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Human Colon Carcinoma Ki-*ras*2 Oncogene and Its Corresponding Proto-Oncogene

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We isolated cDNA clones corresponding to the normal human Ki-*ras*2 gene and to the transforming allele of the Ki-*ras*2 gene present in the human colon carcinoma cell line SW480. These two cDNAs encode p21 proteins which differ only at the amino acid at position 12. The normal cDNA encodes a glycine at this position, and the transforming allele encodes a valine. Expression of these cDNAs indicates that this amino acid 12 alteration confers oncogenic activity on the mutated gene. Analysis of the relationship of the cDNAs and Kirsten sarcoma virus *ras* gene to a genomic clone allowed us to identify two alternative 3′ coding exons for the Ki-*ras*2 gene, suggesting that the Ki-*ras*2 gene encodes two p21 proteins which differ at their carboxy termini. Our data also show that only one of the p21s is necessary to convert cells to a tumorigenic phenotype.

DNAs from a variety of human tumor cell lines and human tumor tissues transform NIH 3T3 cells (for reviews, see references 3 and 32). Transfection of such DNAs into NIH 3T3 cells has led to the identification of three independent genes that are frequently activated in human tumors. All three of the genes belong to the *ras* gene family (5, 17, 19, 24), some of whose members were first identified by virtue of their presence in the genomes of the Harvey and Kirsten sarcoma viruses. The *ras* oncogenes present in these various human tumors appear to arise via somatic mutation of preexisting proto-oncogenes residing in the cellular genome (1, 18, 23, 27–29, 33).

Previous analysis of the oncogenic Ha-*ras*1 allele present in the EJ/T24 human bladder carcinoma cell line indicated that this oncogene was activated by a point mutation which affected the codon specifying the 12th amino acid of the 21,000-dalton ras protein. The glycine residue normally present was replaced by a valine in the oncogene-encoded protein (18, 27, 29). In the present work, we extended this analysis to a Ki-*ras*2 oncogene present in the DNA of the human colon carcinoma cell line SW480 (12).

MATERIALS AND METHODS

Synthesis of cDNA. Polyadenylated RNA was isolated by the guanidine isothiocyanate protocol as described by Schwarzbauer et al. (20), and by selection on oligodeoxythymidylate-cellulose (Collaborative Research, Inc.). We utilized a cDNA synthesis protocol which was generously provided by Parmjit Jat (personal communication). The RNA to be reverse transcribed was first denatured in 12.5 mM methylmercury hydroxide. Subsequently, the components of the first strand synthesis reaction were added so that the reaction contained 50 mM Tris-hydrochloride (pH 8.3), 100 mM KCl, 10 mM $MgCl_2$, 30 mM β-mercaptoethanol, 2.5 mM methylmercury hydroxide (initially 12.5 mM), 40 μg of oligodeoxythymidylate per ml, RNasin (3 U per μg of RNA; Boehringer-Mannheim Biochemicals), 0.5 mM each dATP, dGTP, dCTP, and dTTP, 1 mg of RNA per ml, and 10 U of reverse transcriptase (Life Sciences, Inc.) per μg of RNA. The reaction mixture was incubated at 42°C for 35 min, then boiled, quenched on ice, and spun for 3 min in a microcentrifuge. The supernatant was diluted with 10× Klenow buffer (100 mM $MgCl_2$, 100 mM Tris-hydrochloride [pH 7.5], and 500 mM NaCl), 5 mM each dATP, dGTP, dCTP, and dTTP, and 10 mM dithiothreitol, so that the final mixture contained 1× Klenow buffer, 1 mM each dATP, dGTP, dCTP, and dTTP, and 1 mM dithiothreitol. Two units of Klenow fragment (New England BioLabs) were added for each microgram of input RNA, and the reaction mixture was incubated at 16°C for 20 h.

After the addition of an equal volume of 5 M ammonium acetate, the reaction was extracted twice with phenol-chloroform and ethanol precipitated. The pellet was taken up in 0.3 M sodium acetate, ethanol precipitated, washed with 70% ethanol, and taken up in water. S1 buffer (5×) was added until the final concentration was 1× S1 buffer. S1 nuclease (500 U; Sigma Chemical Co.) was added for each microgram of input RNA, and the reaction was incubated at 37°C for 60 min. One-tenth volume of 1 M Tris-hydrochloride (pH 8) and 1 volume of 5 M ammonium acetate were added. The mixture was extracted twice with phenol-chloroform and ethanol precipitated. After another ethanol precipitation and washing with 70% ethanol, the pellet was taken up in water. The tailing of the cDNA was carried out in 1 mg of bovine serum albumin per ml–0.1 mM dCTP–100 mM potassium cacodylate (pH 7.2)–2 mM $CoCl_2$–0.2 mM dithiothreitol–terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) at 37°C for 2 min. The tailed cDNA was annealed into G-tailed pBR322 (New England Nuclear Corp.) according to the manufacturer's instructions. The plasmids were then transformed into *Escherichia coli* HB101 LM1035 with a protocol kindly provided by Constance Cepko.

Screening of cDNA libraries. The cDNA libraries were screened as described by Hanahan and Meselson (8).

Sequencing. Nucleotide sequences were determined by the method of Maxam and Gilbert (11).

Plasmid construction. A derivative of pCD (16) was constructed which had only one *Pst*I site. This was accomplished by replacing the pCD *Bgl*I-*Pvu*I fragment containing the *Pst*I site that we wished to remove with the corresponding *Bgl*I-*Pvu*I fragment from pUC13 (31), which lacks *Pst*I sites but nonetheless can form part of a functional ampicillin resistance gene. The modified pCD was partially cleaved with *Bam*HI and then cleaved (at its unique *Pst*I site) with *Pst*I. The pSW11-1 insert containing the cDNA derived from the transforming allele was then ligated into the vector. The

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insert was prepared by subcloning it into pUC13 (31) and then deleting the 3′ *Pst*I site with T4 polymerase to remove the *Pst*I-generated 3′ overhanging nucleotides. The pSW11-1 insert was then ligated into the pCD derivative after its excision, utilizing the 5′ *Pst*I site and a 3′ *Bam*HI site present in the pUC13 polylinker.

DNA transfection. DNA transfections were carried out as previously described (27) with 75 μg of NIH 3T3 carrier DNA, 1 μg of chimeric pCD plasmid, and 100 ng of pSV2 Neo per 2×10^6 cells. Colonies resistant to G418 were then selected.

Tumorigenicity assays. Cells assayed for their tumorigenicity in mice were treated with trypsin, washed twice with phosphate-buffered saline, and injected subcutaneously into 7-week-old CD-1 mice. An inoculum of 6×10^6 cells was injected into each mouse. The mice were examined for tumors 10 to 14 days after injection.

Immunoprecipitations. Cells to be immunoprecipitated were labeled in methionine-free Dulbecco modified Eagle medium containing 2% dialyzed calf serum. [^{35}S]methionine (250 μCi; New England Nuclear) was used for each 100-mm plate. The cells were harvested and lysed in RIPA buffer (10 mM Tris-hydrochloride [pH 7.2], 150 mM NaCl, 0.1 mM $MgCl_2$, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% sodium dodecyl sulfate). After lysis, cellular debris was removed by centrifugation and the lysates were precleared with *Staphylococcus aureus* protein A and normal rat serum. After the preclearing step, the lysates were immunoprecipitated with either p21 monoclonal antibody Y13-259 (7) or normal rat serum. Then goat anti-rat antibodies and *S. aureus* protein A were added. Finally, the pellets were washed with high-salt RIPA buffer (RIPA buffer containing 750 mM NaCl) and then normal RIPA buffer. After the addition of sample buffer and boiling, the samples were subjected to electrophoresis on a 15% sodium dodecyl sulfate-polyacrylamide gel (9). Autoradiography of the gels was enhanced with PPO (2,5-diphenyloxazole) and acetic acid (25).

Isolation of genomic clone. The genomic segments, termed φ9, were isolated by screening a normal human genomic library, kindly provided by Tom Maniatis (10), with a derivative of the single-copy probe p640. p640 is a unique sequence that is derived from a 2.6-kilobase (kb) *Eco*RI fragment that is present in all transfectants containing the SW480 oncogene (12). The p640 derivative was constructed by cloning a *Pst*I-*Hin*dIII fragment from p640 (the *Pst*-*Eco*RI portion is from pBR322, and the *Eco*RI-*Hin*dIII portion is p640) into the miniplasmid πvx. Screening of the human library cloned in the amber lambda phage Charon 4A with the chimeric πvx plasmid led to the isolation of the wild-type (non-amber) bacteriophage φ9, which had acquired the πvx suppressor tRNA gene via homologous recombination in the p640 sequences of the miniplasmid (21). We verified that φ9 was derived from the cKi-*ras*2 gene by cleaving the phage DNA with the *Eco*RI and hybridizing the resulting DNA fragments with p640. Since we knew that p640 was originally derived from a 2.6-kb *Eco*RI fragment, we reasoned that in φ9, p640 should hybridize with a 2.6-kb *Eco*RI fragment. Indeed, φ9 was found to contain a 2.6-kb RI fragment which hybridized to p640 (data not shown). Further verification of φ9 was provided by the sequence data discussed below.

RESULTS

We wished to ascertain the molecular alterations responsible for creating the Ki-*ras*2 oncogene present in the SW480 human colon carcinoma cell line (12). Molecular analysis of the Ki-*ras*2 gene has been hampered by the fact that the gene spans a DNA segment greater than 40 kb, a size which precludes the isolation of a biologically active genomic clone (12, 15). Following the analogy with the Ha-*ras*1 oncogene, we suspected that activation of the Ki-*ras* gene could be traced to a lesion present in the protein-encoding region of the Ki-*ras*2 proto-oncogene. This offered the possibility of great simplification in our analysis, since such a lesion would be found in the Ki-*ras*2 cDNA, whose sequence can be 30-fold less complex than that of the gene itself. Therefore, we chose to compare cDNA clones of the RNAs encoded by the colon carcinoma oncogene with the cDNA derived from the corresponding Ki-*ras*2 proto-oncogene.

Isolation of a cDNA clone corresponding to the transforming allele was facilitated by a finding concerning the RNAs transcribed from this oncogene: whereas in the SW480 colon carcinoma cell line the major Ki-*ras*2 RNA is greater than 5,000 nucleotides in length, the major human Ki-*ras*2 RNA present in several derived transfectants is only approximately 1,200 nucleotides in length (data not shown). At present we do not understand the molecular basis for this difference in length, but we do have evidence that the smaller Ki-*ras* RNA species occurs naturally in at least one human cell line.

Polyadenylated RNA was isolated from a transfectant (SW-2-3) that had been derived by two sequential cycles of transfer of the SW480 oncogene (15) and contained a 1,200-nucleotide-long Ki-*ras* mRNA (data not shown). This RNA was used as a template for cDNA synthesis and cloning as described above. Screening 50,000 of the resulting recombinant plasmids with the vKi-*ras* HiHi3 probe (6) led to the isolation of two Ki-*ras*–homologous plasmids. All analyses described were carried out on the longer of the two inserted cDNAs, which is approximately 1,050 base pairs in length and is designated pSW11-1. A restriction map of this insert is shown in Fig. 1b, along with the strategy that was utilized to determine its nucleotide sequence.

A cDNA corresponding to the normal allele was isolated by screening a cDNA library prepared from RNA of the simian virus 40-transformed human cell line GM637. This library had been constructed and generously provided by Hiroto Okayama and Paul Berg (16). Screening of this library with the vKi-*ras* probe HiHi3 also led to the isolation of two plasmids. Once again, all analyses were carried out on the longer of these two inserted cDNAs, which was approximately 1,000 base pairs long and designated pcKi-*ras*2-76. The restriction map and sequencing strategy for this clone are shown in Fig. 1a.

Activation of the oncogene. Examination of the coding sequences of the normal and transforming cDNAs (Fig. 2) revealed that the proteins encoded by these cDNAs differed in only a single amino acid residue: the cDNA from the normal allele encoded a glycine residue at amino acid residue 12, whereas the sequence from the transforming gene encoded a valine residue. This amino acid replacement is identical to that which led to activation of the Ha-*ras*1 gene in the EJ/T24 human bladder carcinoma cell line (18, 27, 29).

We wished to prove that this simple alteration did indeed confer oncogenic activity on the mutated gene. Therefore, the transforming abilities of the two cDNAs were examined after insertion into the eucaryotic expression vector pCD (16). The early simian virus 40 promoter carried in this vector drives transcription of any adjacently inserted DNA. The cDNA of the normal allele was already present in pCD, having been isolated from a cDNA library constructed with this vector. Therefore, we had only to insert the cDNA clone of the oncogene allele into the vector. These two chimeric plasmids, representing the oncogene and proto-

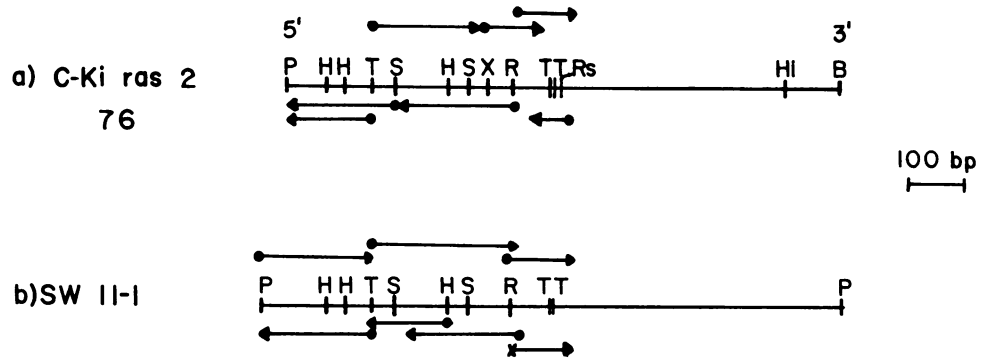

FIG. 1. (a) Restriction map of the insert of pcKi-*ras*2-76, which contains the cKi-*ras*2 cDNA derived from a normal allele present in the simian virus 40 transformed human cell line GM637. (b) Restriction map of the insert of pSW11-1, which contains the cKi-*ras*2 cDNA derived from the transforming allele present in the secondary transfectant SW-2-3. Abbreviations: P, *Pst*I; H, *Hin*fI; T, *Taq*I; S, *Sau* 96I; X, *Xba*I; R, *Eco*RI; Rs, *Rsa*I; Hi, *Hin*dIII. The 5' ends of the inserts are as indicated. ×, Fragments labeled at their 3' ends with Klenow fragment. ●, Fragments labeled at their 5' ends with polynucleotide kinase.

oncogene alleles, were cotransfected with the pSV2 Neo plasmid (26) into NIH 3T3 cells. We utilized transfection conditions to ensure that those cells that had acquired the pSV2 Neo gene and resistance to the drug G418 also contained copies of the cotransfected pCD chimera (see above).

We observed that the G418-resistant colonies carrying the plasmid encoding the normal Ki-*ras*2 protein were composed of cells that were flat, nonrefractile, and nontumorigenic in mice (none of six mice developed tumors). In contrast, the neomycin-resistant colonies bearing the gene that encodes the altered Ki-*ras*2 protein were composed of cells that were refractile, spindle-shaped, and tumorigenic in mice (9 of 12 mice developed tumors). These results indicate that the introduction of a valine residue at position 12 in the Ki-*ras*2–encoded protein is sufficient to confer transforming capacity.

The *ras* genes encode proteins of molecular weight 21,000, termed p21 (6, 22). When lysates of neomycin-resistant cell lines were immunoprecipitated with monoclonal antibodies reactive with the p21 protein (7), novel p21 proteins not present in untransfected NIH 3T3 cells were observed (Fig. 3, lanes B and F). The p21 encoded by the transforming cDNA comigrated with the p21 observed in secondary transfectants derived by passage of the uncloned, genomic version of the oncogene (lanes D and F). The p21 encoded by this transforming cDNA differed slightly in mobility from that encoded by the normal cDNA (Fig. 3, lanes F and G). Analogous observations have been made when comparing the normal Ha-*ras*1–encoded p21 with the p21 encoded by the related oncogene present in the EJ/T24 human bladder carcinoma cell line (27).

It appears that the difference in phenotype induced by the normal and oncogenic alleles is due to the structural difference in the encoded p21 proteins rather than to differences in levels of expressed protein. Thus, the cells transfected by the clone representing the normal allele are flat, nonrefractile, and nontumorigenic and express p21 in levels seen in Fig. 3 (lane G). In contrast, comparable levels of p21 are seen in cells transfected with the oncogene clone (Fig. 3, lane M), yet these cells are rounded, highly refractile, and highly tumorigenic.

Alternative splicing in the cKi-*ras*2 gene. We undertook a comparison of the amino acid sequence of the p21 proteins encoded by the cDNAs with the corresponding Ki-*ras* gene present in the genome of Kirsten sarcoma virus (KiSV; Fig. 2B). It appears that the cDNAs of the human transcripts encode p21s that are one amino acid shorter than the p21 encoded by KiSV. In addition to the residue 12 differences, we noted the presence of 21 amino acid differences between the KiSV- and cDNA-encoded p21s. This large number of amino acid differences was somewhat surprising in light of the observation that there are only three amino acid differences between the Harvey sarcoma virus p21 and the human Ha-*ras*1-encoded p21. It was also curious that 16 of these amino acid changes were found in a region of the p21 protein between amino acids 165 and 184. Since many of these amino acid replacements appear to be due to greater than a single nucleotide change, it appeared that clustering of amino acid changes in the cKi-*ras*2 gene was not likely to have been caused by an accumulation of simple point mutations occurring since the divergence of rodents and primates. These observations, when taken together, led us to believe that the differences among these various p21 proteins were due to another mechanism that was able to create a more drastic change in coding sequence. Such a mechanism might involve the use of an alternative splicing pathway.

To investigate this possibility, we used the human cDNAs and the HiHi3 subclone of the KiSV genome (6) as probes in Southern blot analysis of a genomic clone containing the 3' end of the human Ki-*ras*2 gene. The isolation of this genomic clone, and verification of its proposed structure, are described in detail above. DNA from this genomic Ki-*ras*2 clone was cleaved with the restriction endonuclease *Eco*RI, transferred to nitrocellulose, and incubated with ^{32}P nick-translated Ki-*ras*2 cDNA or KiSV DNA probes. We found that homology with the KiSV subclone was present in a genomic 3.2-kb *Eco*RI fragment, and homology with the cDNA was localized to a 2.4-kb *Eco*RI genomic segment. Sequence analysis of the exon present in the 3.2-kb *Eco*RI fragment indicated that this exon encoded the carboxy-terminal 37 amino acids of a p21 protein. This carboxy terminus is virtually identical to that of the KiSV p21 protein. In Fig. 4, we compare the carboxy termini of the p21s encoded by KiSV and its homologous human exon with the carboxy terminus of the cDNA-encoded p21.

These data allow several conclusions. The human Ki-*ras*2 gene contains at least two alternative fourth coding exons. The first exon, termed 4A (1, 23), is embedded in a 3.2-kb *Eco*RI fragment. It is represented in homologous form in the KiSV genome but not in the colon carcinoma oncogene cDNA that we constructed. Instead, an alternative fourth exon, termed 4B (1, 23), carried in a 2.4-kb *Eco*RI genomic

(A)

	1									10
cKi-*ras*2-76	ATG	ACT	GAA	TAT	AAA	CTT	GTG	GTA	GTT	GGA
	11									20
cKi-*ras*2-76	GCT	GGT	GGC	GTA	GGC	AAG	AGT	GCC	TTG	ACG
SW-11-1		GTT								
	21									30
cKi-*ras*2-76	ATA	CAG	CTA	ATT	CAG	AAT	CAT	TTT	GTG	GAC
	31									40
cKi-*ras*2-76	GAA	TAT	GAT	CCA	ACA	ATA	GAG	GAT	TCC	TAC
	41									50
cKi-*ras*2-76	AGG	AAG	CAA	GTA	GTA	ATT	GAT	GGA	GAA	ACC
	51									60
cKi-*ras*2-76	TGT	CTC	TTG	GAT	ATT	CTC	GAC	ACA	GCA	GGT
	61									70
cKi-*ras*2-76	CAA	GAG	GAG	TAC	AGT	GCA	ATG	AGG	GAC	CAG
	71									80
cKi-*ras*2-76	TAC	ATG	AGG	ACT	GGG	GAG	GGC	TTT	CTT	TGT
	81									90
cKi-*ras*2-76	GTA	TTT	GCC	ATA	AAT	AAT	ACT	AAA	TCA	TTT
	91									100
cKi-*ras*2-76	GAA	GAT	ATT	CAC	CAT	TAT	AGA	GAA	CAA	ATT
	101									110
cKi-*ras*2-76	AAA	AGA	GTT	AAG	GAC	TCT	GAA	GAT	GTA	CCT
	111									120
cKi-*ras*2-76	ATG	GTC	CTA	GTA	GGA	AAT	AAA	TGT	GAT	TTG
	121									130
cKi-*ras*2-76	CCT	TCT	AGA	ACA	GTA	GAC	ACA	AAA	CAG	GCT
SW-11-1		TCC								
	131									140
cKi-*ras*2-76	CAG	GAC	TTA	GCA	AGA	AGT	TAT	GGA	ATT	CCT
	141									150
CKi-*ras*2-76	TTT	ATT	GAA	ACA	TCA	GCA	AAG	ACA	AGA	CAG
	151									160
cKi-*ras*2-76	GGT	GTT	GAT	GAT	GCC	TTC	TAT	ACA	TTA	GTT
	161									170
cKi-*ras*2-76	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG
	171									180
cKi-*ras*2-76	AGC	AAA	GAT	GGT	AAA	AAG	AAG	AAA	AAG	AAG
	181							188		
cKi-*ras*2-76	TCA	AAG	ACA	AAG	TGT	GTA	ATT	ATG	TAA	

(B)

	1									10
cKi-*ras*2-76	Met	Thr	Glu	Tyr	Lys	Leu	Val	Val	Val	Gly
	11									20
SW-11-1		Val								
cKi-*ras*2-76	Ala	Gly	Gly	Val	Gly	Lys	Ser	Ala	Leu	Thr
vKi-*ras*		Ser								
	21									30
cKi-*ras*2-76	Ile	Gln	Leu	Ile	Gln	Asn	His	Phe	Val	Asp
	31									40
cKi-*ras*2	Glu	Tyr	Asp	Pro	Thr	Ile	Glu	Asp	Ser	Tyr
	41									50
cKi-*ras*2-76	Arg	Lys	Gln	Val	Val	Ile	Asp	Gly	Glu	Thr
	51									60
cKi-*ras*2-76	Cys	Leu	Leu	Asp	Ile	Leu	Asp	Thr	Ala	Gly
vKi-*ras*									Thr	
	61									70
cKi-*ras*2-76	Gln	Glu	Glu	Tyr	Ser	Ala	Met	Arg	Asp	Gln
	71									80
cKi-*ras*2-76	Tyr	Met	Arg	Thr	Gly	Glu	Gly	Phe	Leu	Cys
	81									90
cKi-*ras*2-76	Val	Phe	Ala	Ile	Asn	Asn	Thr	Lys	Ser	Phe
	91									100
cKi-*ras*2-76	Glu	Asp	Ile	His	His	Tyr	Arg	Glu	Gln	Ile
vKi-*ras*										Leu
	101									110
cKi-*ras*2-76	Lys	Arg	Val	Lys	Asp	Ser	Glu	Asp	Val	Pro
	111									120
cKi-*ras*2-76	Met	Val	Leu	Val	Gly	Asn	Lys	Cys	Asp	Leu
	121									130
cKi-*ras*2	Pro	Ser	Arg	Thr	Val	Asp	Thr	Lys	Gln	Ala
	131									140
cKi-*ras*2-76	Gln	Asp	Leu	Ala	Arg	Ser	Tyr	Gly	Ile	Pro
vKi-*ras*		Glu								
	141									150
cKi-*ras*2-76	Phe	Ile	Glu	Thr	Ser	Ala	Lys	Thr	Arg	Gln
	151									160
cKi-*ras*2-76	Gly	Val	Asp	Asp	Ala	Phe	Tyr	Thr	Leu	Val
vKi-*ras*	Arg		Glu							
	161									170
cKi-*ras*2-76	Arg	Glu	Ile	Arg	Lys	His	Lys	Glu	Lys	Met
vKi-*ras*					Gln	Tyr	Arg	Leu		Lys
	171									180
cKi-*ras*2-76	Ser	Lys	Asp	Gly	Lys	Lys	Lys	Lys	Lys	Lys
vKi-*ras*	Ile	Ser	Lys	Glu	Glu		Thr	Pro	Gly	Cys
	181								189	
cKi-*ras*2-76	Ser	Lys	Thr		Lys	Cys	Val	Ile	Met	
vKi-*ras*	Val		Ile	Lys						

FIG. 2. (A) Nucleotide sequences of the coding regions of cDNAs corresponding to the Ki-*ras*2 proto-oncogene (cKi-*ras*2-76) and the Ki-*ras*2 transforming allele present in SW480 (SW-11-1). The sequence of SW-11-1 is indicated only where it differs from the cKi-*ras*2-76 sequence. (B) Predicted amino acid sequences of the p21s encoded by SW-11-1, cKi-*ras*2-76, and KiSV (vKi-*ras*). The sequences of the transforming p21s (SW-11-1, vKi-*ras*) are indicated only where they differ from the normal p21. The sequence of the vKi-*ras* gene was determined by Tsuchida et al. (30).

segment, is present in the cDNAs. This alternative fourth exon is represented as well in the KiSV genome (23) but is not used in translation because of its position downstream of a termination codon. These data confirm the work of others (1, 23) published during the course of these studies.

DISCUSSION

We previously identified three distinct oncogenes activated in three different human tumor cell lines. These oncogenes were present in the EJ bladder carcinoma cell line, the SW480 colon carcinoma cell line, and the HL-60 promyelocytic leukemia cell line (15). It is now apparent that these three distinct oncogenes are related to one another in that they are all members of the *ras* gene family (5, 12, 14, 17, 19, 24). In the EJ bladder carcinoma cell line, we and others have shown that the cHa-*ras*1 gene has been activated via a point mutation that alters the encoded p21. The glycine residue present at amino acid 12 in the normal gene is replaced by a valine residue in the transforming gene (18, 27, 29). In the present report, we have shown that the Ki-*ras*2

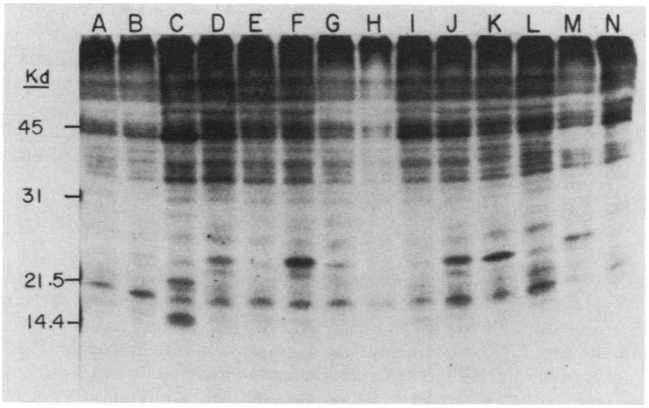

FIG. 3. Immunoprecipitation of p21 from transfectant cell lines. ^{35}S-labeled lysates of cells were prepared as described in the text and immunoprecipitated with either normal rat serum (lanes A, C, E, H, I, L, and N) or p21 monoclonal Y13-259 (7) (lanes B, D, F, G, J, K, and M). The immunoprecipitated lysates were subjected to electrophoresis through a 15% sodium dodecyl sulfate-polyacrylamide gel and fluorographed as described in the text. Lysates were from NIH 3T3 cells (lanes A and B), secondary transfectant SW-2-3 (lanes C and D), transfectants containing the pCD transforming allele plasmid (lanes E, F, I, J, M, and N), transfectants containing the pCD normal allele plasmid (lanes G and H), and transfectant containing a plasmid in which the normal allele is transcribed off of a Moloney murine leukemia virus long terminal repeat (lanes K and L).

gene in the SW480 colon carcinoma cell line is activated in precisely the same manner. Work by others on the Ki-*ras*2 allele in the human lung carcinoma cell line Calu 1 indicates that the associated Ki-*ras*2 oncogene is activated via a point mutation that altered the amino acid residue present at position 12. However, in this instance, the transforming allele encodes a cysteine residue at amino acid 12 (1, 23). Thus, it appears that the mutations in the codon for amino acid 12 are often the activating lesion in the *ras* oncogenes present in human tumors. This is not, however, the only site whose alteration can lead to oncogenic activation. Others have shown that in the human lung carcinoma cell line Hs 242, the Ha-*ras*1 gene has been activated via an alteration that changes the glutamine residue normally present at position 61 in the p21 protein to a leucine residue in the transforming p21 (33). In addition, studies of two N-*ras* oncogenes by others and ourselves have found point mutations in residue 61, leading to a substitution of lysine (28) and a leucine (J. M. Cunningham and R. A. Weinberg, unpublished data) in place of the normally present glutamine.

Additional experiments, not described here, investigated the transforming activity of the normal Ki-*ras*2 cDNA when its transcription was driven by the Moloney murine leukemia virus long terminal repeat, a potent transcriptional promoter. These experiments suggest that even very high levels of the normal p21 protein are able at best to induce marginal morphological transformation and weak tumorigenicity (one of six mice developed tumors). These experiments confirm earlier work showing that structural alteration of the p21 is more important for oncogenic activation than are high levels of expression (2, 4, 27). Thus, we believe that the amino acid 12 alteration alone is sufficient for the conversion of the Ki-*ras*2 proto-oncogene into an active oncogene.

Finally, we have shown that the cKi-*ras*2 gene contains two alternative fourth exons. One exon, which others have termed 4A (1, 23), encodes carboxy-terminal amino acids virtually identical to those present in KiSV. The other exon, termed 4B, encodes C-terminal amino acids very different from those present in the 4A exon and in KiSV. It appears that the cKi-*ras*2 gene encodes two p21s which differ only at their carboxy termini. Indeed, RNA blots hybridized with probes specific for the 4A and 4B exons indicate that both exons are present in RNA (data not shown). This result is in agreement with data previously published by others (1).

Our data suggest that expression of only one of the two alternative fourth exons, the 4B exon, is sufficient for the ability to induce foci on monolayers of NIH 3T3 cells and to form tumors in mice. The foci induced by the oncogene cDNA construct are only readily seen 17 to 20 days after transfection, and these foci are induced at a rate of approximately 400 per μg of transfected cloned DNA. This contrasts with the behavior of the cloned Ha-*ras* bladder carcinoma oncogene that yields approximately 10^4 foci per μg of cloned DNA, these foci being already apparent 10 to 14 days posttransfection. We are unable to provide an explanation of this reproducible difference in behavior. In contrast to results reported by others (1), we do not find that the presence of both fourth exons is required for focus formation.

In conclusion, our data and those of others (1, 13, 23)

(A)

	151									160
vKi	AGA	GTG	GAG	GAT	GCT	TTT	TAT	ACA	TTG	GTG
ϕ9 vKi homology (4A)	AGA	GTG	GAG	GAT	GCT	TTT	TAT	ACA	TTG	GTG
cDNAs (4B)	GGT	GTT	GAT	GAT	GCC	TTC	TAT	ACA	TTA	GTT
	161									170
vKi	AGA	GAG	ATC	CGA	CAG	TAC	AGA	TTA	AAA	AAA
ϕ9 vKi homology (4A)	AGA	GAG	ATC	CGA	CAA	TAC	AGA	TTG	AAA	AAA
cDNAs (4B)	CGA	GAA	ATT	CGA	AAA	CAT	AAA	GAA	AAG	ATG
	171									180
vKi	ATC	AGC	AAA	GAA	GAA	AAG	ACT	CCT	GGC	TGT
ϕ9 vKi homology (4A)	ATC	AGC	AAA	GAA	GAA	AAG	ACT	CCT	GGC	TGT
cDNAs (4B)	AGC	AAA	GAT	GGT	AAA	AAG	AAG	AAA	AAG	AAG
	181								189	
vKi	GTA	AAA	ATT	AAA	AAA	TGC	GTT	ATA	ATG	TAA
ϕ9 vKi homology (4A)	GTG	AAA	ATT	AAA	AAA	TGC	ATT	ATA	ATG	TAA
cDNAs (4B)	TCA	AAG	ACA	AAG		TGT	GTA	ATT	ATG	TAA

(B)

	151									160
vKi	Arg	Val	Glu	Asp	Ala	Phe	Tyr	Thr	Leu	Val
4A	Arg	Val	Glu	Asp	Ala	Phe	Tyr	Thr	Leu	Val
4B	Gly	Val	Asp	Asp	Ala	Phe	Tyr	Thr	Leu	Val
	161									170
vKi	Arg	Glu	Ile	Arg	Gln	Tyr	Arg	Leu	Lys	Lys
4A	Arg	Glu	Ile	Arg	Gln	Tyr	Arg	Leu	Lys	Lys
4B	Arg	Glu	Ile	Arg	Lys	His	Lys	Glu	Lys	Met
	171									180
vKi	Ile	Ser	Lys	Glu	Glu	Lys	Thr	Pro	Gly	Cys
4A	Ile	Ser	Lys	Glu	Glu	Lys	Thr	Pro	Gly	Cys
4B	Ser	Lys	Asp	Gly	Lys	Lys	Lys	Lys	Lys	Lys
	181								189	
vKi	Val	Lys	Ile	Lys	Lys	Cys	Val	Ile	Met	TER
4A	Val	Lys	Ile	Lys	Lys	Cys	Ile	Ile	Met	TER
4B	Ser	Lys	Thr	Lys		Cys	Val	Ile	Met	TER

FIG. 4. (A) Nucleotide sequence of the alternative fourth exons present in the cKi-*ras*2 gene: the 4A exon, which represents the ϕ9 vKi-*ras* homologous sequences, and the 4B exon, which is present in the cDNAs, as compared with the equivalent region of vKi-*ras* gene present in KiSV. (B) Predicted amino acid sequences of the carboxy termini of the p21 proteins encoded by the KiSV *ras* gene (vKi) and the cKi-*ras*2 4A and 4B exons.

leave two as yet unexplored hypotheses. First, although the bulk of the oncogene transcripts serve as template for synthesis of an exon 4B-encoded terminus, it is possible that concomitant expression of small amounts of the p21 protein carrying a 4A-encoded terminus is important for the phenotype induced by the uncloned oncogene present in the colon carcinoma cells. Second, alterations within the promoter or the introns of this 40-kb gene may have effects on its ability to transform cells.

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LITERATURE CITED

1. **Capon, D. J., P. H. Seeberg, J. P. McGrath, J. S. Hayflick, U. Edman, A. D. Levinson, and D. V. Goeddel.** 1983. Activation of Ki-*ras* 2 gene in human colon and lung carcinomas by two different point mutations. Nature (London) **304**:507–513.
2. **Chang, E. H., M. E. Furth, E. M. Scolnick, and D. R. Lowy.** 1982. Tumorigenic transformation of mammalian cells induced by a normal gene homologous to the oncogene of Harvey murine sarcoma virus. Nature (London) **297**:479–483.
3. **Cooper, G. M.** 1982. Cellular transforming genes. Science **217**:801–806.
4. **DeFeo, D., M. A. Gonda, H. A. Young, E. H. Chang, D. R. Lowy, E. M. Scolnick, and R. W. Ellis.** 1981. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **78**:3328–3332.
5. **Der, C., T. G. Krontiris, and G. M. Cooper.** 1982. Transforming genes of human bladder and lung carcinoma cell lines are homologous to the *ras* genes of Harvey and Kirsten sarcoma viruses. Proc. Natl. Acad. Sci. U.S.A. **79**:3637–3640.
6. **Ellis, R. W., D. DeFeo, T. Y. Shih, M. A. Gonda, H. A. Young, N. Tsuchida, D. R. Lowy, and E. M. Scolnick.** 1981. The p21 *src* genes of Harvey and Kirsten sarcoma viruses originate from divergent members of a family of normal vertebrate genes. Nature (London) **292**:506–511.
7. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick.** 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular *ras* gene family. J. Virol. **43**:294–304.
8. **Hanahan, D., and M. Meselson.** 1980. A protocol for the high density screening of plasmids in X1776. Gene **10**:63–67.
9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
10. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, and D. Quon.** 1978. The isolation of structural genes from libraries of eukaryotic DNA. Cell **15**:687–701.
11. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing and end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
12. **McCoy, M. S., J. J. Toole, J. M. Cunningham, E. H. Chang, D. R. Lowy, and R. A. Weinberg.** 1983. Characterization of a human colon/lung carcinoma oncogene. Nature (London) **302**:79–81.
13. **McGrath, J. P., D. J. Capon, D. H. Smith, E. Y. Chen, P. Y. Seeberg, D. V. Goeddel, and A. D. Levinson.** 1983. Structure and organization of the human Ki-*ras* proto-oncogene and a related processed pseudogene. Nature (London) **304**:501–506.
14. **Murray, M. J., J. M. Cunningham, L. F. Parada, F. Dautry, P. Lebowitz, and R. A. Weinberg.** 1983. The HL-60 transforming sequence: a *ras* oncogene coexisting with altered *myc* genes in hematopoietic tumors. Cell **33**:749–757.
15. **Murray, M. J., B. Shilo, C. Shih, D. Cowing, H. W. Hsu, and R. A. Weinberg.** 1981. Three different human tumor cell lines contain different oncogenes. Cell **25**:355–361.
16. **Okayama, H., and P. Berg.** 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol. Cell. Biol. **3**:280–289.
17. **Parada, L. F., C. J. Tabin, C. Shih, and R. A. Weinberg.** 1982. Human EJ bladder carcinoma oncogene is homologue of Harvey sarcoma virus *ras* gene. Nature (London) **297**:474–478.
18. **Reddy, E. P., R. K. Reynolds, E. Santos, and M. Barbacid.** 1982. A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature (London) **300**:149–152.
19. **Santos, E., S. R. Tronick, S. A. Aaronson, S. Pulciani, and M. Barbacid.** 1982. T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB and Harvey-MSV transforming genes. Nature (London) **298**:343–347.
20. **Schwarzbauer, J. E., J. W. Tamkun, I. R. Lemischka, and R. O. Hynes.** 1983. Three alternative fibronectin mRNAs arise by alternative splicing within the coding region. Cell **35**:421–431.
21. **Seed, B.** 1983. Purification of genomic sequences from bacteriophage libraries by recombination and selection in vivo. Nucleic Acids Res. **11**:2427–2445.
22. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1979. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. **31**:546–556.
23. **Shimizu, K., D. Birnbaum, M. A. Ruley, O. Fasano, Y. Suard, L. Edlund, E. Taparowsky, M. Goldfarb, and M. Wigler.** 1983. The structure of the K-*ras* gene of the human lung carcinoma cell line Calu-1. Nature (London) **304**:497–500.
24. **Shimizu, K., M. Goldfarb, Y. Suard, M. Perucho, Y. Li, T. Kamata, J. Feramisco, E. Stavnezer, J. Fogh, and M. H. Wigler.** 1983. Three human transforming genes are related to the viral *ras* oncogenes. Proc. Natl. Acad. Sci. U.S.A. **80**:2112–2116.
25. **Skinner, M. K., and M. D. Griswold.** 1983. Fluorographic detection of radioactivity with polyacrylamide gels with 2.5-diphenyloxazole in acetic acid and comparison with existing procedures. Biochem. J. **209**:281–284.
26. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
27. **Tabin, C. J., S. M. Bradley, C. I. Bargmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, D. R. Lowy, and E. H. Chang.** 1982. Mechanisms of activation of a human oncogene. Nature (London) **300**:143–149.
28. **Taparowsky, E., K. Shimizu, M. Goldfarb, and M. Wigler.** 1983. Structure and activation of the human N-*ras* gene. Cell **34**:581–586.
29. **Taparowsky, E., Y. Suard, O. Fasano, K. Shimizu, M. Goldfarb, and M. Wigler.** 1982. Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. Nature (London) **300**:762–765.
30. **Tsuchida, N., T. Ryder, and E. Ohtsubo.** 1982. Nucleotide sequence of the oncogene encoding the p21 transforming protein of Kirsten murine sarcoma virus. Science **217**:937–939.
31. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
32. **Weinberg, R. A.** 1982. Oncogenes of spontaneous and chemically induced tumors. Adv. Cancer Res. **36**:149–163.
33. **Yuasa, Y., S. Srivastava, C. Dunn, J. Rhim, E. Reddy, and S. A. Aaronson.** 1983. Acquisition of transforming properties by alternative point mutations within the *cbas1has* human proto-oncogene. Nature (London) **303**:775–779.