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Activation of a human c-K-*ras* oncogene

Fumiichiro Yamamoto and Manuel Perucho

Department of Biochemistry, State University of New York, Stony Brook, NY 11794, USA

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ABSTRACT

The human lung carcinomas PR310 and PR371 contain activated c-K-ras oncogenes. The oncogene of PR371 was found to present a mutation at codon 12 of the first coding exon which substitutes cysteine for glycine in the encoded p21 protein. We report here that the transforming gene of PR310 tumor contains a mutation in the second coding exon. An A→T transversion at codon 61 results in the incorporation of histidine instead of glutamine in the c-K-ras gene product. By constructing c-K-ras/c-H-ras chimeric genes we show that this point mutation is sufficient to confer transforming potential to ras genes, and that a hybrid ras gene coding for a protein mutant at both codons 12 and 61 is also capable of transforming NIH3T3 cells. The relative transforming potency of p21 proteins encoded by ras genes mutant at codons 12, 61 or both has been analyzed. Our studies also show that the coding exons of ras genes, including the fourth, can be interchanged and the chimeric p21 ras proteins retain their oncogenic ability in normal rodent established cell lines.

INTRODUCTION

Considerable interest has been recently focused in ras genes since they were found altered in some human tumor cells by mutations which activated their transforming potentiality in rodent established cell lines (for review see 1). Ras genes represent a family of highly conserved genes in eukaryotic cells (2) encoding structurally and probably functionally related proteins which in mammalian cells have been designated p21 for their approximate molecular weight (3). Although the physiological role of p21 ras proteins remain unknown, it appears that they are involved in some aspects of cell growth and that specific alterations in their structure is associated to the development of the tumor phenotype "in vitro" and "in vivo."

Human ras oncogenes have been isolated by molecular cloning techniques and the mechanisms of their activation in the tumor cells have been determined as single amino acid substitutions in their protein coding regions (4-10). These studies have revealed the existence in ras genes of

two hot spots for their mutational activation, codons 12 and 61. In vitro mutagenesis studies also show that mutations in regions around these codons preferentially activate the c-H-ras gene (11).

The human oncogene homologue to the Kirsten sarcoma virus oncogene (human c-K-ras) has been found activated in a broad variety of human tumor cell lines (12-17) and primary tumors (16-17). We previously isolated the c-K-ras oncogene of two human lung tumors, PR310 and PR371, propagated into athymic mice, and determined the mutation which activated the transforming gene in PR371 tumor (18). We report here the analysis of the activating mutation of the PR310 c-K-ras gene.

METHODS

Construction of recombinant plasmids and DNA purification

Recombinant plasmids were constructed by restriction endonuclease (Bethesda Research Laboratories or New England Biolabs) digestions, ligation in vitro with T4 DNA ligase and transformation of the E. coli strain DH1 according to the procedure of D. Hanahan as described (19).

When necessary, synthetic DNA linkers (New England Biolabs), were kinased with T4 polynucleotide kinase (New England Biolabs) and ligated with T4 DNA ligase (Bethesda Research Laboratories) to restriction endonuclease digested plasmid DNA. After extraction with phenol, chloroform-isoamyl alcohol (24:1) and ethanol precipitation, the DNA was digested with the appropriate restriction endonuclease and electrophoresed through 1% agarose gels. Restriction endonuclease fragments were purified by cutting the desired bands and extracting the DNA by the freezing-thawing method (20). Briefly, the gel fragments were frozen at -70°C for twenty minutes and then thawed at 37°C. The process was repeated once more and the thawed gel fragments were centrifuged for fifteen minutes in an Eppendorf microfuge. The supernatant was extracted with phenol and chloroform-isoamyl alcohol and DNA was precipitated with ethanol. The purified DNA fragments were ligated with T4 ligase and used to transform E. coli. Recombinant plasmids were identified by their size and tested for the diagnostic restriction endonuclease sites. Plasmid DNAs for cloning were purified from 2 ml overnight cultures of DH1 by the alkaline lysis method (19). For DNA sequencing and transfection of animal cells, plasmid DNAs were purified from 250 ml cultures of DH1 by lysis with SDS and CsCl/ethidium bromide equilibrium centrifugation as described (19). Recombinant phage DNAs were purified for cloning and transfection of NIH3T3 cells from 500 ml cultures

of E. coli 803 SupF by centrifugation of phage particles through CsCl step gradients as described (19).

DNA sequence analysis

Plasmid DNAs were digested with various restriction endonucleases and were labelled at their 3' ends by using the Klenow fragment of the E. coli DNA polymerase I (Bethesda Research Laboratories) and α^{32}P-deoxynucleotides (Amersham). End labelled DNA fragments were further digested with appropriate restriction endonucleases and the specific fragments were purified by polyacrylamide gel electrophoresis and used for sequencing analysis according to the procedure of Maxam and Gilbert (21).

DNA transfection experiments

DNA transfection assays using NIH3T3 cells were performed by the calcium phosphate coprecipitation method (22) as previously described (23). For focus assays using in vitro ligated DNA (experiment of Figure 2) restriction fragments were purified from recombinant phages or plasmids after agarose gel electrophoresis by the KI/glass powder method (24) as described (18) and ligated with T4 DNA ligase at a DNA concentration of 100 µg/ml. The ligated DNA was diluted with 20 µg of NIH3T3 carrier DNA and added to culture dishes of NIH3T3 cells. Foci were scored after 14-20 days. To estimate the approximate amount of the correct ligated DNA fragments, aliquots of ligated DNA were digested with EcoRI, electrophoresed through agarose gels and stained with ethidium bromide. Transfections were performed using approximately 50 ng of the correct constructs.

For transfection assays using transforming chimeric genes cloned in recombinant plasmids, (experiments of Table I) 20 to 100 ng of linearized plasmid DNAs were added to 1 ml aliquots of a mixture of NIH3T3 carrier DNA (30 µg/ml) and pSV2gpt (20 ng/ml), a plasmid containing the bacterial xanthine guanine phosphoribosyl transferase (gpt) gene (25). The DNA mixture was precipitated with calcium-phosphate, and added to NIH3T3 cultures. After transfection cells were trypsinized and transferred to three plates containing DMEM with 5% calf serum, and to three plates of mycophenolic acid containing medium (200 µg/ml xanthine, 14 µg/ml hypoxanthine, 86 µg/ml azaserine and 100 µg/ml mycophenolic acid). gpt positive colonies were scored at 14 days and foci of morphologically transformed cells were scored at 14-18 days. Cotransformation efficiencies were calculated by scoring gpt positive colonies showing a transformed morphology.

RESULTS

Functional analysis of the mutation of PR310 oncogene

We have previously isolated human sequences which span over 40 Kbp of the c-K-ras oncogene from two lung tumors propagated into nude mice (18). While the oncogene of one of these tumors, PR371, was found to contain a point mutation in the first coding exon, similar to the mutation which activated the c-H-ras protooncogene in the T24 bladder carcinoma cell line (4-6), the c-K-ras oncogene from the PR310 tumor was normal in its first coding exon (18). In an effort to find the mutation responsible for the activation of the c-K-ras protooncogene in PR310 tumor cells, we sequenced the second and the third coding exons of the cloned oncogenes of the PR310 and PR371 tumors. The sequences of the third coding exons were identical to the reported sequence of the same region of the c-K-ras protooncogene (26). However, the sequences of the second coding exons differed in a single nucleotide. While the PR371 oncogene contained a deoxyadenosine as the third base of codon 61, the PR310 oncogene showed a thymidine at this position. Thus, the PR371 oncogene encodes glutamine at position 61 of the p21 protein, like the c-K-ras protooncogene (26). The thymidine present at this position in the oncogene of the PR310 tumor predicts the incorporation of histidine instead of glutamine in the c-K-ras gene product. Based on previous studies (7, 8), it is reasonable to assume that the presence of this thymidine in the PR310 oncogene is the result of a mutational event, which could be responsible for its activation.

Due to the complexity and large size of the c-K-ras locus, a rigorous functional analysis of the mechanisms resulting in its activation in human tumors has not yet been reported because it has not been possible to clone the entire gene in a single molecular vector. By constructing chimeric c-K-ras/c-H-ras genes, we have designed a functional assay to investigate if the mutation at the second coding exon is sufficient to activate the transforming potential of the PR310 oncogene. Recombinant plasmids containing the second and third coding exons of the c-K-ras oncogene from PR371 and PR310 tumors and the fourth coding exon of the c-H-ras oncogene were constructed (Figure 1). The HindIII-KpnI fragments of these chimeric plasmids were purified by agarose gel electrophoresis and ligated *in vitro* to similarly gel purified BglII/HindIII fragments of λLDH15 and λLNH16 (18) which contain the first coding exon of the PR371 or PR310 c-K-ras oncogenes respectively. We have found that the 5' end sequences contained in these fragments are sufficient for the efficient expression of the c-K-ras

oncogene (unpublished results). Thus, eight different DNA fragments were generated each containing a chimeric gene composed of the first three coding exons of the PR310 and PR371 c-K-ras, and the fourth coding exon of the T24 c-H-ras oncogenes in the correct orientation and in all possible combinations (Figure 2b).

The ligated DNA was added to NIH3T3 cells in the usual conditions of the calcium-phosphate transfection assay. The different chimeric genes generated in the ligation experiment were coded and added to NIH3T3 cells in such a way that the foci were scored without knowing the nature of the corresponding chimeric genes. The results of this experiment are summarized in Figure 2b. Only the chimeric genes containing the first coding exon of the PR371 oncogene and/or the second coding exon of the PR310 oncogene were able to induce morphological transformation of NIH3T3 cells. However the chimeric genes containing both the first coding exon of the PR310 and the second of the PR371 oncogenes failed to induce foci. Therefore, the HindIII-XhoI 0.56 Kbp fragment containing the second coding exon of the PR310 c-K-ras oncogene was sufficient to confer transforming activity to these chimeric genes, while the same fragment of the PR371 tumor was not (Figure 2b, constructs I through IV).

The HindIII-XhoI DNA fragments of p2N3N4H and p2D3D4H (Figure 1) were sequenced in their entirety, by end labeling at the restriction sites indicated (Figure 2a). The sequences of these fragments were identical to the reported sequence of the same region of the c-K-ras protooncogene (26) with the exception of the single base change at codon 61 in the second coding exon of the PR310 oncogene (Figure 3).

Therefore, we conclude from these experiments that the c-K-ras oncogene of the PR310 tumor contains at least one genetic alteration, the point mutation at codon 61, which is sufficient to activate the transforming potential of the gene. Taken together, our results indicate that the same protooncogene, c-K-ras, can be activated in naturally occurring human lung adenocarcinomas by single base substitutions in two distinct regions of the gene, codons 12 and 61 which appear to be hot spots for the mutational activation of ras genes in human tumors.

Analysis of the transforming potency of different mutant chimeric ras genes.

The results of the transfection experiment (Figure 2b) also demonstrate that a p21 ras protein containing cysteine and histidine at positions 12 and 61 respectively (constructs V and VI) is capable of inducing morphologically transformed foci in the NIH3T3 assay with efficiencies similar to the

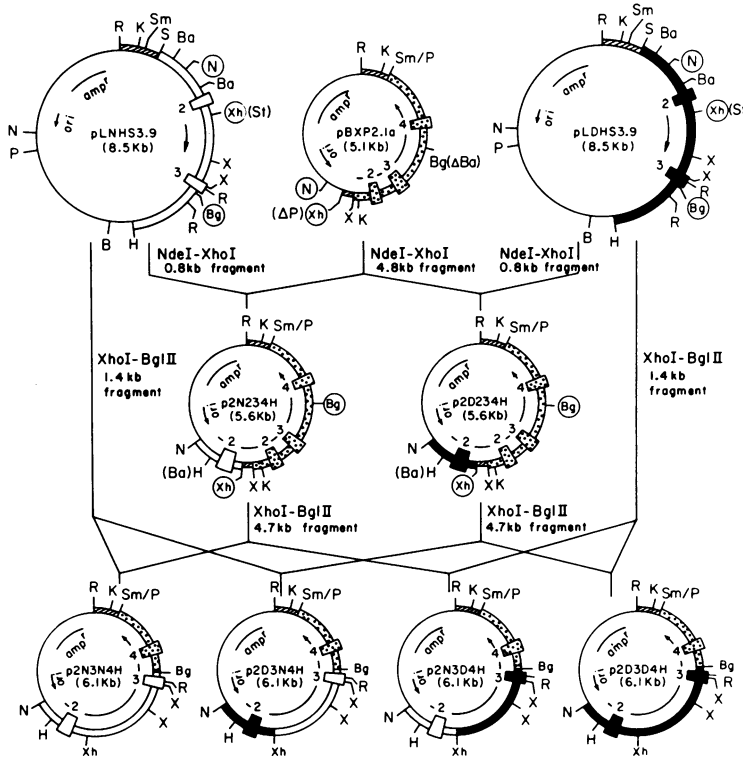

Figure 1. pBXP2.1a is a derivative of pBXP2.1 (27), which is the 2.1 Kbp XbaI-PvuII fragment containing the last three coding exons of the T24 c-H-ras oncogene, cloned into the XbaI-SmaI sites of pchtk2, a plasmid containing the chicken thymidine kinase (tk) gene (28) which was used as a cloning vector. The PvuII sites of pBXP2.1 located in the PBR sequences and near the XbaI site of the chicken tk gene, and the BalI sites between the third and fourth coding exons of the T24 oncogene, were replaced sequentially by XhoI and BglII sites respectively by molecular linker insertion. The 3.9 Kbp HindIII-SstI fragments of λLNH14 and λLDH14 (18), recombinant phages containing the second and third coding exons of PR310 and PR371 c-K-ras oncogenes respectively (see Figure 2a), were inserted into the HindIII-SstI sites of pchtk2. Subsequently, pLNHS3.9 and pLDHS3.9 were generated by changing the StuI site located between the second and third coding exons of the PR310 and PR371 oncogenes respectively (27) to an XhoI site by StuI digestion and linker ligation. The 0.8 Kbp NdeI-XhoI fragments of pLNHS3.9 and pLDHS3.9 were inserted into the NdeI-XhoI sites of pBX2.1a generating p2N234H and p2D234H plasmids respectively, after the BalI site located between the NdeI site and the second coding exon of the c-K-ras gene (26) was changed to a HindIII site by molecular linker ligation. The 1.4 Kbp XhoI-BglII fragments of pLNHS3.9 and pLDHS3.9 were inserted into the XhoI-BglII sites of p2N234H and p2D234H plasmids in the four possible combinations, thus generating the plasmids p2N3N4H, p2D3N4H, p2N3D4H, and p2D3D4H (bottom of figure). In the nomenclature used, N and D indicate sequences of PR310 and PR371 oncogenes respectively. Thin lines and

stippled areas represent PBR322 and chicken tk sequences respectively. Open, closed and dotted areas represent PR310, PR371 c-K-ras, and T24 c-H-ras oncogene sequences, respectively. The boxes indicate the exons of the c-H-ras and c-K-ras oncogenes. The arrows along the oncogene sequences show the direction of transcription. The restriction endonuclease sites are B: BamHI; Ba: BalI; Bg: BglII; H: HindIII; K: KpnI; N: NdeI; P: PvuII; R: EcoRI; S: SstI; Sm: SmaI; St: StuI; X: XbaI and Xh: XhoI. The encircled restriction sites delineate the fragments which were purified for plasmid constructions. The restriction sites in parentheses represent the original sites before replacement by molecular linkers. The triangles represent deletions of the corresponding restriction endonuclease fragments.

protein containing only one of these mutations (constructs I, II, VII and VIII). DNA from several of these primary transformants showed similar transforming activity in another transfection cycle, and Southern blot hybridization experiments revealed the presence of human oncogene sequences in NIH3T3 primary and secondary transformants induced by each of these chimeric genes (data not shown). Therefore, the uptake of a single copy of either of the chimeric genes appears to be sufficient to induce morphological transformation of NIH3T3 cells.

In order to accurately compare the relative transforming potency of the single or double mutant ras genes, we have constructed recombinant plasmids containing the first three coding exons of the c-K-ras gene of PR310 and PR371 tumors and the last fourth coding exon of the T24 c-H-ras oncogene (Figure 4, Set III). Four different plasmids, p1N2N3N4H, p1N2D3D4H, p1D2N3N4H and p1D2D3D4H, containing the first and second coding exons of PR310 and PR371 oncogenes in all four combinations, and the third c-K-ras and fourth c-H-ras coding exons, were thus generated. These plasmids also contain the BglII-XhoI 2.9 Kbp fragment upstream of the first coding exon, containing the 5' end nontranslated exon of the human c-K-ras gene previously identified by analysis of cDNAs synthesized from c-K-ras specific mRNAs (10, 26). At the same time, these constructs present a deletion of the XhoI-PvuII 3.5 Kbp fragment located between the 5' nontranslated exon and the first coding exon of the c-K-ras gene.

These chimeric plasmids were linearized at the SalI site of PAT153 plasmid vector, and added to NIH3T3 cells as a calcium phosphate coprecipitate. As controls we included the plasmids of sets I and II (Figure 4) which contain the first mutant exon of PR371 c-K-ras oncogene and the last three coding exons of the c-H-ras gene of the T24 cell line, with or without the XhoI-PvuII 3.4 Kbp intron fragment, as well as the entire T24 c-H-ras oncogene contained in pTBG-I (27). Foci of transformed NIH3T3 cells were scored 14-18 days after DNA transfection (Table I).

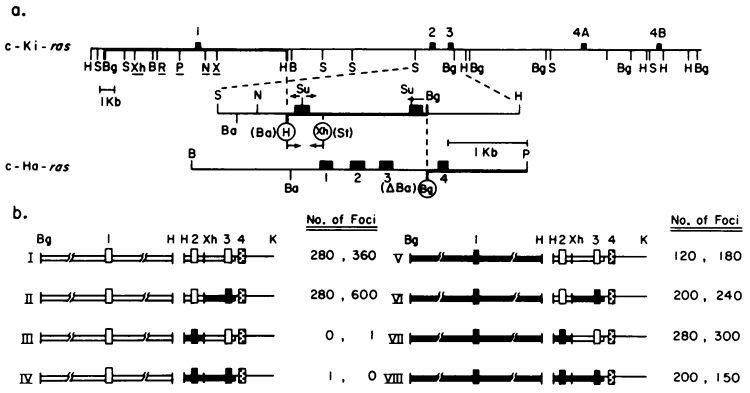

Figure 2. a) Partial restriction maps of the human c-K-ras and c-H-ras oncogenes and structure of the chimeric genes generated in our studies. The oncogene fragments present in the chimeric genes are indicated by a thick line. The solid boxes indicate the position of the coding exons. The arrows indicate the areas of the c-K-ras genes that were sequenced by the Maxam-Gilbert method. Restriction endonuclease sites are the same as in Fig. 1. Sau96I (Su) and the underlined restriction sites are not mapped through all the gene. The restriction sites generated by molecular linker insertion are represented in circles.

b) Schematic representation of the chimeric genes and their transforming efficiencies. The BglII-HindIII fragments of λ LNH16 and λ LDH15, containing the first coding exon of PR310 and PR371 c-K-ras oncogenes respectively (18) were ligated in equimolar ratio to the HindIII-KpnI fragments of p2N3N4H, p2N3D4H, p2D3N4H and p2D3D4H plasmids (Figure 1, bottom panel), which contain the second and third coding exons of the c-K-ras, and the fourth exon of the c-H-ras genes, in all possible combinations. The open, closed and dotted blocks indicate the coding exons of PR310, PR371 and T24 ras genes respectively. The numbers to the right indicate the approximate total number of foci obtained in two independent transfection experiments.

In contrast with the ligation experiment (Figure 2), this experiment allowed a direct comparison of the transforming potency of the different chimeric ras genes because it was possible to estimate the precise amount of DNA added to the NIH3T3 cultures. Again, the chimeric genes containing non mutated exons (plasmids p1N234H and p1N2D3D4H) failed to induce foci of morphologically transformed cells. It could be argued that these constructs were negative in the transfection assay due to some rearrangements occurred during their construction which could impair their functionality at the transcriptional or translational level. However, some colonies of NIH3T3 cells selected for the gpt vector (see Methods) and cotransfected with these normal plasmids developed a distinguishable transformed morphology after some time in culture. Southern and Northern blot analysis revealed that these morphologically altered clones contained substantially more copies of

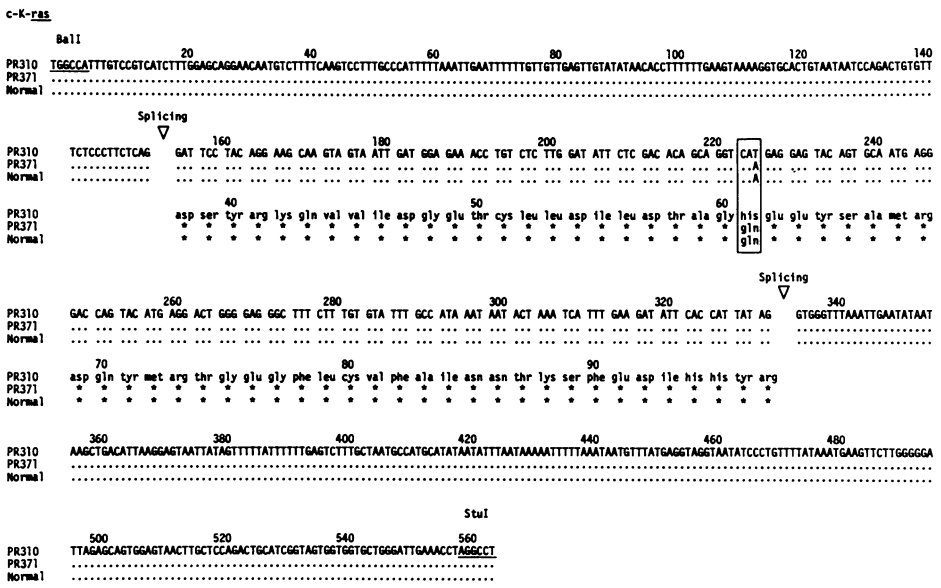

Figure 3. The sequence of the BalI-StuI 560 bp fragment containing the second coding exon of PR310 and PR371 oncogenes is compared with the previously reported sequence of the same region of the c-K-ras protooncogene (26). The predicted amino acid sequence is also indicated. Dots and asterisks indicate identical nucleotide and amino acid sequences respectively.

exogenous human ras gene DNA and RNA sequences than those which still showed a normal morphology (unpublished results). This is in agreement with the observation that increased expression of the normal c-H-ras gene may also lead to phenotipic transformation of NIH3T3 cells (6,29).

The transforming efficiencies of the different mutant chimeric ras genes were lower (two-three fold) than that of pTBG-I. However, the transforming potency of the chimeric genes containing only the first (sets I and II), or the first three coding exons of the c-K-ras gene (set III) was essentially the same. At the same time, the deletion of the XhoI-PvuII 3.5 Kbp intron fragment had no apparent effect on the oncogenic capacity of these chimeric ras genes (compare constructs I and II). The plasmids of set III showed slight differences in their transforming activity. The chimeric gene containing the mutant 61 codon was approximately two fold less potent than that containing the mutation at codon 12, while the double mutant showed an intermediate transforming potency.

We conclude from this experiment that first, the interchange of coding

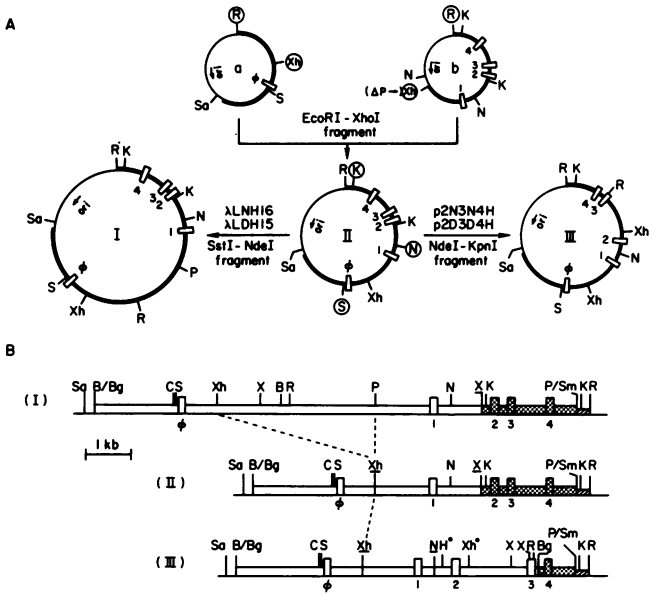

Figure 4. A) Generation of c-K-ras/c-H-ras chimeric plasmids. The 4.5 Kbp BglII-EcoRI fragments of λLNH15 and λLDH15 (18 and our unpublished results), recombinant phages containing the 5' end 15 Kbp HindIII fragments of the PR310 and PR371 c-K-ras oncogenes respectively, were cloned into the BamHI-EcoRI sites of the PAT 153 plasmid vector. Thus the plasmids of set a, pLNBgR and pLDBgR, containing the 5' end nontranslated exon of PR310 and PR371 oncogenes respectively, were generated. The PvuII sites of pLNX2P and pLDX2P (27), recombinant plasmids containing the first coding exons of PR310 and PR371 c-K-ras oncogenes respectively, and the last three coding exons of the T24 c-H-ras oncogene, were changed to a XhoI site by PvuII digestion and XhoI linker ligation, thus generating the plasmids of set b. Next, the 4.9 Kbp XhoI-EcoRI fragments of plasmids b were inserted into the XhoI-EcoRI sites of plasmids a, yielding plasmids of set II, p1N234ΔXhP and p1D234ΔXhP. To restore the entire 5' end sequences of the c-K-ras gene, the 6.3 Kbp SstI-NdeI fragments of λLNH16 and λLDH15 (18), were inserted into these sites of set II plasmids, thus generating the plasmids of set I, p1N234H and p1D234H. Next, the 3.3 Kbp NdeI-KpnI fragment of p2N3N4H and p2D3D4H (Figure 1), were inserted into these sites of plasmids of set II, generating the plasmids of set III, p1N2N3N4H, p1N2D3D4H, p1D2N3N4H and p1D2D3D4H.

B) Detailed restriction endonuclease map of transforming chimeric ras plasmids. The linear maps of the oncogene sequences of the chimeric plasmids of sets I, II and III are represented. Symbols are the same as in Figure 1. Additional restriction endonucleases are Sa: SalI; C: ClaI; ϕ: 5' nontranslated exon. The underlined restriction sites represent the artificial boundaries present in the hybrid introns. The asterisks represents restriction sites generated during the cloning protocol. Dotted and open areas represent c-H-ras and c-K-ras oncogene sequences respectively.

Table I. Relative Transforming Potency of Chimeric ras Genes Containing Different Mutations.

Plasmid	(set)	Amino acids at codons[a] 12	61	Transforming efficiency[b] foci/ng	Relative transforming potency[c]
p1N234H	(I)	gly	gln	<0.01	<0.001
p1D234H	(I)	cys	gln	10.0	0.83
p1D234HΔXhP	(II)	cys	gln	12.0	1.0
p1N2N3N4H	(III)	gly	his	7.0	0.58
p1N2D3D4H	(III)	gly	gln	<0.01	<0.001
p1D2N3N4H	(III)	cys	his	10.0	0.83
p1D2D3D4H	(III)	cys	gln	12.0	1.0
pTBG-I	--	val	gln	25.0	2.05

[a] Underlined are represented the mutant amino acids.
[b] The amount of DNA added to the cultures was estimated by electrophoresis in agarose and acrylamide gels and staining with ethidium bromide. The values were corrected for variability in the individual experimental groups within the same experiment by comparison to the number of colonies selected in mycophenolic acid containing medium (see Materials and Methods).
[c] Transforming activity relative to that of p1D2D3D4H.

exons of c-K-ras and c-H-ras genes does not significantly affect the transforming potentiality of the encoded p21 chimeric proteins. Second, the double mutant p21 protein artificially generated in our constructs shows essentially the same oncogenic activity as the single mutant proteins which were presumably selected in the generation of the naturally occurring tumors. Third, considerable deletions in the introns or the generation of artificial introns in ras genes have no apparent effect in the transforming activity in NIH3T3 cells of the resulting chimeric genes.

DISCUSSION

Activation of ras genes probably plays a role in mammalian tumorigenesis. In tumors from rodent and human origin, the mutational mechanisms involved in the activation of the transforming potential of ras genes have been shown to be point mutations in their protein coding regions which result in the expression of structurally altered p21 proteins. Two hot spots for mutagenesis have become apparent from these studies, codons 12 and 61 at the first and second coding exons of ras genes.

Although activated c-K-ras genes have been found in numerous tumors, all so far analyzed have been shown to contain mutations at codon 12 (9, 10, 17, 18). The apparent preferential activation of the c-K-ras gene in human tumors by mutations at codon 12 could be a reflection of some inherent

constraints for mutagenesis in the sequences around codon 61 or simply a consequence of biased detection in the NIH3T3 transfection assay if mutations in or around codon 61 confer less transforming potency to the mutant genes. By constructing chimeric c-K-ras/c-H-ras genes, we have demonstrated the transforming potentiality of the mutant codon 61 of an activated c-K-ras oncogene, and we have analyzed the relative transforming potency of mutations at codons 12 and 61 of the c-K-ras oncogene present in two human lung adenocarcinomas. The transforming activity of the double mutant p21 protein encoded by the artifically generated chimeric oncogenes support the hypothesis that the mutational activation of ras proteins occurs by disruption of some aspects of their normal function.

Our results also show that the interchange of coding exons of ras genes does not alter the response of NIH3T3 cells to these chimeric transforming genes. In this respect, it is noteworthy that although the first and second coding exons of the human c-K-ras and c-H-ras protooncogenes encode identical amino acid sequences, the third and fourth coding exons of these genes contain two regions of amino acid divergency, encompassed by positions 121-128 and 166-185 respectively (9). Thus, it has been postulated that the specific interaction of these two variable domains could be responsible for the putative distinct physiological function of individual ras proteins in the same species (8).

However, our results show that p21 proteins encoded by chimeric genes containing the third coding exon of the c-K-ras and the fourth of the c-H-ras, remain functionally active in the NIH3T3 assay. This can be explained if mutant ras proteins could induce the oncogenic transformation of NIH3T3 cells in the absence of their putative c-terminus dependent physiological function. It is also possible that the intraspecies specificity of individual ras proteins could be dependent exclusively on their carboxy terminal amino acid sequences encoded by the last exon of ras genes.

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