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Concomitant K- and N-*ras* Gene Point Mutations in Clonal Murine Lymphoma

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We have surveyed a panel of induced murine lymphomas for c-*ras* gene mutations. The K-*ras* gene seems to be preferentially activated in our system, and there are at least two examples of concomitant K- and N-*ras* gene mutations in the same tumor. This indicates that in some cases additional *ras* mutations may contribute to tumorigenesis and is evidence for a role of *ras* activation in tumor progression.

Transforming counterparts of all three members (H-, N-, and K-*ras*) of the c-*ras* gene family have been identified in a significant proportion of spontaneous and induced tumors. Activation of the c-*ras* oncogenes in vivo has been attributed to single point mutations localized to codon 12, codon 61, or, less frequently, codon 13 (2, 5, 12, 15). Additionally, codon 117 mutations in the H-*ras* gene of rodent liver tumors have been reported (15). However, whether a mutated *ras* gene alone is sufficient to transform cells in vivo is unclear.

We have been using a murine model system of thymic lymphoma induction to study the contribution of c-*ras* gene point mutations to tumorigenesis. In a previous report we described the activation of N- and K-*ras* genes in mice obtained from genetic crosses (8). It was necessary to study a large series of induced tumors from inbred strains of mice to investigate the existence of any correlations between the mutagenic agent, target tissue, genetic background, and *ras* gene activation.

In this report we describe the results of a survey of 92 induced thymic lymphomas in terms of the distribution of activated *ras* genes, the spectrum of point mutations detected in these genes, and the allelic composition of the primary tumors with respect to normal and mutated *ras* alleles.

Thymic lymphomas were induced in RF/J mice by fractionated doses of either whole-body gamma irradiation or intraperitoneal injection of the chemical carcinogen *n*-nitrosomethylurea (NMU), and in 129/J mice by NMU injection. The 129/J strain is apparently resistant to radiation-induced development of thymic lymphomas (an observation previously reported [13]), as no tumors were obtained from 55 irradiated 129/J mice. The T-cell origin of all tumors was verified by monitoring for expression of Thy-1 and TL (6), which are markers for T cells and thymocytes, respectively, as well as by Southern blot analysis of T-cell receptor gene rearrangement (L. E. Diamond, S. R. Sloan, A. Pellicer, and A. C. Hayday, Immunogenetics, in press). Establishment of the T-cell origin of the tumors allowed determination of tumor clonality as judged by the T-cell receptor gene rearrangement patterns of individual tumors. The above analyses indicated that between 90 and 98% of the cells comprising each tumor were of T-cell origin and that all tumors (with possibly one exception) represented monoclonal expansion of a single cell.

Transforming activity of thymic lymphoma DNA is predominantly due to the c-*ras* genes. Activation of c-*ras* genes in primary tumor DNAs was detected by using a combination of approaches, including the NIH 3T3 focus-forming assay, oligonucleotide mismatch hybridization to sequences corresponding to codons 12 and 61 of the N- and K-*ras* genes, and gene cloning and sequencing. Analysis of 55 NMU-induced and 37 radiation-induced tumors revealed the presence of transforming N- or K-*ras* genes in approximately 40% (37 of 92) of the tumors, with no significant variation between tumors obtained from different mouse strains (data not shown). NMU tumor induction appears to result in a higher frequency (51%) of *ras* oncogene activation than does induction by gamma rays (24%). Of the 37 tumors, 31 (23 NMU induced; 8 gamma ray-induced) carried an active K-*ras* gene while 6 tumors (5 NMU induced; 1 gamma ray induced) were positive for the transforming N-*ras* gene. It is noteworthy that H-*ras* involvement was never detected in over 90 tumors. This has been the transforming *ras* gene most consistently implicated in other animal model systems of carcinogenesis which involve tumors of epithelial origin (1, 4, 18), suggesting a tissue specificity for susceptibility of a particular *ras* gene to mutagenesis. It has in fact been established that the H-*ras* gene is expressed at relatively higher levels in murine epithelial tissues and that K- and N-*ras* gene expression are more prevalent in tissue of hematopoietic origin (11).

When the induced tumors carrying transforming *ras* genes were compared, the frequency of K-*ras* gene activation by either inducing agent was similar (23 of 28 NMU-induced tumors and 8 of 9 radiation-induced tumors were positive for a K-*ras* gene mutation). N-*ras* gene involvement occurred at an overall lower frequency, with only 1 of 9 radiation-induced tumors resulting in N-*ras* gene activation and 5 of 28 NMU-induced tumors carrying transforming N-*ras* genes. While the sample number for gamma-ray induction is somewhat lower than that obtained with NMU, it appears that between the K- and N-*ras* genes no striking target gene specificity can be strictly correlated to these two inducing agents in this system.

Six of the tumors which were positive in the 3T3 focus-forming assay did not appear to carry activated forms of any of the three *ras* genes. These tumors were able to reproducibly transform 3T3 cells, the DNA of which gave rise to secondary and tertiary transformants, and were positive for

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TABLE 1. *ras* gene point mutations

Gene and codon	Point mutation	Frequency[a] in tumors induced with:	
		NMU	Gamma rays
K-*ras*, codon 12	GGT → GAT Gly Asp	22	7
N-*ras*			
Codon 12	GGT → GAT Gly Asp	1[b]	1
Codon 13	GGT → GAT Gly Asp	1	
Codon 61	CAA → AAA Gln Lys	2	
	CAA → CTA Gln Leu	1[b]	

[a] *n* for K-*ras*: NMU, 23 tumors; gamma rays, 8 tumors. *n* for N-*ras*: NMU, 5 tumors; gamma rays, 1 tumor.

[b] Tumors in which a K-*ras* point mutation was subsequently detected.

the cotransfected sequences routinely included in each transfection. The 3T3 transformant DNAs were screened by Southern analysis with oncogenes known to be active in the focus-forming assay (*fos*, *raf*, *sis*, and *neu*) as well as with additional oncogene probes (*myc*, *myb*, *erbB*, *fgr*). No additional restriction fragments or gene amplification corresponding to any of these oncogenes were detected (data not shown), indicating that copies of these genes were not segregating with the transformed phenotype.

Identification of *ras* gene base mutations. *ras* gene point mutations were identified first in the 3T3 transformant DNAs and then in the primary tumor DNA from which each had been derived (Table 1). The brain DNA of each animal served as a control to verify the somatic nature of all mutations detected. By oligonucleotide mismatch hybridization, it was found that 29 of 31 tumors carrying transforming K-*ras* genes were positive for a GGT (glycine)-to-GAT (aspartate) transition in the K-*ras* codon 12 (Table 1). Twenty-two of these 29 tumors were induced by NMU, and 7 of the 29 were derived from gamma irradiation. Thus, two completely diverse mutagenic agents result in identical activating mutations in the K-*ras* gene, suggesting a biological selection for this particular mutation during tumor development. These findings may be comparable to those of other animal model systems of carcinogenesis, in which identical *ras* mutations are consistently detected (14, 19).

In contrast to the homogeneous nature of the K-*ras* gene base substitutions detected, four different N-*ras* mutations were identified among only six tumors (Table 1). Two NMU-induced tumors were positive for a codon 61 CAA (glutamine)-to-AAA (lysine) substitution, and one NMU-induced tumor contained a codon 13 GGT-to-GAT transition. Analysis of the gamma irradiation-induced tumor (tumor no. 4) which transferred active N-*ras* sequences in the focus-forming assay revealed that the two independent 3T3 transformants obtained (Fig. 1c) were positive for a GGT-to-GAT mutation in the 12th codon (Fig. 1a, lanes B and C). The mutation could not, however, be detected in the original tumor DNA (Fig. 1a, lane D). The identification of the same base substitution in independent 3T3 transformants makes it highly unlikely that the mutations occurred as an in vitro artifact. To address the possibility that the mutation existed in a small subpopulation of cells, under the level of detection of this assay, the sensitivity of the mismatch hybridization assay was determined. In our hands a minimum of 10% of the cell population bearing a mutation can be detected (unpublished results). This suggests that less than 10% of the cells in tumor 4 carry the N-*ras* gene mutation which was transferred (at a low efficiency of 2 foci per 150 μg of DNA) in the 3T3 focus-forming assay. As was the case with all tumors, tumor 4 was clonal, as determined by T-cell receptor gene rearrangement analysis (Fig. 1d), and greater than 90% of the cells comprising the tumor were of T-cell origin (data not shown). Another NMU-induced tumor (tumor 34) and its corresponding 3T3 transformant were also positive for the GGT-to-GAT transition in N-*ras* codon 12 (Fig. 1a, lanes F and G). The final N-*ras* mutation was localized to codon 61 as a CAA (glutamine)-to-CTA (leucine)

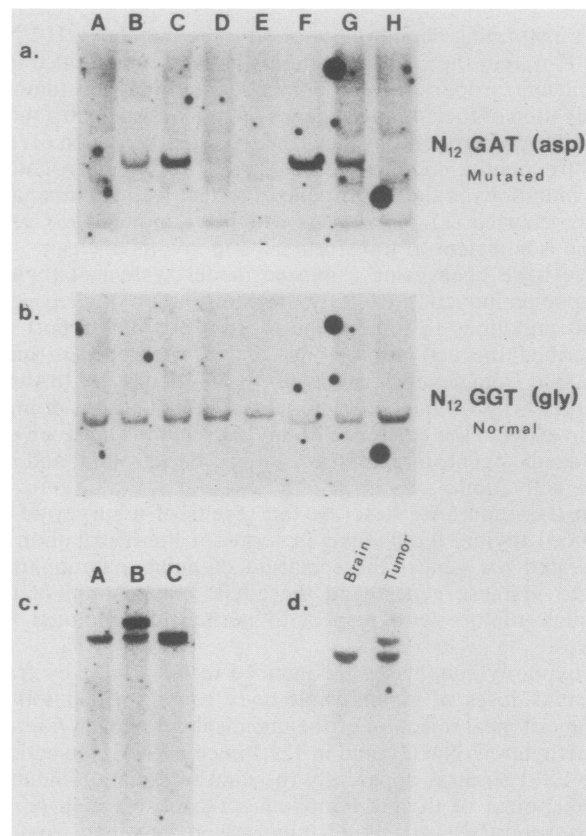

FIG. 1. Detection of N-*ras* codon 12 point mutation. (a and b) DNAs from 3T3 cells (lanes A), two independent 3T3 transformants (lanes B and C), the gamma ray-induced tumor (tumor 4) from which they were derived (lane D), the brain of that animal (lane E), a 3T3 transformant derived from an NMU-induced tumor (tumor 34) (lanes F and G, respectively), and the brain of that animal (lane H) were digested with *Pvu*II and hybridized with (a) the synthetic 19-mer (5'-TTGGAGCAGATGGTGTTGG-3'), specifying a GGT-to-GAT mutation in codon 12 of N-*ras*, or with (b) the corresponding wild-type sequence (5'-TTGGAGCAGGTGGTGTTGG-3'), as described by Kidd et al. (10). (c) 3T3 transformants obtained from tumor 4 are independent as judged by Southern analysis (17) of rearranged N-*ras* sequences. DNAs from 3T3 cells (lane A) and two 3T3 transformants (lanes B and C) were digested with *Eco*RI and hybridized to the murine N-*ras* first exon. (d) Clonality of tumor 4 as judged by T-cell receptor gene rearrangement analysis. DNAs from the brain and tumor were digested with *Pvu*II and hybridized with plasmid PHDS11, a $beta_2$ constant-region cDNA probe which recognizes a 6.1-kilobase germ line restriction fragment (16).

substitution. The DNA of this NMU-induced tumor (tumor 6) gave rise to four foci. However, the codon 61 mutation was detectable in only two of the four 3T3 transformants, and the tumor DNA appeared to be negative for this mutation. Again, this suggests a small population of cells in tumor 6 which carry the mutation.

Concomitant *ras* gene point mutations in two tumors. In contrast to the high proportion of tumors containing identical K-*ras* mutations, transforming N-*ras* genes were detected infrequently and the mutations identified were relatively heterogeneous. Of the six tumors which gave rise to 3T3 transformants by transferring active N-*ras* genes, two (tumors 4 and 6) appeared to have a very low percentage of cells carrying the mutated N-*ras* allele. In particular, only two of the four transformants derived from tumor 6 were positive for the same codon 61 base substitution (Fig. 2b). The possibility existed that the remaining two 3T3 transformants were spontaneous events, or that, as had been reported recently (7, 9a), an additional mutated *ras* gene existed in a single tumor. Therefore, the six tumors which were positive for transforming N-*ras* genes were screened for the more prevalent K-*ras* codon 12 GGT-to-GAT mutation by using oligonucleotide mismatch hybridization.

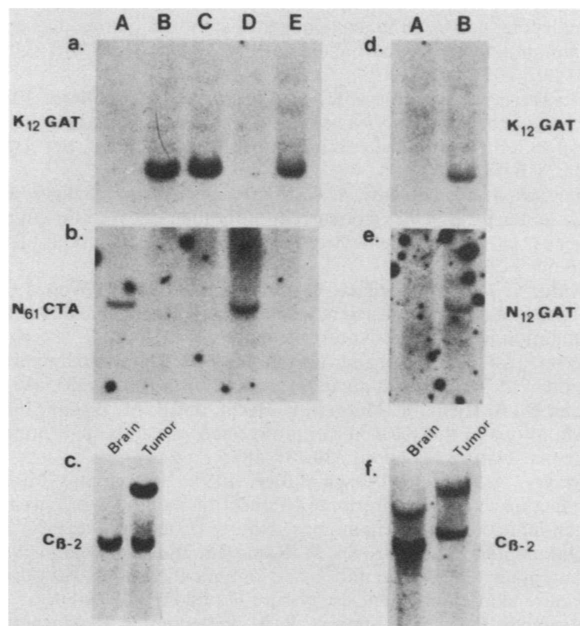

FIG. 2. Concomitant K- and N-*ras* gene point mutations in two clonal tumors. (a) DNAs from four independent 3T3 transformants derived from tumor 6 (lanes A through D) and DNA from tumor 6 itself (lane E) were digested with *Hin*dIII and *Pst*I and hybridized to the oligonucleotide (5'-TTGGAGCT<u>GAT</u>GGCGTAGG-3') specifying a GGT-to-GAT mutation in K-*ras* codon 12. (b) The gel was rehybridized to the oligonucleotide (5'-CAGCTGGA<u>CTA</u>GAGGAGTA-3') specifying a CAA-to-CTA mutation in N-*ras* codon 61. (c) Southern analysis of T-cell receptor gene rearrangement of tumor 6. Brain and tumor DNAs were digested with *Pvu*II and hybridized to the beta$_2$ constant-region probe as described in the legend to Fig. 1. (d) DNAs from the brain (lane A) and tumor 34 (lane B) were digested with *Hin*dIII and *Pst*I and hybridized to the K-*ras* codon 12 GGT-to-GAT oligonucleotide. (e) The gel was rehybridized to an oligonucleotide (5'-TTGGAGCA<u>GAT</u>GGTGTTGG-3') specifying an N-*ras* codon 12 GGT-to-GAT mutation. (f) T-cell receptor gene rearrangement analysis of tumor 34. Tumor and brain DNAs were digested with *Hpa*I, which restricts a 6.1- and 11.6-kilobase germ line fragment, and hybridized with the beta$_2$ constant-region probe.

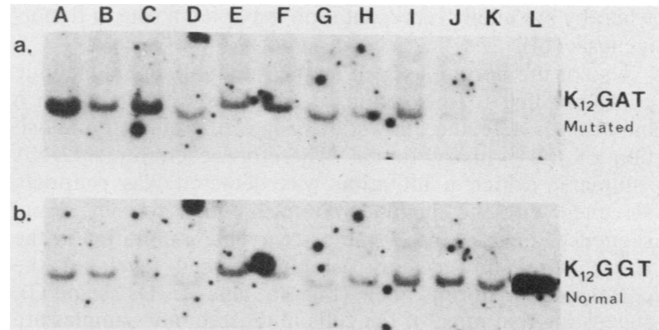

FIG. 3. Loss of the normal K-*ras* allele. DNA from a K-*ras* 3T3 transformant (lanes A), DNA from tumors from which K-*ras* 3T3 transformants had been derived (lanes B through K), and 10 ng of a clone containing the normal K-*ras* exon 1 (which represents approximately 14 times the gene copy number present in genomic DNA) (lane L) were analyzed for the (a) mutated and (b) normal K-*ras* codon 12 sequences.

The results showed that tumor 6 contained the K-*ras* codon 12 mutation, as did the remaining two 3T3 transformants (Fig. 2a). The clonality of tumor 6 was verified by analysis of T-cell receptor beta$_2$ gene rearrangement (Fig. 2c). The germ line beta$_2$ restriction fragment observed in the tumor DNA represents one unrearranged allele (as opposed to contamination of the tumor sample with normal surrounding tissue), since analysis of beta$_1$ gene rearrangement revealed complete deletion of both alleles (data not shown). Tumor 34, which was positive for an N-*ras* codon 12 mutation, also contained a mutation in codon 12 of the K-*ras* gene (Fig. 2d). Rehybridization of the gel with the N-*ras* codon 12 mutated oligomer demonstrated that the tumor DNA harbored two *ras* gene mutations (Fig. 2e), and the clonality of tumor 34 was confirmed by T-cell receptor gene rearrangement analysis (Fig. 2f). Both beta$_1$ alleles (corresponding to the higher-molecular-weight germ line *Hpa*I fragment) were completely deleted in tumor 34 (Fig. 2f and data not shown), and the two *Hpa*I restriction fragments seen in the tumor DNA correspond to two rearranged beta$_2$ alleles. Although unlikely, the presence of two independent clones in tumor 34, each with both beta$_1$ and one beta$_2$ allele deleted and the second beta$_2$ allele rearranged, cannot be formally excluded. The remaining tumors which contained transforming N-*ras* genes were negative for the K-*ras* codon 12 GGT-to-GAT mutation. This does not, of course, rule out the possibility that alternative *ras* gene substitutions may exist in these tumors.

Thus we have evidence in at least two cases for two individual transforming *ras* gene point mutations occurring in the same primary tumor, providing support for the notion that at least one of these base mutations does not represent an initial event.

Loss of the normal allele in primary tumor DNA. Previous results in our laboratory showed complete loss of the normal N-*ras* allele in one thymic lymphoma which carried an N-*ras* codon 61 mutation (9). Complete or partial loss of the normal allele in primary tumors containing a mutated *neu* (3) or H-*ras* (14) gene has also been reported. One interpretation of these findings is that the relative representation of normal and mutated alleles in a cell plays a role in cell transformation; i.e., both alleles of a gene must be mutated, or the normal allele must be eliminated, to observe a transformed phenotype. The observed amplification of a mutated *ras* allele in tumor cells suggests an additional mechanism

whereby *ras* allelic representation may play a role in tumorigenesis (14).

Use of the normal as well as the mutated oligonucleotide corresponding to the codon of each *ras* gene in which a mutation is detected allows analysis of the allelic composition of the primary tumor. Therefore each tumor DNA sample in which a mutation was detected was routinely screened with the oligomer complementary to the normal sequence. Four of the 29 tumors carrying a mutation in the K-*ras* gene showed considerably reduced levels of the corresponding normal allele (Fig. 3b, lanes C, D, F, and G), suggesting that most of the cells in these tumor samples are homozygous or hemizygous for the mutated allele.

An additional point (Fig. 3a) is that in some instances (for example, tumors J and K) only a small proportion of the cells comprising the tumor appear to bear the GAT mutation.

The four tumors which showed loss of the normal K-*ras* allele represent approximately 11% of the total number of tumors (37) in which *ras* gene activation was detected in this study. The two tumors carrying double mutations account for another 5% of the tumor samples. Based upon the data presented, it is possible to speculate that loss of the normal allele or mutation of a second *ras* gene may represent second steps which contribute to tumor development in vivo.

In comparing induced murine lymphomas from RF/J and 129/J strains, the genetic background does not seem to play a role in *ras* gene activation (although it does apparently contribute to susceptibility to tumor induction). Among the tumors which do carry transforming *ras* genes, both inducing agents (gamma rays and NMU) result predominantly in activation of the K-*ras* gene, suggesting that this may reflect a biological selection for tumor development. The results presented here do not preclude the possibility, however, that K-*ras* activation may represent an initiating event in the development of some thymic lymphomas. The inference that N-*ras* gene activation may be a secondary event that is correlated with tumor progression in this system would be consistent with the following experimental observations: (i) the polymorphic mutation pattern of N-*ras* gene activation, (ii) the fact that the majority of cells of at least two different clonal tumors did not contain the activating N-*ras* mutation detectable by the 3T3 focus-forming assay, and (iii) the identification in some tumors of N-*ras* activation occurring concomitantly with the more prevalent K-*ras* mutation.

Our observations underscore the significance of secondary events in addition to single *ras* gene point mutations in tumor DNA. The fact that clonality of the tumors can be verified in this system supports the rationale to search for additional genetic events in tumors in which *ras* mutations have been identified. These results also indicate that alternative model systems can supply quite different information about the role of *ras* activation in the tumorigenic process and that the complex picture presented here could be instrumental in interpreting human oncogenesis.

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