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Human Hypervariable Sequences in Risk Assessment: Rare Ha-*ras* Alleles in Cancer Patients

by Theodore G. Krontiris,*† Nancy A. DiMartino,‡ H. David Mitcheson,§ Joyce A. Lonergan,*† Colin Begg,|| and David R. Parkinson*

A variable tandem repeat (VTR) is responsible for the hyperallelism one kilobase 3′ to the human c-Ha-*ras*-1 (Ha-*ras*) gene. Thirty-two distinct restriction fragments, comprising 3 allelic classes by frequency of occurrence, have thus far been detected in a sample size of approximately 800 caucasians. Rare Ha-*ras* alleles, 21 in all, are almost exclusively confined to the genomes of cancer patients ($p < 0.001$). From our data we have computed the relative cancer risk associated with possession of a rare Ha-*ras* allele to be 27.

To understand the molecular basis for this phenomenon, we have begun to clone Ha-*ras* fragments from nontumor DNA of cancer patients. We report here the weak activation, as detected by transfection and transformation of NIH 3T3 mouse cells, of two Ha-*ras* genes which were obtained from lymphocyte DNA of a melanoma patient. We have mapped the regions that confer this transforming activity to the fragment containing the VTR in one Ha-*ras* clone and the fragment containing gene coding sequences in the other.

Introduction

Several recent studies have reported that a collection of rare allelic fragments at the highly polymorphic c-Ha-*ras*-1 (Ha-*ras*) locus appeared only in cancer patients (*1–3*). This association has not been obtained in studies of patients with preleukemia (*4*), lung cancer (*5*), or melanoma (*6*). In an expanded population survey, we have now sampled approximately 1600 alleles by Southern blot analysis of DNA from 424 cancer-free controls and 369 cancer patients with a wide variety of tumors. Three groups of fragments (common, intermediate, and rare) have been defined by allelic frequency. We have again observed an increased prevalence of rare alleles in cancer patients that is highly significant ($p < 0.001$). A family history of cancer in our controls has no apparent influence on this result. From these data we have calculated the relative risk of cancer in individuals possessing a rare Ha-*ras* allele to be 27, which is comparable to the relative risk of cigarette smoking (*7*). Since 10% of all cancer patients demonstrated such alleles, the Ha-*ras* locus may have general application in risk assessment.

To understand the molecular basis for the anomalous population behavior of Ha-*ras* alleles, we have begun to clone these genes from the normal tissue of cancer patients. If rare Ha-*ras* alleles represent abnormal genes, we should be able to characterize the defect(s). Such an outcome would demonstrate rigorously that inherited lesions of oncogenes occur that are responsible for an increased lifetime risk of developing cancer. Furthermore, we could then exploit this knowledge to refine strategies for predicting risk. We report here our initial investigation of the transforming activity possessed by Ha-*ras* genes cloned from the lymphocytes of a familial melanoma patient.

*Department of Medicine, Hematology/Oncology, Tufts-New England Medical Center, Boston, MA 02111.
†Department of Molecular Biology and Microbiology, Tufts-New England Medical Center, Boston, MA 02111.
‡Department of Medicine, Hematology/Oncology, Veterans Administration Medical Center and Boston University School of Medicine, Boston, MA 02115.
§Department of Urology, Tufts-New England Medical Center, Boston, MA 02111.
||Dana-Farber Cancer Institute and Harvard School of Public Health, Boston, MA 02115.

Material and Methods

Study Population

As before, our study population consisted of unrelated caucasians with and without cancer. The tumors were carcinomas of the head and neck (approximately 1% of cases); lung (5%); breast (16%); gastrointestinal tract (5%), including esophagus, stomach, pancreas, liver,

(5%), including esophagus, stomach, pancreas, liver, colorectum; genitourinary tract (22%), including kidney, bladder, prostate, testis; and female reproductive tract (8%), including ovary, uterus, cervix, endometrium; as well as lymphoma (18%); melanoma (4%); sarcoma (2%); and myeloma (1%). Acute and chronic leukemia, of lymphocytic and nonlymphocytic origin, accounted for 11%; brain tumors accounted for 2%, nonmelanoma skin cancers for 3%, and tumors of unknown primary for 2%. Thirty ethnic/nationality groups were equally represented in both the cancer and control populations. We have shown that the distribution of rare alleles was not influenced by this factor, since the fraction of rare alleles contributed by any one ethnic group was proportional to the representation of that group in cancer patients (*2*). In this study we have expanded the data from cancer-free controls to include those with a positive history of cancer in first degree relatives.

Ha-*ras* Genotype Determination

Leukocyte and tumor DNAs were prepared and digested with MspI/HpaII as previously described (*1,2*). Samples were subjected to electrophoresis through 0.7% agarose gels. Southern transfer (*8*), hybridization to Ha-*ras* probe, washing, and autoradiography were performed as before (*1,2*). Fragment sizes were determined by coeletrophoresis of HindIII-digested lambda DNA, and a1, a2, a3, and a4 markers (the four most common Ha-*ras* alleles) on each gel. After this preliminary electrophoresis, a putative new allele was run against previous isolates of similar size. This process confirmed the existence of a new allele and, at the same time, allowed the relative ordering of all the allelic fragments, even though the determination of fragment length was only reproducible to ± 50 bp. Thus, fragment lengths given below represent interpolations.

Cloning of Ha-*ras* Alleles

DNA was purified from EBV-transformed lymphocytes of a patient with familial melanoma (*1*). Following digestion with BamHI, the DNA was ligated into the BamHI site of the lambdoid bacteriophage, L47.1 (*9*) and packaged using a commercial extract (Gigapack, Stratagene). Recombinant phage was plated on a P2 lysogen, and plaques were screened directly by the method of Benton and Davis (*10*). The BamHI fragment of Ha-*ras* from the plasmid pEC (*11*) was nick-translated (*12*) for probe.

DNA Transfection

For direct transfection, a precipitate of 1000 ng of plasmid DNA containing the patient's Ha-*ras* fragments and 20 μg of carrier NIH 3T3 DNA was applied to 5×10^5 NIH 3T3 cells in a 60-mm tissue culture dish following the method of Graham and van der Eb (*13*). pEJ (33.3 ng), which contained the activated Ha-*ras* gene from the EJ bladder carcinoma cell line (*14*), was applied with carrier as a positive control. Cotransfection of Ha-*ras* clones and pSV2neo (*15*) was performed as follows: 0.8 μg of pSV2neo + 1 μg of an Ha-*ras* plasmid were coprecipitated as above and incubated with 5×10^5 NIH 3T3 cells per 60-mm dish. Two days after transfection, selection with the antibiotic G418 (Geneticin, GIBCO), at 400 μg/mL, was begun. Ten days after transfection, each plate was trypsinized and the cells transferred to a 100-mm tissue culture dish. Two to three weeks after transfection, the proportion of transformed cells (fusiform, refractile morphology) was noted.

Construction of Ha-*ras* Chimers

The patient's two allelic fragments, a1 and a2.1, were subcloned into the BamHI site of pBR322. The resulting plasmids were designated pGDa1 and pGDa2.1, respectively. For the construction of chimeric clones involving various fragments of pGDa1, pGDa2.1, pEC, and pEJ, plasmids were digested with NotI, which released a 2.5-kb fragment containing the four coding exons. The NotI fragment was exchanged among the dif-

Table 1. Ha-*ras* allelic frequencies.[a]

Allele	Detected in family member	MspI/HpaII size (bp)	No. detected	Frequency
a1	Yes	1000	975	0.612
a2	Yes	1500	192	0.121
a3	Yes	2160	154	0.097
a4	Yes	2560	140	0.088
a1.2	Yes	1110	34	0.021
a1.1	Yes	1060	16	0.010
a4.1	Yes	2610	11	0.007
a0.1		980	8	0.005
a1.3		1230	8	0.005
a1.4		1450	7	0.004
a5		2800	7	0.004
a3.2*	Yes	2280	4	0.002
a2.2*	Yes	1820	3	0.002
a2.3	Yes	1880	3	0.002
a2.4		2100	3	0.002
a3.1*	Yes	2220	3	0.002
a3.3*	Yes	2330	3	0.002
a3.4*		2410	3	0.002
a1.35*		1280	2	0.001
a2.01*		1560	2	0.001
a3.5*		2480	2	0.001
a4.2*		2710	2	0.001
a0.15*		880	1	0.0006
a0.2*	Yes	810	1	0.0006
a1.25*		1180	1	0.0006
a2.015*		1590	1	0.0006
a2.02*		1650	1	0.0006
a2.025*		1680	1	0.0006
a2.1*	Yes	1710	1	0.0006
a2.11*		1750	1	0.0006
a2.12*		1790	1	0.0006
a5.2*		3080	1	0.0006
			Total 1592	

[a]To accommodate the new intermediate-frequency allele, a1.4, the cancer-associated rare allele, previously called a1.4, was reassigned the designation a1.35. Rare alleles were detected in patients' first degree relatives in the nine instances in which family members were available for testing (seven rare alleles). Asterisks denote alleles found only in cancer patients.

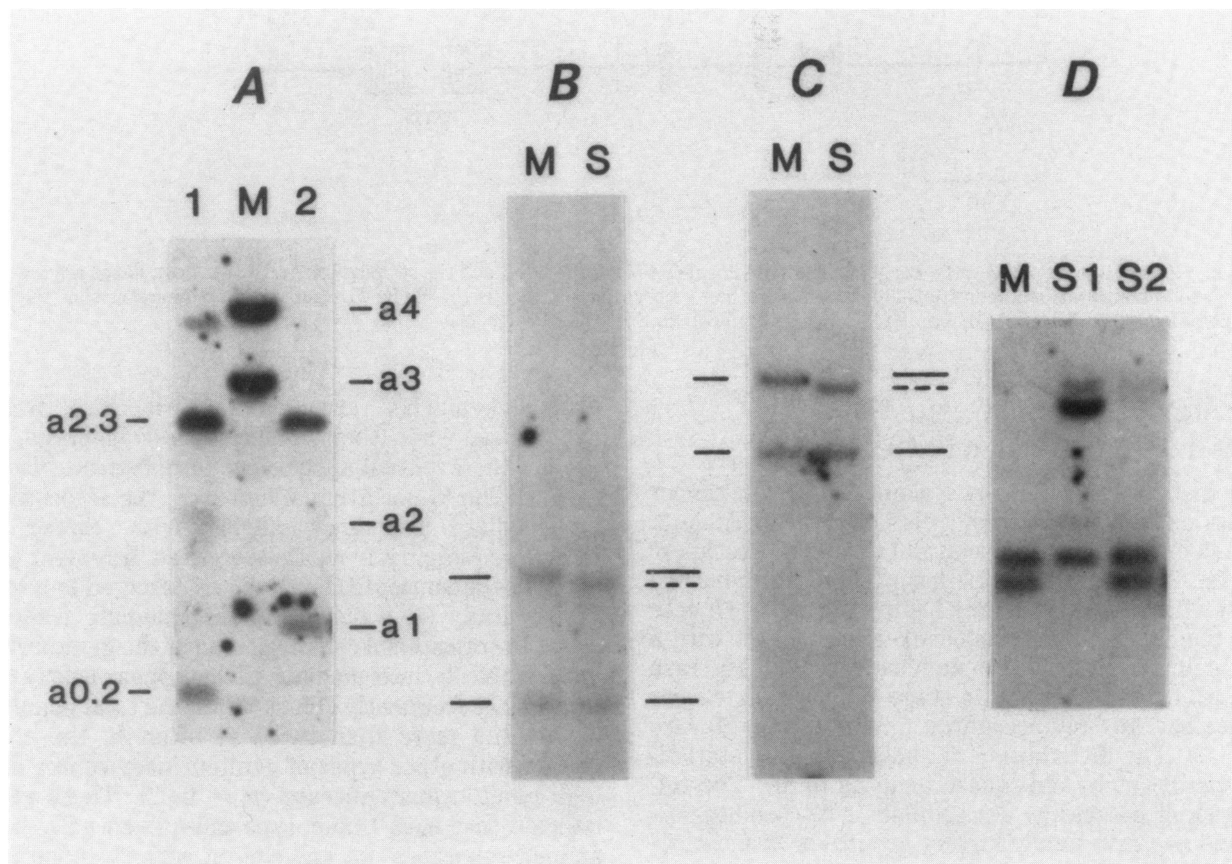

FIGURE 1. Resolution of Ha-*ras* allelic fragments. WBC DNAs were digested with MspI/HpaII (*A*, *B*, and *C*) or PstI (*D*), blotted and hybridized to Ha-*ras* probe. Bars in *B* and *C* represent the migration of markers; dotted line is the migration of patients' intermediate or rare alleles. (*A*) Rare a0.2 and a2.3 alleles in two members of a melanoma kindred (lanes 1 and 2). M is a marker DNA with a3 and a4 alleles. Migration of a1 and a2 alleles, from another gel lane not included in the photo, is also indicated. (*B*) M is a marker DNA with a1 and a2 alleles; S is TCC patient WBC DNA with a1.4 allele. (*C*) M is a marker DNA with a3 and a4 alleles; S is TCC patient WBC DNA with a3.5 allele. (*D*) PstI confirmatory digest. M is marker DNA with a1 and a1.2 alleles. S1 and S2 are WBC DNA samples from two TCC patients with a1.2 alleles by MspI/HpaIII.

to determine the influence of coding sequences on transforming activity of the patient clones. To determine the influence of the VTR, the SphI fragment containing this structure was exchanged. Finally, to differentiate the influence of coding exons 1/2 versus exons 3/4, NcoI/BglII fragments were exchanged (see "Results" and Fig. 2).

Results

VTR Polymorphism at the Ha-*ras* Locus

The remarkable degree of hyperallelism at the Ha-*ras* locus is evident, as shown in Table 1. Thirty-two fragments, in three allelic classes by frequency of occurrence, have been detected thus far. As previously noted (*1*,*2*), Ha-*ras* polymorphism is generated by the variation of a tandemly repetitive sequence, designated the VTR, 3′ to the gene coding exons. For the most part, rare alleles are easily distinguished from their common counterparts (Fig. 1*A*). However, very minor variations, or microheterogeneity, can be reproducibly demonstrated with the appropriate technical precautions (Fig. 1*B*,*C*) (*2*). The isoschizomers MspI/HpaII, which produce the greatest resolution of polymorphic fragments, are required; several other enzymes, AvaI (*4*), PvuII (*5*), and BamHI (*16*), produce successively larger fragments and are correspondingly insensitive. [Any of these enzymes, or PstI, can be used to confirm that rare alleles are not the result of partial digestion with, or site polymorphism of, MspI/HpaII (Fig. 1*D*).] Since minor differences in salt concentration of electrophoresed samples can produce artifactual migration differences, internal migration controls, such as those denoted by the solid bands in panels *B* and *C* of Figure 1, are required. Mixing of DNA samples and coelectrophoresis can be performed, although this is not usually necessary because informative internal control bands are nearly always present in the samples being tested. Finally, sample DNA concentrations are carefully monitored, since both overloading and inequality between samples generate irreproducible variation (*4*).

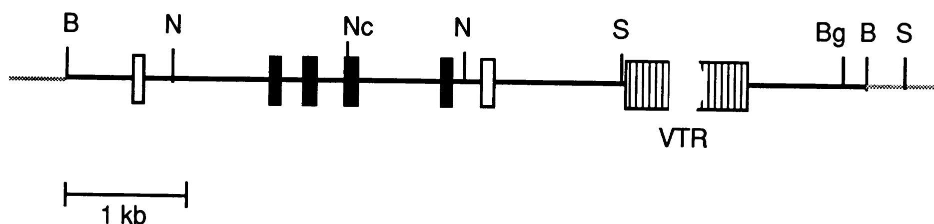

FIGURE 2. Restriction map of the Ha-*ras* gene. The solid line denotes Ha-*ras* DNA and the stippled line, pBR322. Open boxes are noncoding exons; solid boxes are the four translated exons. The box with vertical stripes is the VTR. The gap within it indicates that the size is variable (from 800–3100 bp). B, BamHI, N, NotI, Nc, NcoI, S, SphI, Bg, BglII.

Rare Ha-*ras* Alleles Occur Nearly Exclusively in Cancer Patients

The distribution of Ha-*ras* allelic classes in cancer patients and cancer-free controls is shown in Table 2. Rare alleles are almost exclusively confined to cancer patients. Thus far, only a2.3 has occurred in a normal control with a negative family history of cancer. Of note is the finding that even cancer-free individuals with a positive family history have demonstrated but one rare allele, a2.4. At a comparable stage of collection, cancer patients had already accounted for more than 20 rare alleles (*2*). The distribution of alleles is highly statistically significant by chi-square analysis (6 df). The relative risk of possessing a rare allele is 27 (pooling positive and negative family history groups) or 36 (controls without family history of cancer).

Distribution of Ha-*ras* Alleles in Bladder Cancer Patients

In the population-at-large, intermediate frequency alleles are not associated with cancer to a statistically significant degree. Within particular subgroups, however, patterns may vary. Most of the unusual Ha-*ras* alleles we detected in a small group of myelodysplastics (*1*) have proved to be of intermediate frequency. As more of these patients were accumulated, the association of Ha-*ras* alleles with disease has not been maintained (*4* and unpublished observations). Interestingly, several of these myelodysplastics who progressed to frank leukemia had rare alleles (*1*). Therefore, rare Ha-*ras* alleles, when they occur in myelodysplasia, may predict the eventual appearance of malignant disease.

In certain tumor groups, however, the association of intermediate frequency alleles with cancer was stronger. Judging from the reported fragment sizes, many of the unusual Ha-*ras* alleles detected in a breast cancer study (*3*) were in the intermediate frequency class. In transitional cell carcinoma of the genitourinary tract (Table 3), intermediate alleles appear nearly three times more frequently (16.4%) than the total population (5.7%) and more than twice as often as the control groups with other types of genitourinary cancer or benign genitourinary disease ($p = 0.02$). These results indicate that each tumor type must eventually be examined separately for association with rare and intermediate Ha-*ras* alleles.

Table 2. Distribution of Ha-*ras* allelic subgroups in normal individuals and cancer patients.[a]

DNA source	Common (a1–a4)	Intermediate (a1.2–a.5)	Rare (a3.2–a5.2)	Total
Cancer patient WBC	481	38	31	550
Cancer-free WBC				
Family history negative	540	33	1	574
Family history positive	266	7	1	274
Patient tumors	174	13	7	194

[a]The distribution of alleles in WBC and tumor DNAs is statistically significant (chi-square, 6 df) to $p < 0.001$. The rare allele in the family-history-negative group is a2.3; in the family-history-positive group, a2.4.

Analysis of a Unique Ha-*ras* Allele by Molecular Cloning

We have cloned BamHI fragments containing the Ha-*ras* transcriptional controls, coding sequences, and variable tandem repeat from the normal DNA of a melanoma patient belonging to a kindred with the dysplastic

Table 3. Ha-*ras* alleles in genitourinary (GU) malignancy.[a]

WBC DNA from patients with	Common	Intermediate	Rare
Transitional cell cancer (61)	96	20	6
Other GU malignancy (42)	77	4	3
Benign GU disease (35)	65	5	0

[a]Leukocyte (WBC) DNA from peripheral blood of patients with transitional cell carcinoma (TCC; two from the kidney, the remainder bladder), other GU malignancy (prostate, renal, and testicular) and benign conditions of the GU tract (prostatic hypertrophy, infections, infertility, impotence and renal calculi) were prepared and blotted as described in Table 1. Numbers in parentheses represent total patients in each group. All intermediate alleles were detected in TCC patients; only a1.1 and a1.2 were detected in the other two groups. Rare alleles in TCC patients were a2.4, a3.4, a3.5, and a5.2. Rare alleles in other GU tumor patients were a2.01, a2.2, and a3.4. The proportion of rare alleles in each category conforms roughly to that expected from Tables 1 and 2. Statistical significance of the distribution of intermediate alleles ($p = 0.02$) was computed by chi-square analysis from the 2 by 3 table in which common + rare alleles were compared to intermediate alleles in each of the three patient groups.

nevus syndrome and cutaneous melanoma (*1*). The patient's brother had melanoma, and a niece presented with metastatic ovarian carcinoma at age 22. All these individuals share an Ha-*ras* fragment that is unique to the family. From DNA of EBV-transformed lymphocytes of the patient, GD, we have obtained his common a1 allele, as well as the unique a2.1 allele (see Table 1). The BamHI fragments have been subcloned into pBR322 and have been designated pGDa1 and pGDa2.1, respectively. Our structural characterization of these nontumor clones thus far has localized the major difference between them to the VTR, as expected. Furthermore, fine structure mapping with purified VTRs from both clones indicates that the entire region is composed of 28 bp tandem repeats. Consequently, at this level of resolution, no DNA has been inserted or transposed into the VTR (data not shown).

Transforming Activity of Patient's Ha-*ras* Genes

Activation of *ras* protooncogenes in tumors occurs by the frequent mutation of codons 12 and 61 (*17*). Other mutations, as well as changes which augment transcription, can activate Ha-*ras* (*17*), although the phenotype in these cases is usually far less dramatic than that of 12/61 mutations. When *ras* genes are activated, they become capable of tumorigenic transformation of growth-regulated cell lines, and the immortalization of normal diploid cell strains (*17*). Therefore, we wished to determine if the Ha-*ras* genes we had cloned from normal tissue of the melanoma patient demonstrated any evidence of lesions which could activate the protooncogene.

To this end, the plasmid subclones containing the common and unique Ha-*ras* fragments of the melanoma patient were used to transfect NIH 3T3 cells. In the standard assay, 3 μg plasmid DNA + 60 μg carrier is precipitated and incubated with cells in three 60-mm culture dishes; 500,000 cells per dish. The dishes are incubated for 2 weeks and transformed foci are counted. Under such conditions, neither patient clone transformed NIH 3T3 cells (Table 4).

For further studies on spontaneous and induced transformation of cells bearing a1 and a2.1 fragments, the same subclones were cotransfected with a selectable marker, pSV2neo (1.7 μg *neo* + 2.0 μg *ras* clone + 36.3 μg carrier precipitated and incubated with cells from two 60-mm dishes). Plates transfected only with pSV2neo were also included. In this way, we intended to introduce the apparently phenotypically "silent" patient genes into NIH 3T3 cells as passengers with pSV2neo, which conferred resistance to the antibiotic

Table 4. Direct transfection of patient Ha-*ras* clones.

Ha-*ras* DNA	Total foci/no. plates
pEJ	575/3
pGDa1	0/3
pGDa2.1	0/3

Table 5. Cotransfection with pSV2neo.

	Primary transfectants		After cell transfer	
Ha-*ras* DNA	Colonies/ plate	Colonies transformed	Plates transformed/ total plates	Extent of transformation
None	335	None	0/2	0
pGDa1	410	None	2/2	1+
pDGa2.1	300	None	2/2	2+

[a] 1+ = 25% transformed; 2+ = 50% transformed.

Table 6. Cotransfection with pSV2neo and pEC.

	Primary transfectants		After cell transfer	
Ha-*ras* DNA	Colonies/ plate	Colonies transformed	Plates transformed/ total plates	Extent of transformation[a]
None	350–400	None	0/2	0
pEC	350–400	None	0/2	0
pGDa1	350–400	None	2/2	2+
pGDa2.1	350–400	None	2/2	2+

[a] 2+ = 50% transformed.

G418. When resistant colonies of NIH 3T3 cells first appeared (50–100 cell stage), the plates were trypsinized. Cells were replated at limiting dilution to obtain cell clones that were resistant to G418 and, potentially, contained one or a few copies of either a1 or a2.1. We subsequently have checked by Southern blotting of DNA from the cloned NIH 3T3 sublines that this cotransfer did indeed occur. At the same time, the remainder of the cells from this transfection were replated and allowed to become confluent.

The results of this first cotransfection are presented in Table 5. Although cells transfected only with pSV2neo remained flat, 25 to 50% of cells transfected with either the common a1 or unique a2.1 fragment became transformed under the conditions of the assay. This level of transformation represented a relatively high frequency event and should not be viewed as the simple comparison of 0/2 and 2/2 events. We have two independent confirmations of this assertion. First, all 10 NIH 3T3 subclones we have examined by Southern blotting (5 of which are transformed) are independent transfectants. No common bands are observed except for the expected 2.9-kb SacI fragment containing Ha-*ras* coding sequences (data not shown). Second, several subclones were chosen because their originating colonies from limiting dilution were flat. These were maintained by passaging at subconfluence until Southern blotting confirmed one to two copies of a1 or a2.1. Then 10,000 cells from each of three sublines (*neo* only, *neo* + a1 and *neo* + a2.1) were plated and allowed to become confluent. At 2 weeks, both the a1- and a2.1-containing clones had several hundred transformed foci per plate. The *neo* control demonstrated none. Thus, cell clones bearing either of the patient's Ha-*ras* genes exhibited high frequency spontaneous transformation.

Because the normal Ha-*ras* gene will transform NIH 3T3 cells at low efficiency if gene expression is augmented by increased transcription or gene amplification, we wished to rule out the possibility that our result

was simply the artifactual activation of normal Ha-*ras* clones. This seemed unlikely, since the amount of cloned Ha-*ras* DNA required to produce this effect is more than an order of magnitude greater than that of our protocol. Also, several transformants do not have amplified Ha-*ras* by Southern blot analysis. However, the pSV2neo we employed contains the SV40 enhancer, so it was possible that increased transcription was obtained by the cotransfection. Therefore, we repeated the experiment described above with two changes. First, no limiting dilution was performed because clones were not desired. Second, another cotransfection pair was added: pSV2neo + the nontransforming Ha-*ras* protooncogene, pEC. This plasmid is a normal Ha-*ras* BamHI fragment cloned into pBR322. Once again, cotransfection of a1 and a2.1 led to transformation of NIH 3T3 cells (Table 6). Cells from both the pSV2neo control and the cotransfection involving the protooncogene clone pEC remained untransformed. Therefore, the transformation phenomenon was specific to clones from the patient. Since these clones were obtained from his lymphocyte DNA, not his tumor DNA, we have isolated Ha-*ras* genes bearing potentially inherited lesions. This result, including transfection with pEC, has now been reproduced in multiple experiments.

Mapping the Regions Responsible for Transforming Activity in pGDa1 and pGDa2.1

Finally, we have constructed a series of chimeric plasmids in which the coding sequences, promoter/exon (− 1) and VTR of a2.1, a1, and pEC have been recombined. These exchanges have allowed us to map the region of each Ha-*ras* clone responsible for the transforming activity detected by cotransfection. In this set of experiments, each chimer was transfected directly or cotransfected with pSV2neo. pEC controls were always present. The results to date are summarized in Table 7. The transforming activity of a2.1 resided in the VTR-containing fragment. For a1, the relevant region was the coding sequences, particularly the fragment containing exons 3 and 4. For a1, an unusual coding sequence mutation may, therefore, exist. The constructs involving pEC indicated that all regions of this clone may be activated by the appropriate regions of a1 and a2.1. Hence, our negative control was not artifactually inactive in these assays as the result of some unknown lesion we introduced during, say, pEC DNA purification. Additional information from the chimer experiments was the finding that the a2.1 VTR was active in both orientations, suggesting an enhancerlike function.

Table 7. Chimer transfections.[a]

Promotor/ exon (−1)	Coding sequences	VTR	Transforming activity[b] Direct	Transforming activity[b] Cotransfection
Exchanges of BamHI-NotI, NotI-NotI, and NotI-BamHI fragments				
EJ	EJ	EJ	3–4+	4+
EC	EC	EC	0	0
a2.1	a2.1	a2.1	0	2+
a1	a1	a1	0	1–2+
a1	a2.1	a1	0	0
a1[c]	a2.1	a2.1	0	2–3+
EC	a1	EC	0	2+
a2.1	EC	a2.1	0	3+
a2.1	a1	a2.1	0	2–3+
Exchanges of SphI fragments, which involve only the VTR				
EC	EC	a2.1	ND	2–3+
EC[d]	EC	a2.1	ND	2–3+
Exchange of NcoI-BglII fragments, which result in the separation of exons 1/2 from exons 3/4				
EC	EC(1/2) a1(3/4)	a1	ND	2–3+
a1	a1(1/2) EC(3/4)	EC	ND	0–1+

[a] Transfections were performed as described in the text. pEC negative control was included in each set of assays (a total of six separate experiments).
[b] 0 = no transformed cells; 1+ = 25% transformed; 2+ = 50% transformed; 3+ = 75% transformed; 4+ = 100% transformed; ND = not done.
[c] SphI fragment containing the a2.1 VTR was exchanged for the a1 VTR in the chimer a1/a2.1/a1.
[d] Denotes a clone in which the orientation of the VTR has been reversed.

Discussion

The basis for the association we have detected, rare Ha-*ras* alleles in cancer patients, remains obscure. Distinct underlying mechanisms may be responsible in different tumors. We have observed segregation of the disease locus and Ha-*ras* in a Von Hippel Lindau kindred (*2* and unpublished observations) and in families with the dysplastic nevus syndrome/melanoma or familial melanoma (T.G.K. and S.J. Bale, National Cancer Institute, unpublished; *6*). Nevertheless, in 19 such melanoma families, we have detected 7 rare alleles (Fig. 1) (*1*). This would imply that tandem repeat instability, not Ha-*ras*, demonstrates the principal association with familial melanoma. Such instability may secondarily affect Ha-*ras* genes: As we have reported, both the common and unique Ha-*ras* fragments from lymphocyte DNA of a familial melanoma patient demonstrate weak transforming activity in NIH 3T3 cells.

Whether similar results will be obtained in sporadic cancer cases remains to be determined. If Ha-*ras* genes linked to rare VTRs accelerate tumor progression, then linkage should eventually be detected in sporadic cancer. If an entirely distinct mechanism, genetic instability leading to the increased frequency of appearance of rare VTRs, is the basis for our observations, then one might expect an increased rate of appearance of new alleles at VTR loci distinct from Ha-*ras*. Although such did not occur at a VTR locus we have studied on chromsome 10 (*2*), other hypervariable region probes may prove more informative. Since nontumor Ha-*ras* clones from the familial melanoma patient display weak activation, despite lack of Ha-*ras* linkage to the melanoma

gene even within this kindred, we have hypothesized that Ha-*ras* genes upstream from their unstable VTR element may suffer "innocent bystander" lesions during the generation of new VTRs or recombination within/nearby common VTRs (as with our a1 clone). Alternatively, the creation of a new VTR may be associated with some effect on Ha-*ras* expression, such as mRNA stabilization or transcriptional enhancement (as with our a2.1 clone). In sporadic cancer, where no familial disease locus predominates, such lesions may be central to the governance of genetic risk. Further investigation of patient Ha-*ras* clones, combined with a population analysis of tandem repeats throughout the human genome, should clarify these issues.

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