylated hydroxytoluene or BHT. BHT was used as it has been demonstrated to cause lung lesions and hyperplasia when injected into mice [12–14]. In our system it nearly doubles the rate at which tumors develop. Comparison of tumor-induced mortality with age of animals for those receiving ENU alone, and those receiving ENU and promoted with BHT demonstrates that the mean age of tumor-induced mortality decreases from approx. 20 weeks to around 12, and that initial latency decreases. The differences between these groups are significant with a confidence limit greater than 99.99% using a 2-tailed Cox test. In addition, BHT promotion, while increasing the rate at which tumors develop, does not affect the tumor spectrum.

First clues as to potential oncogene involvement in these tumors came from mRNA expression. Northern blot analysis revealed elevated levels of c-*raf*-1, as compared to

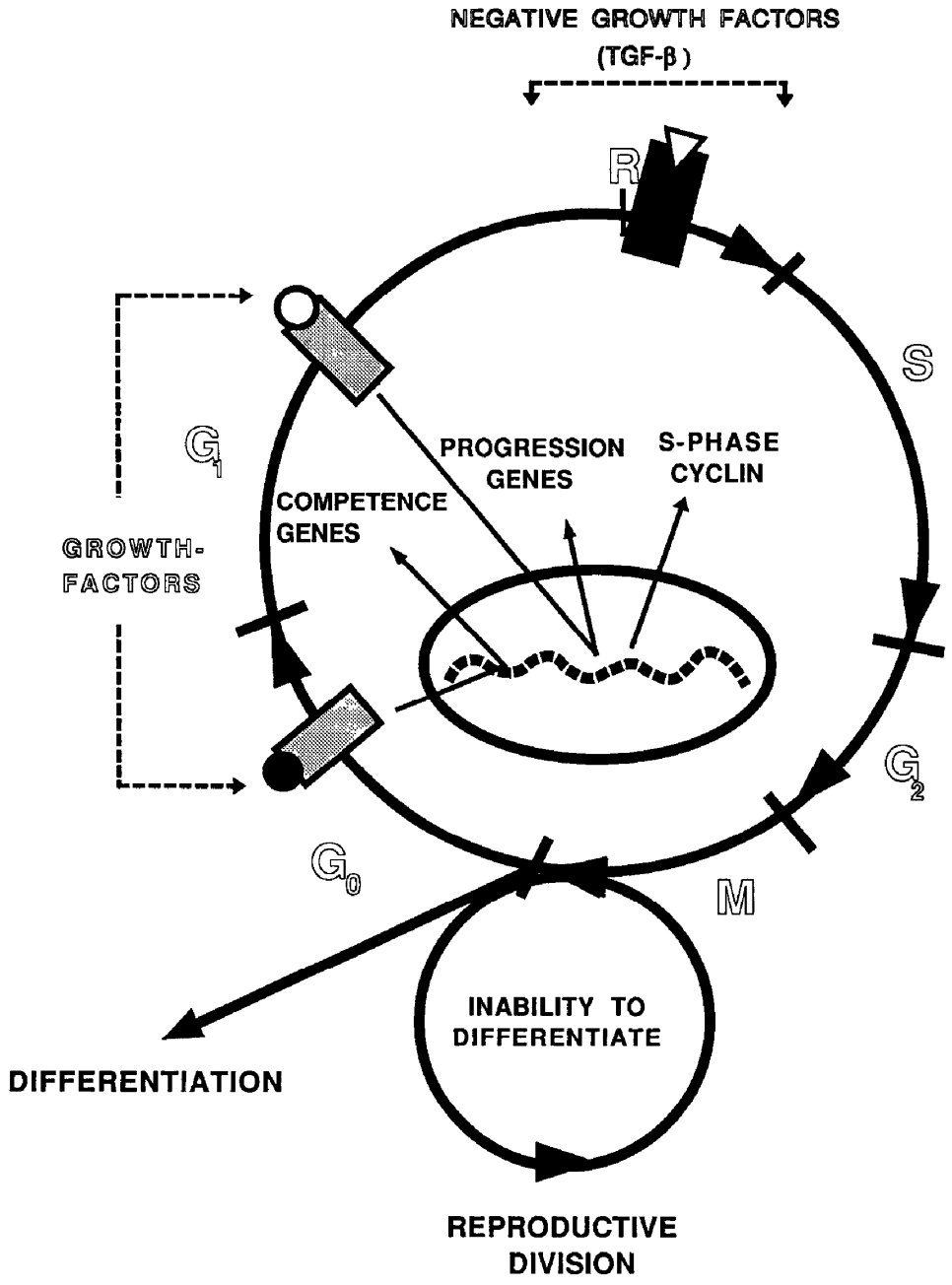


Fig. 1. Schematic of classes of positive and negative growth regulatory genes responsible for homeostatic growth control and their position in the cell cycle.

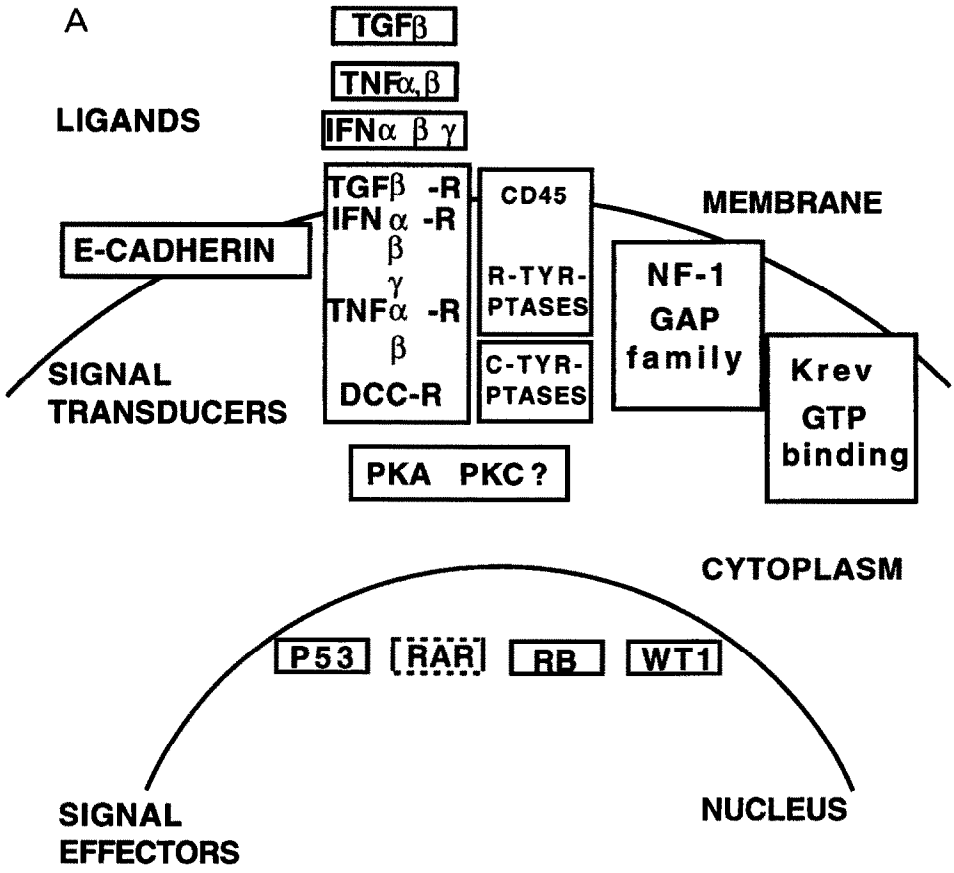
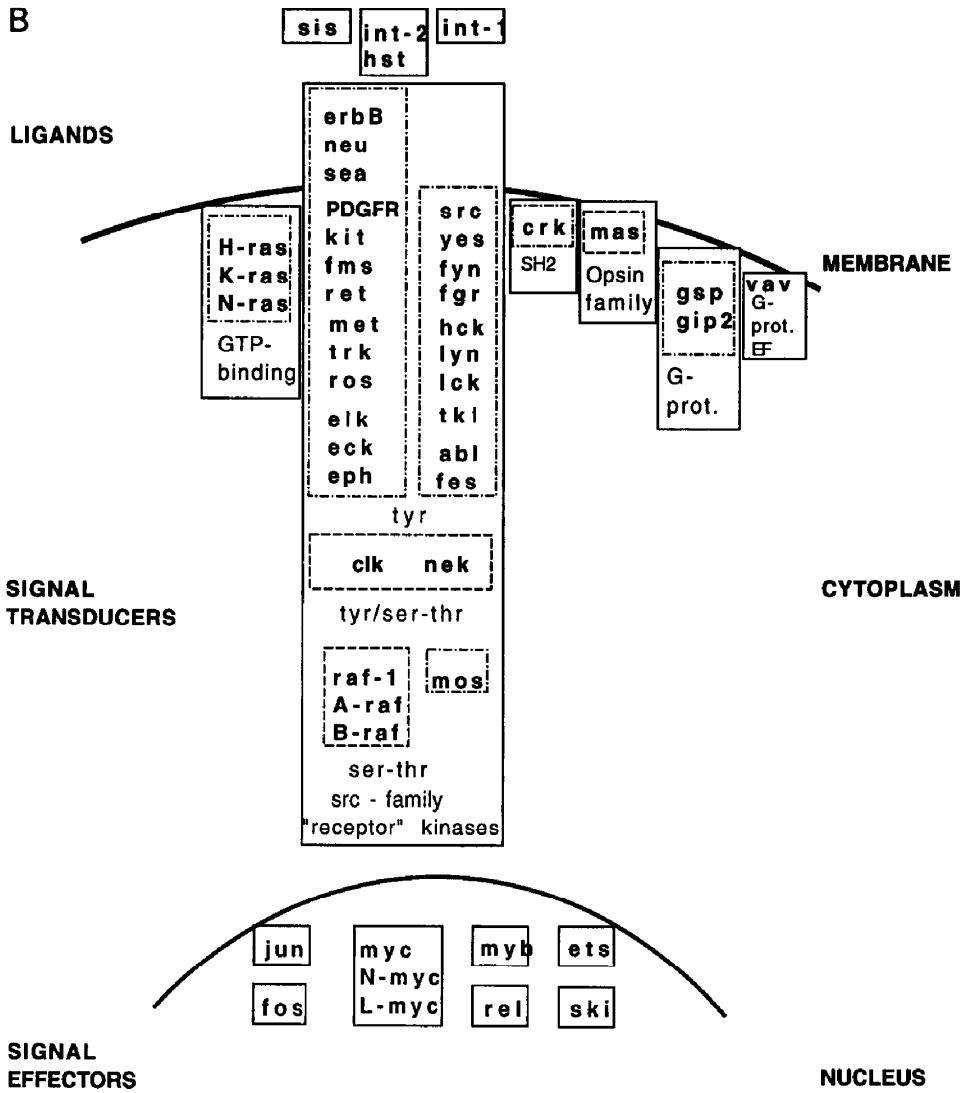


Fig. 2. (A) Growth suppressor (negative regulatory) genes grouped according to subcellular or extracellular location, sequence relatedness, function, and position in signal transduction pathways. (B) Oncogenes (positive regulators) grouped according to subcellular or extracellular location, sequence relatedness, function, and position in signal transduction pathways.

normal tissue, in every tumor examined, and Western blot analysis showed that protein levels correlated with message levels [6]. In addition, in cell lines derived from primary tumors, *Raf*-1 protein kinase activity was shown by immune-complex kinase assays to be constitutive. Further analysis of a wide range of other oncogenes revealed no consistent pattern of expression except for *ras* and *myc* family genes. In the case of the *myc* family, one member (either c-, N-, or L-*myc*), and never more than one, was overexpressed. For the *ras* genes, at least one member (Ki-, Ha-, or N-*ras*), and often more than one, was expressed at high levels when compared with the normal tissue. In addition, all oncogenes examined via Northern analysis exhibited full-length, normal-sized transcripts. Having eliminated gross genetic alterations in the genes exam-



ined (due to the presence of only normal-sized transcripts) we turned to more detailed analysis with methods that allow detection of changes at the single base level.

ras genes were considered likely candidates for mutational activation since oncogenic forms of *ras* have previously been observed in lung tumors [15–17] and lymphomas [18–21], and ENU is a point mutagen [7]. We, therefore, performed a systematic analysis of various *ras* codons known to be involved in oncogenic activation. We examined Ha-, Ki-, and N-*ras* at codons 12, 13, and 61 for potential mutations via RNAse protection assays [22,23], PCR amplification followed by subsequent sequencing [24], and PCR amplification followed by diagnostic restriction digests [25].

TABLE I

SUMMARY OF *RAF* FAMILY ONCOGENE PROPERTIES

Three active genes in man, c-*raf* - 1, B-*raf* and A-*raf* - 1

Distantly related to protein kinase C

c-*raf* -1 on chromosome 3p25, site altered in many epithelial neoplasias, marker for von Hippel-Lindau disease, loss of c-*raf* -1 heterozygosity in small cell lung tumor and renal cancer, A-*raf* -1 on Xp11.2-11.4, B-*raf* on 7q32
c-*raf* -1 and A-*raf* -1 have pseudogenes, c-*raf* -2 and A-*raf* -2, c-*raf* -2 marks Huntington's chorea

D-*raf* -1 on X chromosome and D-*raf* -2 on chromosome 2 in *Drosophila melanogaster*. *D-raf* -1 essential for normal cell proliferation and several developmental pathways

Expression: c-*raf* -1 ubiquitous, A-*raf*-1 urogenital tissues, B-*raf* cerebrum and testes

c-*raf* -1, A-*raf* -1 and B-*raf* encode 74, 68 and 72 kD proteins respectively, predominantly found in the cytoplasm, with associated Ser/Thr kinase activity

Raf kinases synergize in transformation and GF abrogation with *myc*

Raf-1 is coupled to many GF receptors including those for PDGF, EGF, Insulin, FGF and IL-2, IL-3, GMCSF, EPO, NGF as well as the TCR and CD4

Raf-1 functions downstream of Ras in signal transduction

A fraction of GF activated Raf-1 enters the nucleus

Raf-1 is involved in the transcriptional control of *fos*, *jun*, EGR-1, TGFα, etc. Raf response elements include AP1/PEA3 and NFκB motifs

Oncogenic activation can be achieved by truncation, N-terminal extension or site specific mutation

ENU induced murine lung tumors and lymphomas contain consistent c-*raf* -1 point mutations, and vaccination with purified Raf doubles tumor latency.

Of all the tumors (twenty) and cell lines (five) examined by these methods only two *ras* mutations were detected. Both of these mutations were in Ki-*ras* codon 12. One was identified in a lung tumor-derived cell line and the other was shown to be a consequence of transplantation. The most notable point from these data is the conspicuous lack of *ras* mutations. In fact, the number of *ras* mutations is much lower than would

be expected for a sampling of spontaneous tumors [15–17,26]. Having eliminated *ras* genes as playing a primary role in the genesis of these ENU-induced tumors we investigated c-*raf*-1 for possible small or point mutations.

Since no point mutations had been described for *raf* genes in vivo, as had been for the *ras* genes [15–21,27], we screened for point mutations using RNAse protection assays [22,23]. In the case of RNA isolated from normal lung tissue, only one, fully protected, band is detected, while in the case of the tumors two major bands are seen after digestion. Twenty out of 20 tumors analyzed in this fashion showed this extra band, and the same was true for both lung tumors (16/16) and T-cell lymphomas (4/4). These data demonstrate the following major points: (i) There is a tumor-specific alteration in c-*raf*-1 that results in a region of non-homology recognizable by either RNAase A or T1. (ii) The alterations are confined to the same region of one allele as the novel bands are of equal size in the tumor lanes. (iii) Both alleles were expressed

TABLE II

FREQUENCY, SPECTRUM AND LOCATION OF *RAF*-1 MUTATIONS

Mutation	Primary tumors[a]		Transplanted tumors					
	L	T	P1		P2/P3		P10	
			L	T	L	T	L	T
Ser $\xrightarrow[533]{(CG\rightarrow TC)}$ Phe	7/8	9/9	0/5	3/3	3/5	N.T.	1/1	N.T.
Ala $\xrightarrow[543]{(G\rightarrow A)}$ Thr	7/8	9/9	1/5	0/3	0/5	N.T.	0/1	N.T.
Trp $\xrightarrow[511]{(G\rightarrow C)}$ Ser	0/8	0/9	0/5	0/3	1/5	N.T.	0/1	N.T.
Ile $\xrightarrow[517]{(A\rightarrow G)}$ Val	0/8	0/9	1/5	0/3	0/5	N.T.	0/1	N.T.
Phe $\xrightarrow[522]{(T\rightarrow C)}$ Ser	0/8	0/9	0/5	0/3	1/5	N.T.	0/1	N.T.
Asp $\xrightarrow[527]{(G\rightarrow A)}$ Asn	0/8	0/9	2/5	0/3	0/5	N.T.	0/1	N.T.
DVYS $\xrightarrow[530-533]{(-G,\ +C,\ CG\rightarrow TC)}$ TCTF	0/8	0/9	1/5	0/3	0/5	N.T.	0/1	N.T.
Wild-type[b]	1/5	0/6	0/5	0/3	0/5	N.T.	0/1	N.T.
Total mutations	16/17				14/14			
Double mutations	16/17				3/14			

[a] One of the primary T-cell lymphomas was tested as a cell line.

[b] The wild-type sequence may be a result of excess normal tissue in the sample.

Mutations are given in the left-hand column with the amino acid changes and positions indicated. The nucleotide changes are given in parentheses. L and T refer to lung tumors and T-cell lymphomas, respectively, and P1, P2/P3, and P10 refer to passage numbers.

at comparable levels as both wild-type and tumor-specific bands are of equivalent intensity. By running these assays with various markers we were able to estimate the approximate site of the alteration(s) to be in the vincinity of the exon 14/exon 15 junction. In order to define the precise genetic alteration or alterations, we designed PCR primers which would generate a 600-bp fragment encompassing this region. cDNAs from tumor-derived RNA were then amplified and cloned for sequencing (five tumors) or sequenced directly following asymmetric amplification (26 tumors). Sequencing through the kinase domain revealed a variety of mutations clustered around the APE site (a conserved sequence located in subdomain VIII of protein kinases [28]). Repeating the cDNA synthesis, PCR amplification, cloning and sequencing gave the same results, as did sequencing from the opposite strand, and adjacent unaffected tissues showed no mutations demonstrating that these alterations are not artefactual. The mutational analysis is summarized in Table II. The most frequently occurring mutation results in a serine to phenylalanine exchange at amino acid 533. The spectrum of mutations observed in the transplanted tumors was wider than that seen in primary tumors. This may be because the animals from which the tumors were obtained came from different experiments and the precise time of carcinogen administration may have been slightly different. However, in both transplanted and primary tumors the alteration at amino acid 533 is the most prevalent mutation. It is interesting that all of these changes occur within a very small region of the raf protein. In fact, the region where these mutations occur overlaps an epitope shared by monoclonal antibodies generated against *raf* [29], and computer modeling of the protein based on the coordinates of cAMP-dependent kinase predicts these mutations to lie within the substrate pocket and alter its structure. This indicates a biologically important region for the molecule and indeed the first three of these mutations tested in NIH3T3 cell assays, after cloning into either a retroviral expression vector (El-neo [30]) or under the control of the normal *Raf*-1 promoter, were found to be weakly transforming. The transformation efficiency was comparable to EC2, a previously characterized mutation of human c-*raf*-1 cDNA [30,31] and ~ 10-fold lower than the v-*raf* oncogene. The transformation efficiency of these mutations correlated well with their transcriptional transactivation activities, which were shown to be equivalent to that of EC2. The weak transforming activity of these mutations in NIH3T3 cells may reflect a need for cooperating oncogenes (i.e., *ras* or *myc*), or alternatively be a consequence of target cell (i.e., lung or T-cell) specificity.

The discovery of consistent point mutations in Raf-1, which most frequently involves an alteration of amino acid 533, has led us to look for similar mutations in human tumors. Initial screening of human lung tumors indicates the occurrence of mutations in the same location (John Lyons, personal communication). The frequency with which these mutations occur and the tumor types in which they are found are currently being evaluated. Our findings that *Raf* belongs to the group of oncogenes which are frequently activated by site-specific point mutations should add to the arsenal of cancer gene probes that the clinician has at his disposal in searching for carcinogenic lesions.

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