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Mechanism of activation of an N-*ras* oncogene of SW-1271 human lung carcinoma cells

(DNA transfection/molecular cloning/point mutation)

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ABSTRACT **An N-*ras*-related transforming gene was detected in the human lung carcinoma cell line SW-1271 and molecularly cloned. The lesion responsible for its acquisition of transforming activity was localized to a single nucleotide transition from A to G in codon 61 of the predicted protein. This lesion in the second exon results in the substitution of arginine for glutamine at this position. These findings, together with previous studies, indicate that the activation of *ras* oncogenes in human tumors is most commonly due to point mutations at one of two major "hot spots" in the *ras* coding sequence.**

Human cellular transforming genes have been detected by DNA-mediated gene transfer techniques by using NIH/3T3 cells (1–10), a continuous murine cell line that is contact-inhibited and highly susceptible to DNA transfection (11). Most transforming genes so far detected are related to a small family of retroviral *onc* genes, designated *ras*. The human cellular *ras* family consists of three proto-oncogenes, c-Harvey(H)-*ras*, c-Kirsten(K)-*ras*, and N-*ras*, which are closely related at nucleotide sequence and protein coding levels (12, 13). The viral oncogene, v-H-*ras*, of Harvey murine sarcoma virus (MSV) derives from a *ras* proto-oncogene, transduced from the rat (14, 15). The K-MSV *onc* gene, v-K-*ras*, originated from a different rat *ras* proto-oncogene (15). No viral counterpart has as yet been found for N-*ras*.

Human c-H-*ras* oncogenes have been detected and molecularly cloned from the T24/EJ bladder carcinoma- and Hs242 lung carcinoma-derived cell lines (16–21). Tumors as diverse as carcinomas, sarcomas, and hematopoietic malignancies have been found to contain oncogenes homologous to c-K-*ras* (8, 10, 18). N-*ras* oncogenes have so far been detected in human neuroblastoma (9, 13) and fibrosarcoma cell lines (22) as well as in a variety of hematopoietic tumors (10, 23). We have previously cloned the N-*ras* proto-oncogene from a human DNA library by hybridization using v-K-*ras* as a probe (10). Using N-*ras* specific probes, we have detected an N-*ras*-related oncogene in a human lung carcinoma cell line, SW-1271. We report here the cloning and identification of the lesion responsible for the activation of SW-1271 oncogene.

MATERIALS AND METHODS

Cells and Transfection Assays. The SW-1271 cell line was initiated as an explant of a lung carcinoma of a 69-year-old Caucasian male. The cells were obtained from J. Fogh (Sloan Kettering Institute, New York). DNA transfection assays using NIH/3T3 cells were performed by the calcium phosphate precipitation method (24, 25), as described previously (6).

DNA Blotting Analysis. Twenty micrograms of high molecular weight DNA was digested with appropriate restriction endonucleases under the conditions suggested by the manufacturer. Digested DNA was electrophoresed in horizontal agarose (1%, wt/vol) gels and blotted to nitrocellulose as described by Southern (26). Filters were hybridized in 50% formamide/0.75 M sodium chloride/0.075 M sodium citrate at 42°C for 24 hr. Human N-*ras* clones were isolated from a human DNA library (10). From this phage DNA, plasmid subclones lacking human repetitive sequences were prepared and used as probes.

Molecular Cloning. The λgt*WES*·λB strain of λ phage was propagated in *Escherichia coli* strain LE 392 (27). Phage DNA and cellular DNA were digested with *Eco*RI and purified independently by preparative sucrose gradient centrifugation. Purified phage arms and cellular DNA fragments were ligated at 1:1 molar ratio by T4 DNA ligase and packaged *in vitro* into phage particles. Positive plaques were identified by *in situ* hybridization of phage plaques (27).

Electron Microscopy. Equal amounts of DNAs were mixed, denatured with 30 mM NaOH, neutralized with 0.1 M Tris·HCl (pH 8.0), and allowed to hybridize in the presence of 50% formamide at 25°C for 4–6 hr. Samples were spread onto a distilled water hypophase and prepared for electron microscopy according to the method of Davis *et al.* (28). Uranyl acetate-stained grids were rotary shadowed with platinum/palladium and examined in an electron microscope.

DNA Sequence Analysis. Nucleotide sequencing was performed by the procedure of Maxam and Gilbert (29). DNA fragments were obtained by using various restriction endonucleases and were labeled either at their 5′ ends by using [γ-^{32}P]ATP and polynucleotide kinase or at their 3′ ends by using cordycepin 5′-[α-^{32}P]triphosphate and terminal deoxynucleotidyl transferase. End-labeled DNA fragments were digested with appropriate restriction endonucleases, isolated by polyacrylamide gel electrophoresis, and used for sequence analysis.

RESULTS

Identification of the SW-1271 Oncogene. When high molecular weight DNA of SW-1271 cells was subjected to transfection analysis using NIH/3T3 cells, we observed a low level of focus formation with independently prepared DNA preparations (Table 1). The specific activity of the transforming DNA was increased 2-fold by a second cycle of transfection using high molecular weight DNA of individual first-cycle

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Abbreviations: H, Harvey; K, Kirsten; MSV, murine sarcoma virus; kbp, kilobase pair(s); bp, base pair(s).

Table 1. Transforming activity of DNA isolated from SW-1271 cells and their NIH/3T3 transfectants

SW-1271 donor DNA	Transfection efficiency (no. of foci/no. of recipient cultures)		
	Primary	Second cycle	Third cycle
Preparation 1	3/8	8/8	8/8
Preparation 2	10/8	14/8	12/8

Two independently prepared DNAs from SW-1271 cells as well as representative first- and second-cycle NIH/3T3 transfectants were used to transfect NIH/3T3 cells. NIH/3T3 cells, plated 24 hr earlier at 1.3×10^5 cells per 10-cm Petri dish, were transfected with 30 μg of high molecular weight DNA. Cells were maintained in culture with twice-weekly changes of Dulbecco's modified Eagle's medium supplemented with 5% calf serum. Foci of transformed cells were scored at 14–21 days. DNA of normal human fibroblasts was not detectably positive in the assay.

NIH/3T3 transformants. Transforming activity also survived a third cycle of transfection (Table 1).

To confirm that the foci observed were induced by human DNA, the DNAs of individual transfectants were subjected to Southern blotting analysis using a probe specific for human *Alu*-family repetitive sequences (30). Each first-cycle transfectant tested demonstrated numerous bands containing human repetitive sequences. Even third-cycle transfectants digested with *Eco*RI invariably retained more than one *Alu*-family-related band (data not shown), implying that human sequences were linked to the transforming sequence.

In view of evidence relating transforming genes of a number of human tumors to *ras* oncogenes (8–10, 13, 18–23), we analyzed SW-1271 transfectant DNAs for sequences homologous to these transforming genes. Neither K-*ras* nor H-*ras* probes detected any bands other than their endogenous mouse related bands (data not shown). However, when *Eco*RI-digested SW-1271 transfectant DNAs were analyzed using N-*ras* specific probes (10), each exhibited additional hybridizing bands not observed in mouse DNA (Fig. 1). The unique sequence probes [*Pvu* II, 900 base pairs (bp), probe A; *Xba* I, 800 bp, probe B] were specific for the N-*ras* 9-kilobase-pair (kbp) and 7-kbp *Eco*RI fragments, respectively (Fig. 1 *A* and *B*, lanes 1). Probe A detected a 7.5-kbp mouse cell specific band (Fig. 1*A*, lane 3). An additional DNA fragment ranging from 7 to 11 kbp was detected in all second- and third-cycle transfectants tested with probe A (Fig. 1*A*, lanes 4 and 5). Probe B detected only a single extra band, ranging from 6 to 13 kbp, in all transfectants (Fig. 1*B*). These

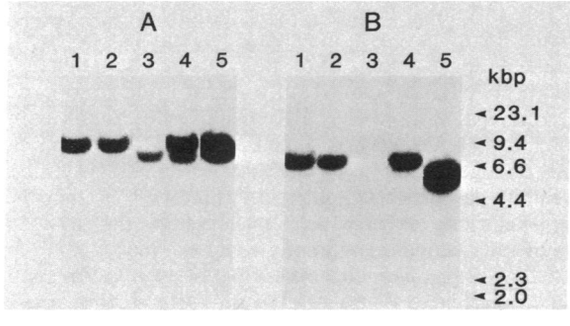

FIG. 1. Detection and identification of the SW-1271 transforming gene. Twenty micrograms of high molecular weight DNA was digested with *Eco*RI, electrophoresed in horizontal agarose (1%, wt/vol) gels, and blotted to nitrocellulose as described (26). Filters were hybridized for 24 hr using N-*ras* specific probes, *Pvu* II fragment (*A*) and *Xba* I fragment (*B*) as shown in Fig. 4. DNAs were obtained from human placenta (lane 1), SW-1271 cells (lane 2), NIH/3T3 cells (lane 3), and two representative transfectants (lanes 4 and 5). Coelectrophoresed DNA fragments of *Hin*dIII-digested λc1857 DNA served as standards (labeled in kbp).

results demonstrated that a human gene related to the N-*ras* proto-oncogene cosegregated with the observed transforming activity and that two *Eco*RI fragments corresponding to the 9-kbp and 7-kbp *Eco*RI fragments of N-*ras* were present in all transfectants. Since SW-1271 DNA itself showed the same sized *Eco*RI fragments as N-*ras* with both probes A and B (Fig. 1 *A* and *B*, lanes 2), it seemed likely that the altered sizes of these fragments in the transfectants resulted from DNA rearrangement during or subsequent to transfection.

Molecular Cloning of the SW-1271 Oncogene. To characterize the N-*ras*-related oncogene associated with SW-1271 cells, we cloned this sequence from a third-cycle NIH/3T3 transfectant, designated 62-2A-1-6. *Eco*RI-digested DNA of this transfectant showed 7.2-kbp and 6-kbp fragments with probes A and B, respectively (Fig. 1 *A* and *B*, lanes 5). 62-2A-1-6 DNA was digested completely with *Eco*RI, and the fragments were separated by sucrose density gradient centrifugation. Fractions that hybridized with probe A or B were ligated separately to phage λgt*WES*·λB purified arms, packaged *in vitro*, and amplified by infecting *E. coli* strain LE 392. From 400,000 plaques screened, 10 and 7 positive clones were obtained by using probes A and B, respectively.

Following plaque purification, the cloned DNAs were digested with *Eco*RI and then subjected to agarose gel electrophoresis and Southern blotting analysis using probes A and B. Clones containing N-*ras*-related *Eco*RI inserts of either 7.2 or 6 kbp were obtained. Clones designated λSW9-7 and λSW9-33 specifically hybridized with probe A, and clones λSW7-11 and λSW7-20 hybridized only with probe B. N-*ras* specific *Eco*RI fragments were subcloned in the plasmid pAT153 (31) for further analysis. These subclones were designated pSW9-7, pSW9-33, pSW7-11, and pSW7-20.

Biologic Activity of the Cloned SW-1271 Gene. The SW-1271 oncogene was cloned as two independent *Eco*RI fragments, neither of which possessed transforming activity in transfection assays (data not shown). To assess the biologic activity of the gene, 7.2-kbp and 6-kbp *Eco*RI fragments purified from clones λSW9-33 and λSW7-11, respectively, were ligated and the resulting concatamers were tested for their ability to transform NIH/3T3 cells (Fig. 2). The reconstituted SW-1271 gene induced morphological transformation of the cells, but with an efficiency reduced to about 1/4 to 1/10 of that of related oncogenes such as T24 and Hs242 (6, 16, 17, 21). This may be explained by the fact that only one combination of the two molecules would be expected to give rise to transforming activity (25% of the possible constructs), assuming ligation was 100% efficient.

To determine the region(s) of the SW-1271 oncogene required for transforming activity, we ligated the λSW9-33 *Eco*RI fragment to the 7-kbp *Eco*RI fragment of N-*ras* and also ligated the 9-kbp *Eco*RI fragment of N-*ras* to the 6-kbp fragment of λSW7-11. The transforming activity of these molecules was compared with that of homologous con-

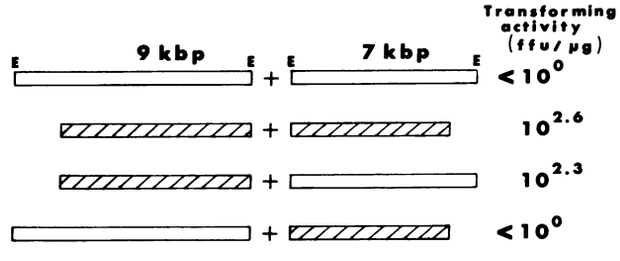

FIG. 2. Transforming activities of ligated N-*ras* fragments. Two purified *Eco*RI fragments from the normal N-*ras* gene (▭) and/or SW-1271 oncogene (▨) were ligated and assayed for transforming activity in the NIH/3T3 transfection assay. ffu, Focus-forming units.

structs. As shown in Fig. 2, the recombinant containing the SW-1271 7.2-kbp and N-*ras* 7-kbp *Eco*RI fragments was active, whereas neither the reciprocal recombinant nor N-*ras* itself showed detectable transforming activity (Fig. 2). Thus, the site of malignant activation of the SW-1271 oncogene could be localized to a region corresponding to the 5′ half of the gene.

Physical Characterization of the Cloned SW-1271 Oncogene. The cloned SW-1271 fragments were compared with the normal N-*ras* gene by heteroduplex analysis. When the normal *Eco*RI 9-kbp fragment was mixed with the purified *Eco*RI fragment from pSW9-33, a homologous region 5.1 kbp long was observed (Fig. 3*A*). Two single-stranded forks of nonhomologous sequences were also present. Similar structures were observed in heteroduplexes between the normal 7-kbp fragment and the purified pSW7-11 *Eco*RI fragment (Fig. 3*B*). These results suggested the rearrangement of human sequences at the ends of the SW-1271 fragments cloned from NIH/3T3 cells.

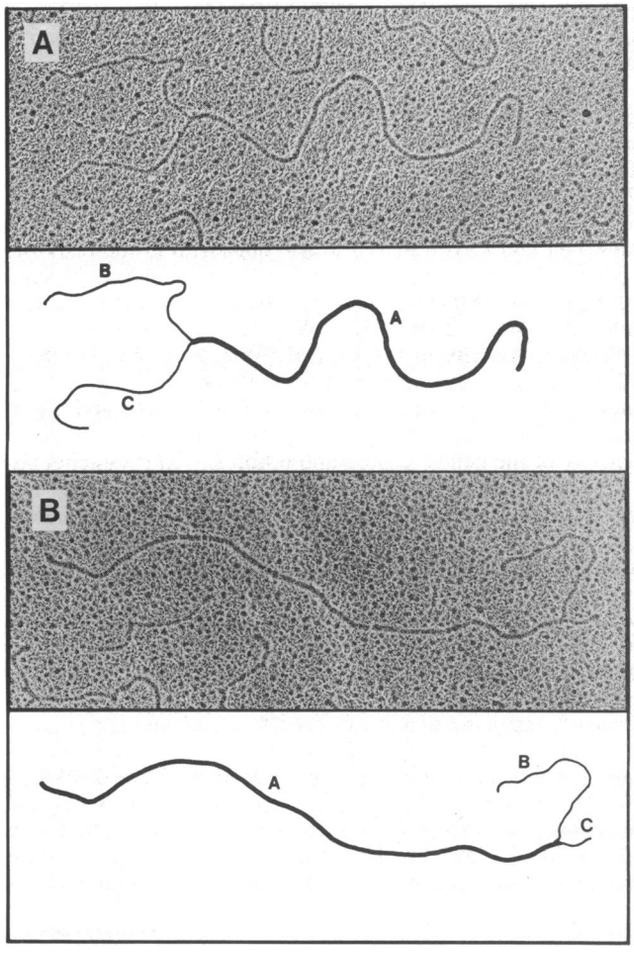

FIG. 3. Comparison of the SW-1271 oncogene with N-*ras* by heteroduplex analysis. The subcloned 7.2-kbp and 6-kbp *Eco*RI fragments of SW-1271 oncogene were annealed with the respective corresponding 9-kbp (*A*) and 7-kbp (*B*) *Eco*RI fragments of the N-*ras* gene. A representative heteroduplex molecule and interpretive sketch is shown in *A* and *B*. The contour lengths (bp) and standard deviation of features are as follows: (*A*) A = 5123 ± 282, B = 3516 ± 164, C = 2016 ± 127; (*B*) A = 5317 ± 309, B = 1883 ± 116, C = 353 ± 53. At least 10 molecules from each reaction were measured. Feature A represents the region of homology, and features B and C are the nonhomologous regions of N-*ras* and SW-1271, respectively. Molecules were measured with the aid of a Tektronix graphics tablet and computer and the lengths of features were compared with those of the double- and single-stranded forms of ϕX174 DNAs.

Restriction enzyme analysis was also carried out to compare normal and SW-1271 N-*ras* genes (data not shown). By using these results together with the heteroduplex analysis, a physical map of the SW-1271 oncogene was determined (Fig. 4). The central 11-kbp sequence was intact, whereas sequences 2-kbp and 0.35 kbp long at the left and right ends, respectively, did not correspond to N-*ras*, indicating that these sequences had been rearranged. These data imply that the central 11-kbp sequences are sufficient for transformation. Previous studies have localized the four exons of the N-*ras* gene to a region encompassed within the 11-kbp sequence (Fig. 4) (32). Since heteroduplex and restriction enzyme analyses did not show major alterations in this region of the SW-1271 oncogene compared to the N-*ras* proto-oncogene, the genetic lesion responsible for its acquisition of transforming activity was likely to be rather subtle.

Identification of the Lesion Responsible for Activation of the SW-1271 Oncogene. Our restriction enzyme analysis demonstrated that the 7.2-kbp *Eco*RI fragment of the SW-1271 oncogene to which its transforming activity was localized (Fig. 4) contained the first and second exons of the N-*ras* p21 coding sequence. To localize the lesion responsible for activation of the SW-1271 oncogene, we performed nucleotide sequence analysis of its first and second exons as well as corresponding exons of the human N-*ras* proto-oncogene. The first (i.e., 5′-most) exon was contained within a 300-bp *Hin*dIII fragment, whereas the second exon resided within a 450-bp *Pst* I–*Bst*EII fragment. No differences were observed in the corresponding sequences of the first exon (Fig. 5). However, analysis of the second exon revealed the presence of a single base change of A to G within the 61st codon of the predicted p21 coding sequence (Fig. 5). This results in a change of glutamine to arginine at this position in the protein. We conclude, therefore, that this single base change and its resultant amino acid change confer transforming activity to the SW-1271 oncogene.

DISCUSSION

A number of human tumors and tumor cell lines have been shown to contain oncogenes capable of transforming NIH/3T3 cells. Among transforming genes thus far detected, a high proportion has been found to be related to the *ras* family of *onc* genes. Oncogenes homologous to K-*ras*, for example, have been detected in tumors as diverse as carcinomas, sarcomas, and hematopoietic malignancies (8, 10, 18). Previous studies have detected N-*ras* oncogenes not only in a neuroblastoma (9) and sarcoma (22) cell lines but frequently in human hematopoietic tumors as well (10, 23). Our detection of an N-*ras* oncogene in a human lung carcinoma line expands the range of tissue types in which this member of the *ras* family can acquire transforming properties. These findings, along with the published evidence that other human lung carcinomas contain either c-K-*ras* (8, 18) or c-H-*ras* (21) oncogenes, demonstrate that each member of the *ras* gene family can be activated in lung tumors. Whether there is any specificity as to which gene is activated in various forms of lung cancer remains to be determined.

The SW-1271 oncogene was cloned as two fragments, which when ligated demonstrated high-titered transforming activity. Moreover, transforming activity of the gene was shown to reside within the left fragment containing exons 1 and 2 of the N-*ras* coding sequence. Comparative sequence analysis of the SW-1271 oncogene with the N-*ras* proto-oncogene revealed a single nucleotide substitution of G for A in the 61st position within the coding sequence. This results in the incorporation of arginine instead of glutamine at this position.

The mechanism by which *ras* oncogenes are activated as transforming genes in human tumors has revealed, in each

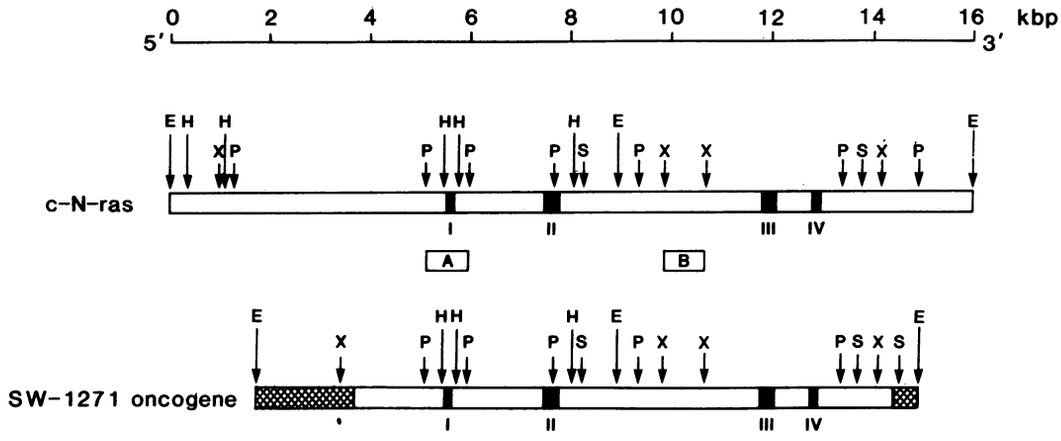

FIG. 4. Restriction endonuclease maps of the human N-*ras* gene (10, 32) (*Upper*) and SW-1271 oncogene (*Lower*). ■, Four exons of N-*ras* gene; □, *Pvu* II (A) and *Xba* I (B) fragments used for probes; ▨, sequences unrelated to human N-*ras* gene. E, *Eco*RI; H, *Hin*dIII; P, *Pvu* II; S, *Sst* I; X, *Xba* I.

case so far analyzed, lesions affecting one of two positions within the coding sequence (Table 2). The genetic lesions responsible for activating T24 and Hs242 oncogenes have been localized to point mutations in codons 12 and 61, respectively, in the c-H-*ras* proto-oncogene (21, 33–35). Point mutations in codon 12 have been described in two c-K-*ras*-related oncogenes isolated from human lung and colon carcinomas, Calu-1 and SW-480, respectively (36, 37). The lesion responsible for acquisition of transforming properties by an N-*ras* oncogene of the SK-N-SH neuroblastoma cell line has recently been localized to position 61 in its coding sequence (32). Thus, accumulating evidence indicates that codons 12 and 61 are the major "hot spots" for activation of members of the *ras* proto-oncogene family as oncogenes in naturally occurring human malignancies.

The occurrence of a point mutation in a gene can sometimes generate restriction site polymorphisms. This has provided an approach to search for the presence of activated *ras* alleles in primary human tumors. Feinberg *et al.* (38) analyzed a series of 29 such tumors (mostly of lung, colon, and bladder origin) for mutations in codon 12 of the H-*ras*-1 allele. Although the results of these studies were negative, they could not rule out either the presence of genetic alterations affecting H-*ras*-1 codon 61 or mutations in the other

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Exon I

1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20
Met Thr Glu Tyr Lys Leu Val Val Val Gly Ala Gly Gly Val Gly Lys Ser Ala Leu Thr
ATG ACT GAG TAC AAA CTG GTG GTG GTT GGA GCA GGT GGT GTT GGG AAA AGC GCA CTG ACA

21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37
Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr Asp Pro Thr Ile Glu
ATC CAG CTA ATC CAG AAC CAC TTT GTA GAT GAA TAT GAT CCC ACC ATA GAG

Exon II

38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57
Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly Glu Thr Cys Leu Leu Asp Ile Leu Asp
GAT TCT TAC AGA AAA CAA GTG GTT ATA GAT GGT GAA ACC TGT TTG TTG GAC ATA CTG GAT

58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76  77
Thr Ala Gly Arg Glu Glu Tyr Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly
ACA GCT GGA CGA GAA GAG TAC AGT GCC ATG AGA GAC CAA TAC ATG AGG ACA GGC GAA GGC
        A
       Gln

78  79  80  81  82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97
Phe Leu Cys Val Phe Ala Ile Asn Asn Ser Lys Ser Phe Ala Asp Ile Asn Leu Tyr Arg
TTC CTC TGT GTA TTT GCC ATC AAT AAT AGC AAG TCA TTT GCG GAT ATT AAC CTC TAC AGG
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FIG. 5. Comparison of the DNA sequence and predicted amino acid sequence of the entire first and second exons of the SW-1271 oncogene and N-*ras* (human). A single base change (A to G) and the consequent amino acid change of glutamine to arginine are boxed. Nucleotide sequence analysis was performed according to the procedure of Maxam and Gilbert (29).

ras gene family members at either positions 12 or 61. In fact, relatively few mutations generate detectable restriction site alterations in these positions. Nonetheless, this approach, as well as others such as hybridization with synthetic oligonucleotides, may provide a more accurate estimate of the frequency with which *ras* proto-oncogenes are activated as oncogenes in different types of human malignancies.

The basis for the striking alteration in function(s) induced in these highly conserved *ras* proto-oncogenes by point mutations at position 12 or 61 can only be speculated upon at present. Computer modeling has suggested that substitution at position 12 induces a major change in secondary structure of the p21 protein, findings consistent with the altered electrophoretic mobility of such proteins (39). The effects of alteration at position 61 have not been subjected to computer analysis. However, we have observed that both SW-1271 and Hs242 oncogene products with position 61 lesions migrate with similar mobilities that differ strikingly from that of *ras* oncogene products possessing position 12 lesions (unpublished data). Thus, lesions affecting position 61 also appear to alter p21 conformation, but in a manner that may be distinguishable from that of position 12 lesions.

It remains to be determined what normal functions are served by *ras* proto-oncogenes as well as how point mutations at position 12 or 61 can so markedly affect their biologic functions. Nonetheless, by means of monoclonal or peptide antibodies, it may be possible to develop immunologic

Table 2. Mutations leading to the activation of *ras* oncogenes in human tumor-derived cell lines

Oncogene	Origin	Codon affected	Base change	Amino acid change	Refs.
H-*ras*					
T24	Bladder carcinoma	12	G→T	Gly→Val	33–35
Hs242	Lung carcinoma	61	A→T	Gln→Leu	21
K-*ras*					
Calu-1	Lung carcinoma	12	G→T	Gly→Cys	36, 37
SW-480	Colon carcinoma	12	G→T	Gly→Val	37
N-*ras*					
SK-N-SH	Neuro-blastoma	61	C→A	Gln→Lys	32
SW-1271	Lung carcinoma	61	A→G	Gln→Arg	

reagents capable of specifically recognizing the altered gene products. Alternatively, oligonucleotide hybridization techniques may provide a method of detecting the changed alleles or transcripts. Such approaches could be used to identify cells with these genetic changes and thus determine whether such alterations are predictive of a particular clinical course.

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