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Genetics

Mutations in *ras* genes in cells cultured from mouse skin tumors induced by ultraviolet irradiation

(point mutation/focus-forming activity/transversion)

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ABSTRACT **Mutations in *ras* oncogenes were detected in cultured cells of mouse skin tumors induced by near-UV irradiation. DNA extracted from the UV-induced tumor cells was transfected to golden hamster embryo cells, and focus-forming ability was confirmed in 22 of 26 cell strains, 15 of which had the repetitive mouse sequence. Mouse *ras* genes were detected in 10 of these 22 cell strains. Point mutations in the *ras* genes were at Ha-*ras* codon 13 (GGC → GTC in two strains, GGC → AGC in one strain), Ki-*ras* codon 61 (CAA → GAA in two strains), and N-*ras* codon 61 (CAA → CAT in two strains, CAA → AAA in two strains). In one tumor cell strain no base change was detected. Most mutations occurred at dipyrimidine sites. Pyrimidine dimers or pyrimidine(6–4)pyrimidone photoproducts are the likely cause of the skin cancers. The base change occurred preferentially at G·C base pairs, and transversions predominated.**

Solar UV light is important in the development of melanoma (1) and nonmelanoma skin cancers (2) in sun-exposed areas of human skin. Cyclobutane pyrimidine dimer and pyrimidine(6–4)pyrimidone photoproducts, which have cytotoxic and mutagenic effects, are the major types of DNA damage induced by far UV (3). The action spectrum for skin carcinogenesis is in the near-UV (UVB) range (4), but the nature and repair of the primary types of DNA damage caused by UVB *in vivo* have been little investigated. Unrepaired or misrepaired UV-induced DNA damage that leads to mutations and alterations in DNA structure during replication may trigger carcinogenesis if it occurs in specific target genes.

Members of the *ras* gene family (c-Ha-*ras*, c-Ki-*ras*, and N-*ras*) are activated in human colon (5) and skin (6) cancers, as well as in animal skin cancers induced by chemicals (7, 8). In human skin cancers, point mutations in codon 61 of the N-*ras* gene have been reported in patients with xeroderma pigmentosum who show defective repair of UV damages (9, 10) as well as in skin cancer patients without xeroderma pigmentosum (11).

Point mutation in codons 12, 13, and 61 of the *ras* genes have been found preferentially in tumor cells that have activated transforming ability. Such mutations are suggested to be a cause of *ras*-related carcinogenesis, including skin cancers, or at the least as being part of the multistage process in carcinogenesis (12). We produced skin tumors on the backs of hairless mice using UVB irradiation (13). We report here results of detailed molecular analyses of the activation of and mutations in *ras* genes of cells cultured from UVB-induced mouse skin tumors. The cause–effect relationship in the development of skin cancers also is discussed.

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MATERIALS AND METHODS

Induction of UVB-caused tumors and establishment of the tumor cell strains have been described (13). Specific-pathogen-free albino hairless mice were irradiated with UVB light from six sun lamps (Toshiba FL-20 SE).

Extraction of DNA from Tumors and Tumor Cells. High-molecular-weight genomic DNA was extracted as described (14) from frozen tumor tissue samples and from cultured cells of those tumors. DNA extracted from the livers of the tumor-bearing mice was used as the normal control.

Focus-Forming Assay. The golden hamster embryo (GHE) cells used for transfection were provided by Fumio Suzuki (Kanazawa University) (15). GHE cells (7×10^5) were seeded on each of 10-cm Petri dishes and then transfected with 30–33 μg of tumor cell DNA by calcium phosphate coprecipitation (16), the medium thereafter being changed every 3 days. After 3 weeks the number of transformed foci was scored, and some were cloned and expanded for subsequent analysis. Only densely stained clear foci were scored, and essentially no such foci appeared in the background. Genomic DNA from T24 bladder cell carcinoma cells (17) was the positive control for efficient transforming activity. The cloned transformed cells were denoted by the serial number of the tumor from which the DNA had been extracted—e.g., TF31 that was transformed by the DNA from tumor cell HL31.

Southern Blot Analysis. DNA from the GHE cells transformed by DNA from the UV-induced skin tumor cells was digested with restriction endonuclease, *Eco*RI or *Bam*HI, as recommended by the manufacturer. The digested DNA was electrophoresed in a 0.7% agarose gel, then transferred, and blotted on a nylon membrane (GeneScreen*Plus*; DuPont) as described (18). DNA was fixed to the membrane by exposure to UV light (1.6 kJ/m^2) from germicidal lamps (Toshiba), after which it was hybridized with probes labeled with [α-^{32}P]dCTP (Amersham) by using a Multiprime labeling kit (Amersham) and then washed according to the manufacturer's instructions. The membrane was dried and exposed for 24–48 hr to Kodak XAR-5 film at −70°C with intensifying screens.

The probes were inserts purified from plasmids v-Ha-*ras* (Takara Shuzo, Kyoto) (19), v-Ki-*ras* (Takara Shuzo) (20), pSPT-Nras-cDNA (human N-*ras*) (21), and p7014 (ref. 22; provided by H. Yamagishi, Kyoto University), which contains four kinds of repetitive mouse sequences.

DNAs isolated from the tumors and tumor cells were digested with *Xba* I, *Pst* I, *Xho* I, *Taq* I, or *Ava* II, electrophoresed on 0.5% agarose gels, then transferred to nylon membranes, and hybridized to the [α-^{32}P]dCTP-labeled Ha-

Abbreviations: RFLP, restriction endonuclease fragment length polymorphism; GHE; golden hamster embryo; UVB, near UV.

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ras probe. These restriction endonucleases are considered to show the point mutations in Ha-*ras* codons 12, 13, and 61 that produce restriction endonuclease fragment length polymorphisms (RFLPs) (7).

DNAs from the tumors and tumor cells also were digested with *Bam*HI, *Eco*RI, or *Hin*dIII. These fragments were hybridized with the v-Ha-*ras*, v-Ki-*ras*, N-*ras*, v-*myc*, v-*erbB*, v-*src*, v-*abl*, or v-*fos* probe by a procedure similar to that used for the DNAs from the transformants.

PCR-Mediated Amplification. Tumor DNA encompassing codons 12, 13, and 61 of Ha-*ras*, Ki-*ras*, and N-*ras* genes was amplified *in vitro* by PCR as described (23). The oligonucleotide primers were Ha-*ras* 12/13-61, 5′-GACAGAATAACAAGCTTGTGG-3′ and 5′-CGCATGTACTGGTCCCGCAT-3′; Ki-*ras* 12/13, 5′-GACTGAGTATAAACTTG-3′ and 5′-CTATCGTAGGGTCGTACTCA-3′; Ki-*ras* 61, 5′-CTCCTACAGGAAACAAGTAG-3′ and 5′-CACAAAGAAAGCCCTCCCCA-3′; and N-*ras* 12/13, 5′-GACTGAGTACAAACTGGTGG-3′ and 5′-CTCTATGGTGGGATCATATT-3′; and N-*ras* 61, 5′-GGTGAGACCTGCCTGCTGGA-3′ and 5′-ATACACAGAGGAACCCTTCG-3′.

One microgram of the genomic DNA and 0.2 μM each of the two primers were added to a reaction mixture containing 1 μM each of dNTP and 1 unit of Ampli*Taq* (Cetus). Thirty cycles of denaturation (94°C, 1 min), annealing (58°C, 1.5 min), and extension (72°C, 2.5 min) were done in an automated DNA thermal cycler (PC-700; Astec, Fukuoka, Japan). The PCR-amplified products from each tumor were run on a 5% polyacrilamide gel, after which fragments of the predicted sizes were isolated and subjected to direct nucleotide sequencing and RFLP analysis.

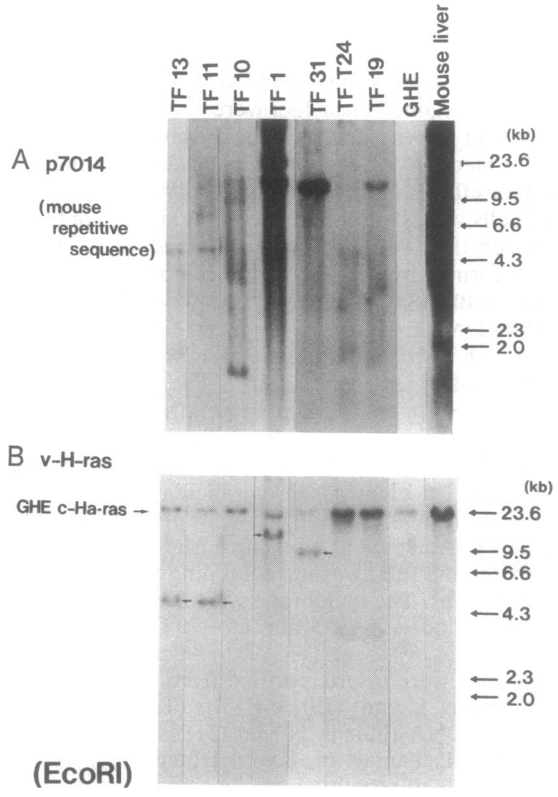

FIG. 1. Detection of repetitive mouse sequence (*A*) and Ha-*ras* (*B*) in transformed GHE cells transfected with DNA from tumor cells. DNA (10 μg) was digested with *Eco*RI and then hybridized by Southern blotting with ^{32}P-labeled random-primed repetitive mouse sequence probe p7014 (*A*) or v-Ha-*ras* probe (*B*). Lanes from left to right: TF13, TF11, TF10, TF1, TF31, TFT24, TF19, GHE cells, normal mouse liver. The filter in *A* was washed to remove probe p7014 and then rehybridized with v-Ha-*ras* probe. A middle-sized arrow indicates the GHE or mouse endogenous Ha-*ras* gene; the small arrows indicate the extra bands of mouse Ha-*ras*.

DNA Sequencing. The mutations were analyzed by direct sequencing of the PCR products. DNA fragments containing codons 12, 13, and/or 61 of the *ras* genes were amplified by PCR using tumor cell DNA. This was followed by DNA sequencing with a Tth DNA polymerase DNA sequencing kit version 2.0 (Toyobo, Osaka), a modification of the dideoxynucleotide chain-terminator method (24), as recommended by the manufacturer. The sequence primer (10 pmol) was end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. The synthetic oligonucleotide primers used in the sequencing were Ha-*ras* 12/13, 5′-CTCTATAGTGGGATCATAC-3′; Ha-*ras* 61, 5′-GACTCCTACCGGAAACAGG-3′; Ki-*ras* 12/13, 5′-CTATCGTAGGGTCGTACTCA-3′; Ki-*ras* 61, 5′-CTCCTACAGGAAACAAGTAG-3′; N-*ras* 12/13, 5′-CTCTATGGTGGGATCATATT-3′; and N-*ras* 61, 5′-ATACACAGAGGAACCCTTCG-3′. Samples were electrophoresed on a 6% polyacrylamide/7 M urea gel and then autoradiographed.

Confirmation of Point Mutation by RFLP. The RFLP test was used to detect base changes in the point mutations that create new restriction sites detectable by a restriction enzyme. The G·C → T·A mutation in codon 13 was confirmed by digestion at 37°C for 6 hr of amplified Ha-*ras* fragments with the restriction enzyme *Hin*fI, after which the digest was electrophoresed in 5% polyacrylamide gels. The C·G → G·C mutation in codon 61 was confirmed by digesting the amplified Ki-*ras* fragments with restriction enzyme *Mbo* II at 37°C for 6 hr and then electrophoresing the digest in 8% polyacrylamide gels. The A·T → T·A mutation of codon 61 was confirmed by digestion of the amplified N-*ras* fragments with restriction enzyme *Nla* III at 37°C for 6 hr and then electrophoresing the digest in 8% polyacrylamide gels.

RESULTS

Transforming Activity of DNA from UV-Induced Skin Tumor Cells and Presence of Mouse *ras* Sequences. We measured the yield of foci in GHE cells as a function assay for screening tumors that might have point mutations in their oncogenes. Of 26 tumor cell strains established from UVB-induced mouse skin tumors, 22 had transforming activity when transfected to GHE cells. The efficiency of transformation varied from 0.3 to 114.0 foci per 100 μg of DNA. The efficiency was

Table 1. Transforming activity of DNA from UV-induced mouse skin tumor cells and the mutations in *ras* oncogenes

Tumor cell line	Focus-forming efficiency, no. of foci per 100 μg of DNA		Activated *ras*	*ras* mutation	
	Primary	Secondary		Position	Mutation
HL1	2.4	3.3	Ha-*ras*	13	GGC(Gly) → GTC(Val)
HL5	1.0	8.0	N-*ras*	61	CAA(Gln) → AAA(Lys)
HL6	1.2	5.0	N-*ras*	61	CAA(Gln) → CAT(His)
HL9	5.5	23	Ki-*ras*	61	CAA(Gln) → GAA(Glu)
HL10	1.8	2.5	N-*ras*	61	CAA(Gln) → AAA(Lys)
HL11	1.0	0.5	Ha-*ras*		
HL13	0.87	25.5	Ha-*ras*	13	GGC(Gly) → AGC(Ser)
HL18	1.5	0.25			
HL19	1.0	5.6	N-*ras*	61	CAA(Gln) → CAT(His)
HL21	1.5	3.2			
HL31	4.5	114	Ha-*ras*	13	GGC(Gly) → GTC(Val)
HL34	9.0	2.7			
HL35	0.71	0.3			
HL41	0.38	1.6	Ki-*ras*	61	CAA(Gln) → GAA(Glu)
HL43	1.3	2.1			

not quantitatively comparable due to fluctuation in repeated experiments, but data clearly indicated the positive transforming activity because background was essentially zero. DNA extracted from T24 bladder cell carcinoma cells was used as the positive control, the transforming efficiency of these cells being 4.0 foci per 100 μg of DNA for primary transformants and 16.7 foci per 100 μg of DNA for secondary ones. DNA extracted from the livers of five mice bearing UVB-induced skin tumors lacked transforming activity.

The GHE cells transformed with DNA from the tumors were tested for the repetitive mouse sequence p7014 (Fig. 1*A*). Fifteen of 22 cell strains that showed various intensities of p7014 are listed in Table 1. Southern blots with the positive repetitive mouse sequence are shown in Fig. 1*A*. GHE cells transformed with DNA extracted from human bladder carcinoma cells (TF T24) do not have the mouse sequence, but normal mouse cells show a heavy smear, as expected. When the mouse sequence was stripped off and the membrane was rehybridized with v-Ha-*ras* gene probe, mouse Ha-*ras* gene was identified (Fig. 1*B*). Bands at the top of the membrane probably represent the endogenous GHE c-Ha-*ras* gene as well as the full size of the *Eco*RI fragment of the mouse c-Ha-*ras* genes, as the sizes of the *Eco*RI-digested fragments resemble those reported for genomic mouse Ha-*ras* genes (20, 25). *Bam*HI digestion identified the GHE Ha-*ras* and mouse *ras* bands separately (data not shown).

The presumably activated mouse Ha-*ras* gene (arrows in Fig. 1*B*) was present in four transformed cell strains: TF1, TF11, TF13, and TF31. Similar tests were done for the Ki-*ras* and N-*ras* genes, the two Ki-*ras* and four N-*ras* genes appearing in presumably activated forms in GHE cells transformed with DNA of cultured cells from UVB-induced mouse skin tumors. Table 1 lists the 15 cell strains that yielded secondary transformants in which the mouse sequence was identified; extra *ras* gene bands appear in the fourth column. Other activated oncogenes than *ras* genes—such as *myc*, *erbB*, *src*, *abl*, and *fos*—were sought, but none were positively identified in the transformants (data not shown).

Digestion with *Bam*HI, *Eco*RI, or *Hin*dIII endonucleases detected no amplification, deletion, or rearrangement of the Ha-*ras*, Ki-*ras*, N-*ras*, v-*myc*, v-*erbB*, v-*fos*, v-*src*, and v-*fos* genes in the original tumor tissues and tumor cells. Nor was RFLP detected by the use of *Taq* I, *Ava* II, *Pst* I or *Xho* I, which detect certain mutations in codon 12 or 61, evidence that no mutation of G → C existed at position 1 or 2 of codon 12, and no mutation of A → G, A → C, or A → T existed at position 2 of codon 61 in the Ha-*ras* gene (7).

Mutations in *ras* Genes in UVB-Induced Skin Tumor Cells. DNA sequences in the *ras* genes of tumor cells identified as having activated mouse *ras* genes are shown in Fig. 2. PCR was done with mouse tumor cells rather than transformed GHE cells because the same primers amplify golden hamster *ras* sequences of similar size. DNA from 10 tumor cell strains with focus-forming activity in GHE cells was analyzed for mutated genes. Three cell strains had one mutation each in the Ha-*ras* gene, two strains had one mutation each in the Ki-*ras* gene, and four strains had one mutation each in the

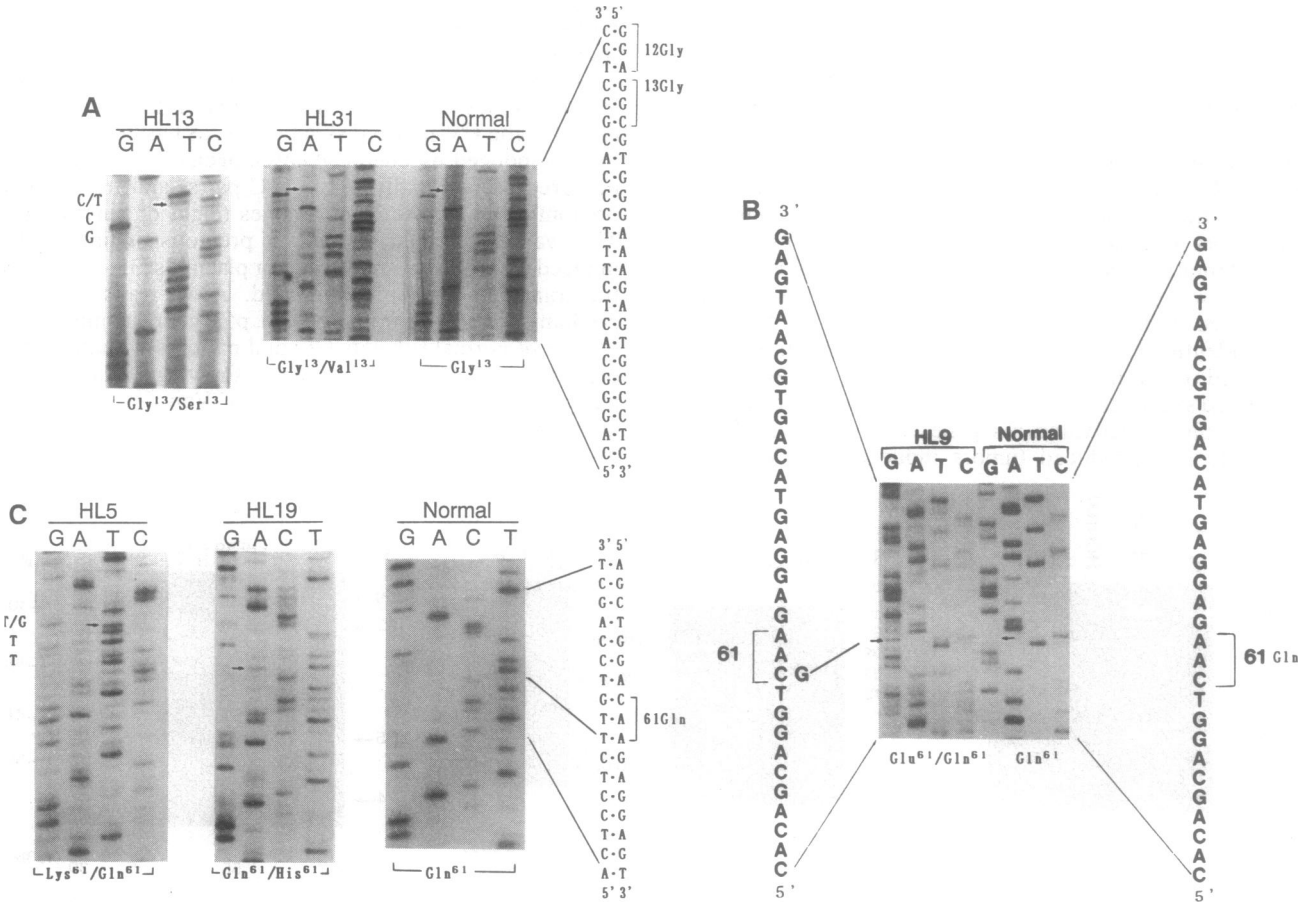

FIG. 2. Mutations in Ha-*ras* (*A*), Ki-*ras* (*B*), and N-*ras* (*C*) genes in UVB-induced tumor cells with focus-forming activity. (*A*) A transversion (G·C → T·A) and a transition (G·C → A·T) (arrows) in codon 13 are present, respectively, in the Ha-*ras* gene of HL31 and HL13 tumor cells. (*B*) A transversion (C·G → G·C) in codon 61 is present in the Ki-*ras* gene of HL9 and HL41. (*C*) A transversion (A·T → T·A) is present in codon 61 in HL6 and HL19, and transversions (C·G → A·T) (arrows) in codon 61 are present in the N-*ras* genes of HL5 and HL10. The coding strand was the sequence primer for Ki-*ras*, and the noncoding strand was the primer for Ha-*ras* and N-*ras*. Arrows indicate the substituted bases (HL cells) or their normal counterparts.

N-*ras* gene. One cell strain (TF11) had no detectable mutations in the sequences encompassing codons 11–26 and 48–66, which include codons 12, 13, and 61 of the Ha-*ras* sequences, despite confirmation of an extra band of the mouse Ha-*ras* gene.

Two types of mutation could be identified in the Ha-*ras* gene; one cell strain (HL13) with the GGC → AGC mutation and two cell strains (HL1 and HL31) with the GGC → GTC mutation in codon 13 (Fig. 2*A*). Similarly, two cell strains (HL9 and HL41) had the CAA → GAA mutation in codon 61 of the Ki-*ras* gene (Fig. 2*B*). Two types of mutations were identified: one in two cell strains (HL5 and HL10) with the CAA → AAA mutation and the other in two cell strains (HL6 and HL19) with the CAA → CAT mutation. Both were in codon 61 of the N-*ras* gene (Fig. 2*C*).

Of the five types of mutation shown in Fig. 2, three that show an additional cleavage site in each of the mutated codons can be identified by RFLP analysis. Differences in RFLP in the normal and mutated *ras* genes for restriction enzymes *Hin*fI, *Mbo* II and *Nla* III are shown in Fig. 3 *A–C*, which respectively represent the Ha-*ras* (GGC → GTC), Ki-*ras* (CAA → GAA), and N-*ras* (CAA → CAT) genes.

For tumors 5, 10, and 13, confirmation of the mutations by RFLP analysis was impossible because the changes did not cause new restriction sites. Another method of confirming base changes is to sequence the other DNA strand. In our experimental system, however, it was not possible to sequence the other strand corresponding to the mutated sites because the mutated sites and the locations of the available primers were too close to obtain any sequence information.

DISCUSSION

ras oncogene activations in UVB-induced mouse skin tumors detected by a focus-forming assay that uses GHE cells were followed by the detection of repetitive mouse sequence and extra-*ras* bands with Southern blot analyses. The presence of presumably activated mouse *ras* genes with extra bands in GHE cells transformed with DNA from UVB-induced mouse skin tumors was confirmed in 10 of 22 transformed cell strains. Mutations in one codon (codon 12, 13, or 61) of the *ras* genes were identified in 9 of the 10 GHE cell strains transformed with mouse-activated *ras* (Table 1). Most reports of identified mutations in *ras* genes in skin tumors have been based on dot hybridization in which the probes carried the mutated sequences. Our approach was to analyze in detail the mutations in activated *ras* genes by sequencing DNA from artificially produced tumors induced by UVB. Our method has two main advantages. It confirms the functionally activated state by use of the focus-forming assay, and it identifies the mutations by direct sequencing.

In cell line HL11, no mutations were found in the Ha-*ras* gene in the sequences encompassing codons 11–26 and 48–66, which include codons 12, 13, and 61, despite the clear presence of mouse Ha-*ras* with an extra band. Undetected changes at other sites or a rearrangement such as that involving the insertion of a promoter (26) or truncation could have caused the activation of Ha-*ras* without a point mutation in the activation-related codons.

Five types of base changes were found: two types in Ha-*ras*, one in Ki-*ras*, and two in N-*ras*. Three are the same as previously reported in tumors: CAA → CAT in human or mouse N-*ras* codon 61 (11, 27, 28), CAA → AAA in human and mouse N-*ras* codon 61 (27–29), and GGC → GTC in mouse Ha-*ras* codon 13 (30). The last type, GGC → GTC, has been found in high frequency in human Ha-*ras* codon 12 in skin tumors on sun-exposed areas (11). Possible involvement of UV exposure also has been suggested in the CAA → CAT in N-*ras* codon 61 in a melanoma from a xeroderma pigmentosum patient (10) and in mouse melanoma induced by UVB (27) or 7,12-dimethylbenz[*a*]anthracene plus UVB (26), as well as in the CAA → AAA in N-*ras* codon 61 from mouse squamous cell carcinoma induced by UVB (27), dermal nevi induced by 7,12-dimethylbenz[*a*]anthracene plus UVB (28), and a plasmid exposed to UVC (31). One mutation in codon 13 of Ha-*ras*, GGC → AGC, is the same as that reported in the transformation of NIH 3T3 cells by a site-directed-mutagenized Ha-*ras* gene (32). One mutation in codon 61 of Ki-*ras*, CAA → GAA, has not been reported in tumor or transformed cells.

No specific prevalent gene (Ha-, Ki-, or N-*ras*) or prevalent mutation site was shown by our results for codons 12, 13, and 61 in the UV-induced skin tumors, as has been shown for tumors induced by chemical carcinogens (7, 30). Mutations occurred preferentially at the G·C pairs, and transversions predominated for the base changes (eight of nine) (Fig. 4). This was unexpected because of previous findings of UV-induced mutations in shuttle vector plasmids (33–35) in which the transition type predominated. Also, transitions were predominant in the mutations in p53 gene in human skin tumors of normal subjects (36) and patients with xeroderma pigmentosum (37). The only report showing a relatively high frequency of transversions is that of Van der Lubbe *et al.* (31), who found that introduction of an *in vitro* UV-irradiated N-*ras* gene to Rat2 cells produced transformed cells with

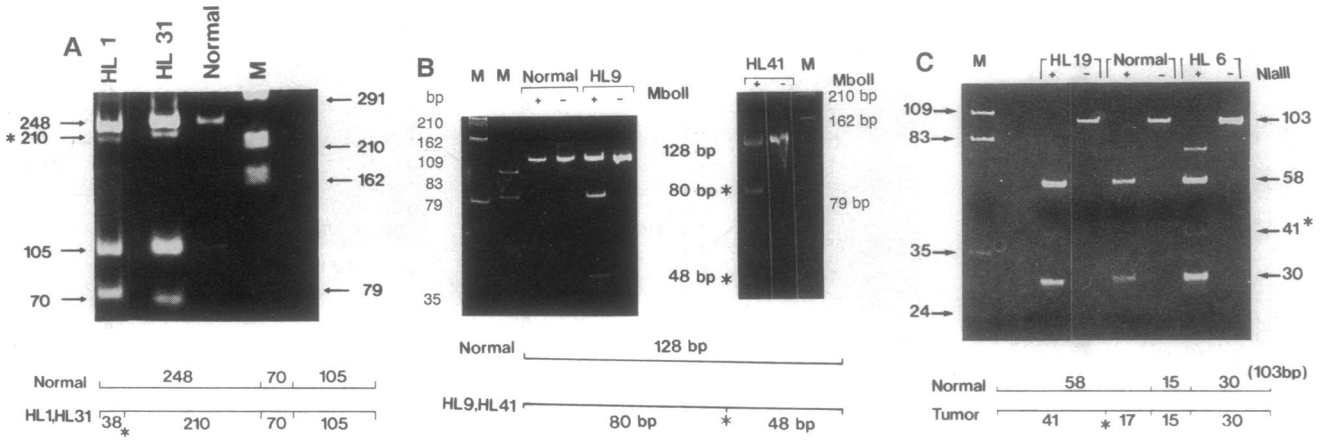

FIG. 3. RFLP confirmation of the mutated sequence. (*A*) RFLP with *Hin*fI for the Ha-*ras* fragments encompassing codons 1–73, showing a new cleavage site in HL1 and HL31 cells. *, Additional site produced by the point mutation. Lane M, size marker ϕX174/*Hin*cII digest. (*B*) RFLP with *Mbo* II for the Ki-*ras* fragment encompassing codons 38–81. Normal DNA yields one band of 128 bp, whereas HL9 and HL41 yield two bands (80 and 48 bp) because of the new cleavage site (*) for *Mbo* II. Lanes M, size marker ϕX174/*Hin*cII digest. (*C*) Transversions in HL6 and HL19 in codon 61 of the N-*ras* gene shown by the new cleavage site (*) at *Nla* III.

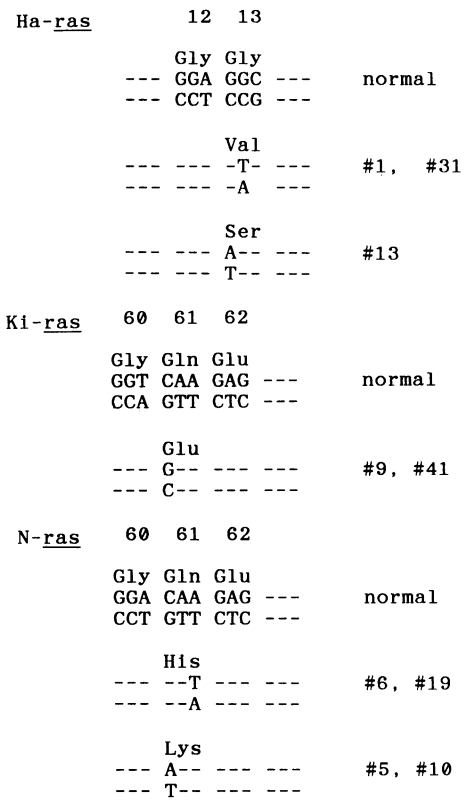

FIG. 4. Base changes detected in mouse tumor cells with positive focus-forming activity.

mutations resembling those found in our tumors, 8 out of 14 (57%) being transversions. The predominance of transversions could relate to changes in *ras* genes that produced the transformed characteristics by which the foci were selected.

Another mechanism could involve formation of 8-hydroxyguanine by the indirect action of UVB, owing to its activation of UV-absorbing biomolecules in the cells. When 8-hydroxyguanine is formed, a G·C base pair would yield a T·A base pair through the pairing of 8-hydroxyguanine with adenine (38). Such changes would explain at least four mutations, those in Ha-*ras* of HL1 and HL31 and in the N-*ras* of HL5 and HL10 (Fig. 4). GC → TA transversions have been found in some mutations in human *ras* genes identified in cancers in sun-exposed skin areas (11).

Formation of pyrimidine dimers or pyrimidine(6–4) pyrimidone photoproducts also could explain the base changes found in mutated sequences, as in the Ha-*ras* codon 13 of HL13 cells—the only transition-type mutation we found. Our results differ markedly from those of *in vitro* experiments done with shuttle vector genes, presumably because of the more complex conditions that exist *in vivo* than *in vitro*. The indirect action of UV action may be less in shuttle vectors in a suspension solution or in cultured cells than under the conditions *in vivo*.

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