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Allele-specific Deletion in Exon I of the HRAS1 Gene

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Summary

We have detected a 6-bp deletion in the untranslated first exon of a unique HRAS1 gene cloned from lymphocyte DNA of a familial melanoma patient. The deletion is without apparent functional consequence. Using an RNase protection assay, we have demonstrated the deletion in leukocyte DNAs of individuals unrelated to the patient. In these cases, the deletion marker is specifically associated with one class of common HRAS1 allele, thereby establishing the origin of the unique allele. We discuss the means by which DNA sequence heterogeneity at other loci may be rapidly analyzed.

Introduction

The protooncogene, c-Ha-*ras1*, encodes a 21,000-dalton GTPase which has been implicated in mitogenic signal transduction (Barbacid 1987). c-Ha-*Ras1* genes bearing "activating" somatic mutations in codon 12, 13, or 61 are capable of tumorigenic transformation of certain cell types, either alone (Reddy et al. 1982; Tabin et al. 1982; Taparowsky et al. 1982) or in combination (Land et al. 1983; Ruley 1983; Thompson et al. 1989) with other oncogenes. The occurrence of c-Ha-*ras1* mutations in a number of human tumors suggests a role for this gene in the pathogenesis of human malignancies.

The human c-Ha-*ras1* locus, HRAS1, is highly polymorphic because of a variable tandem repeat (VTR; minisatellite) located 1,000 bp 3′ to the gene's polyadenylation signal (Capon et al. 1983; Krontiris et al. 1985; see fig. 1). Several dozen allelic fragments have been defined in Caucasians; of these, four common alleles, designated a1 (allelic frequency .61), a2 (.12), a3 (.10), and a4 (.08), constitute 91% of the total. The remainder, a minimum of 26 rare alleles, display individual frequencies ranging from .02 to .0006 (Krontiris et al. 1985, 1986, 1987). No function has as yet been ascribed to the VTR, although it does possess modest enhancer activity (Spandidos and Holmes 1987).

We have reported an increased prevalence of rare VTR alleles in cancer patients (Krontiris et al. 1985). In subsequent studies from our laboratory and others, nearly 5,000 alleles have been typed from roughly equal numbers of cases and controls. An aggregate twofold increase in the prevalence of rare alleles in cancer patients ($P < .001$) is now firmly established (Heighway et al. 1986; Lidereau et al. 1986; Thein et al. 1986; Boehm et al. 1987; Ceccherini-Nelli et al. 1987; Gerhard et al. 1987; Krontiris et al. 1987; Carter et al. 1988; Corell and Zoll 1988; Hayward et al. 1988; Radice et al. 1988; Wyllie et al. 1988). The biological basis for this phenomenon has not yet been determined. Linkage studies have shown that HRAS1 is not the primary disease locus for certain forms of familial melanoma (Sutherland et al. 1986; Gerhard et al. 1987; T. G. Krontiris, unpublished data) or breast cancer (Hall et al. 1988), yet the frequency of rare alleles is increased in such families (Sutherland et al. 1986; Hall et al. 1988; T. G. Krontiris, unpublished data). Whether rare HRAS1 alleles are, therefore, secondary pathogenetic factors

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or the product of an increased frequency of VTR mutation in some individuals must ultimately be decided by family studies which quantitate allele sharing among relatives with cancer, as well as the mutation frequency of HRAS1 and other VTRs (Jeffreys et al. 1988).

To begin the functional characterization of rare HRAS1 alleles and to understand the mechanism by which such alleles are generated, we have cloned the unique allelic fragment, a2.1, and the common allelic fragment, a1, from lymphocyte DNA of a familial melanoma patient. Our analysis of these clones indicates that the a2.1 allele bears a short deletion in the noncoding first exon, which is apparently without functional consequence. Subsequent population analysis has revealed that this deletion is in complete linkage disequilibrium with the second most common HRAS1 VTR allele, a2.

Methods

Cloning of HRAS1 Allelic Fragments

DNA from the Epstein-Barr virus–transformed lymphocytes of the patient, GD, was digested to completion with *Bam*HI, ligated into the *Bam*HI site of L47.1 (Loenen and Brammer 1980), and incubated with a commercial packaging extract (Stratagene). Recombinant phage were screened without prior amplification by in situ plaque hybridization (Maniatis et al. 1982). HRAS1 probe was the nick-translated (Maniatis et al. 1975) *Bam*HI fragment from the plasmid, pEC (Chang et al. 1982). The cloned genomic *Bam*HI fragments, containing the entire HRAS1 gene, transcriptional controls, and VTR region (Capon et al. 1983), were subcloned into pBR322 and maintained in *Escherichia coli* strain HB101. Clone pGDa1 contained the patient's common HRAS1 allele; clone pGDa2.1 contained his unique allele.

Preparation of Hybrid HRAS1 Clones

The 2.5-kb *Not*I fragment contains all HRAS1 coding exons but lacks the promotor, first exon, polyadenylation signal, and VTR (fig. 1). This fragment was exchanged between the activated HRAS1 gene of pEJ (Shih and Weinberg 1982) and the patient clone, pGDa2.1, generating the hybrid clone, pCH3.

Transfection of NIH3T3 Cells

*Eco*RI-digested plasmid DNA was mixed with 20 μg NIH3T3 DNA as carrier and was used to transfect 5 × 10^5 NIH3T3 cells by the method of Copeland et al. (1980). Cells were maintained in Dulbecco's minimal essential medium supplemented with 10% calf serum. Foci were counted at 14–17 d posttransfection. Transforming activity was expressed as foci per μg DNA of the transfected plasmid.

Southern Blotting

Southern (1975) blotting of leukocyte and recombinant DNA was performed as described elsewhere

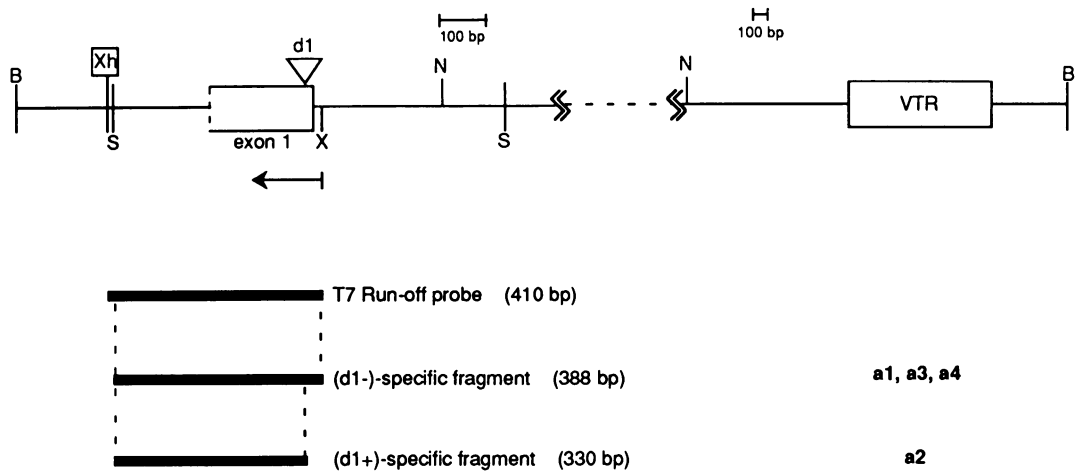

Figure 1 HRAS1 gene. A partial restriction map depicts the upstream region containing exon 1 and the downstream segment containing the VTR. The 5′ extent of exon 1 is variable. Four coding exons and one terminal noncoding exon reside on the intervening *Not*I fragment. The inverted triangle shows the approximate position of the 6-bp deletion. Thick lines represent the *Xma*III probe fragment (see Methods) and the protected fragments expected for HRAS1 alleles containing (d1+) or lacking (d1−) the exon 1 deletion. The arrow denotes the origin and direction of DNA sequencing. The box indicates polymorphism of the *Xho*I site (Xh). B = *Bam*HI; S = *Sac*I; X = *Xma*III; N = *Not*I.

(Krontiris et al. 1986, 1987). Washes were to 0.1 × SET at 65°C (1 × SET = 150 mM NaCl, 10 mM EDTA, 10 mM Tris pH 7.5). Leukocyte DNA samples were obtained from unrelated Caucasians and were purified according to a method described by Krontiris et al. (1985, 1986).

DNA Sequencing

DNA sequence from both strands of exon 1 and intron 1, proceeding in both directions from the *Xma*III (*Eag*I) site in figure 1, was obtained by the Maxam and Gilbert (1980) method.

RNA Probe Preparation

The *Sac*I fragment of pEJ, containing the HRAS1 promotor, exon 1, and the first half of intron 1, was subcloned into pBS+ (Vector Cloning Systems). Plasmid DNA for in vitro transcription was then obtained from routine alkaline lysis preparations (Maniatis et al. 1982) and subjected to polyethylene glycol precipitation. DNA was digested with *Eag*I (New England Biolabs), extracted with phenol and chloroform, then precipitated with ethanol and redissolved in sterile H_2O at 0.4 μg/μl. With this template DNA, uniformly labeled, single-stranded RNA probes were then prepared according to a method described by Myers et al. (1985). T7 RNA polymerase was purchased from New England Biolabs, RNasin from Promega, and (α^{32}P) UTP from NEN. Our run-off transcripts contained approximately 1 ^{32}P/molecule; the specific activity of UTP in the transcription reaction was approximately 200 Ci/mM. After transcription, the reaction mixture was treated with RNase-free DNase (Promega) for 15 min. The RNA probe was then extracted with phenol-chloroform, precipitated, redissolved in running buffer (80% formamide, 1 mM EDTA, 0.05% BPB, 0.05% XCFF), and purified by electrophoresis through an 8 M urea–4% polyacrylamide gel. The 410-bp probe fragment was eluted from the gel, precipitated in ethanol, and redissolved in hybridization buffer (see below).

RNase Protection Assay

The RNase protection assay was performed essentially as described by Myers et al. (1985); for our particular probe-target mismatch, consisting exclusively of G's and C's (see Results), we employed RNase T1. In brief, 1 ng cloned target DNA and 5 μg yeast tRNA were dried together and redissolved in 30 μl hybridization buffer (80% formamide, 40 mM Pipes pH 6.4, 400 mM NaCl, 1 mM EDTA). Following the addition of probe (10^5 cpm), the mixture was sealed in a glass capillary tube, heated at 90°C for 10 min, and transferred directly to a 68°C waterbath. After 5–15 h the hybridization mixture was expelled directly into 300 μl RNase T1 digestion buffer (300 mM NaCl, 100 mM LiCl, 10 mM Tris pH 7.4, 1 mM EDTA, 150 U/ml RNase T1 [Calbiochem]) and incubated at 21°C for 15 min. The reaction was stopped by the addition of 320 μl phenol-chloroform. After a second extraction with chloroform, the protected probe was ethanol-precipitated, dried, and redissolved in running buffer (as above). It was then heated to 90°C for 3 min, chilled briefly on ice, and sized by electrophoresis in 8 M urea–4% polyacrylamide gels, using RNA size markers from Bethesda Research Laboratories. For genomic DNA targets, 10–20 μg leukocyte DNA was dried with the yeast tRNA. Hybridization time was 18–24 h.

Results

Exon 1 Deletion in the Unique HRAS1 Allele of a Familial Melanoma Patient

The unique HRAS1 allele, a2.1, was identified in the index case of a family with dysplastic nevus syndrome and cutaneous malignant melanoma (Krontiris et al. 1985). The a2.1 VTR was 225 bp, or 8 repeat units, larger than the common a2 allele (fig. 2*A*, lane 2) and was present in several family members with cancer or melanoma, including the proband's affected brother (fig. 2*A*, lane 1). For further characterization of this rare HRAS1 allele, we cloned the *Bam*HI fragment containing the entire HRAS1 gene and VTR from lymphocyte DNA of the proband's brother. Examination of several

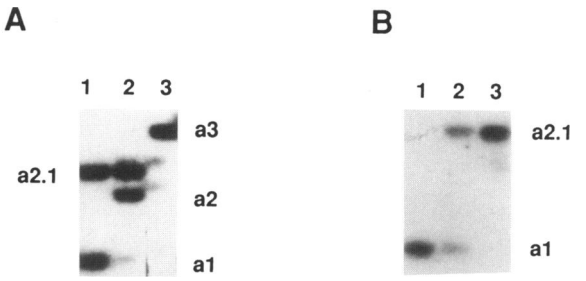

Figure 2 Southern blotting of DNAs containing the unique HRAS1 allele, a2.1. *A*, *Msp*I/*Hpa*II digests of leukocyte DNA, depicting the three most common VTR alleles, a1 (c. 1,000 bp), a2 (1,500 bp) and a3 (2,160 bp), as well as the unique allele, a2.1 (1,725 bp). Lane 2, Proband's DNA; lane 1, his affected brother's DNA; lane 3, An a3/a3 marker DNA. *B*, *Msp*I/*Hpa*II digests of pGDa1, which contained the patient's common HRAS1 allele, a1 (lane 1); lymphocyte DNA from which the clones were obtained (lane 2); and pGDa2.1, which contained his unique allele, a2.1 (lane 3).

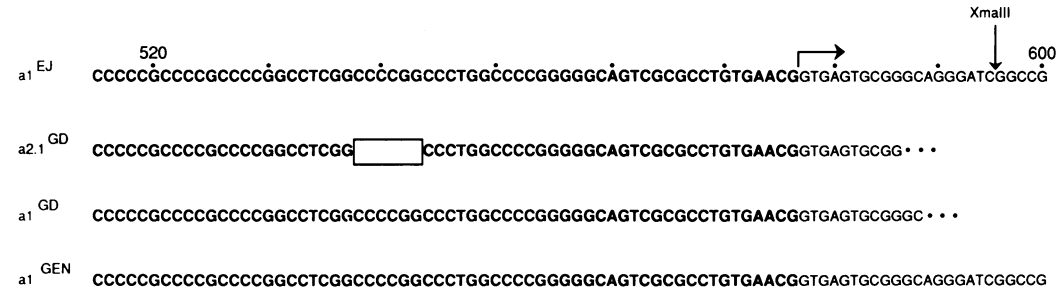

Figure 3 DNA sequence of the exon 1 terminus. Nucleotides in boldface represent the exon. The arrow denotes the splice donor site. Numbering is adopted from Capon et al. (1983). $a1^{GD}$ and $a2.1^{GD}$ are the regions from the patient clones. $a1^{EJ}$ and $a1^{GEN}$ are sequences from transforming and nontransforming HRAS1 clones, respectively (Capon et al. 1983), provided for comparison.

recombinant phage clones revealed that both his common (a1) and unique (a2.1) alleles had been obtained without alteration of the VTR region (fig. 2*B*). Comparison of the two clones by routine mapping of restriction-enzyme sites, however, revealed that the 840-bp *Sac*I fragment of a2.1, containing both the promoter and the first, noncoding exon (Ishii et al. 1985; fig. 1), was slightly, though reproducibly, smaller than the corresponding region of a1. Fine-structure mapping of the 840-bp fragment with *Bst*NI (not shown) confirmed that the a2.1 allele contained two short deletions within or near exon 1. Therefore, we sequenced the region surrounding an *Xma*III (*Eag*I) site 20 bp into the first intron (fig. 1). One deletion, the 6-bp sequence CGGCGG, appeared 100 bp into intron 1, within a repetitive stretch of CGG triplets (not shown). This deletion has previously been reported in another HRAS1 clone (Capon et al. 1983) and will not be considered further. The second a2.1 deletion, also 6 bp in length, was located within exon 1, 35 bp upstream of the splice donor site (fig. 3).

Association of the Exon 1 Deletion with the Common HRAS1 Allele, a2

We employed an RNase protection assay and a uniformly labeled RNA probe representing exon 1 and the proximal region of intron 1 (fig. 1) to examine the a2.1 deletion in genomic DNA. When the assay was first tested with cloned a2.1 DNA, the expected fragment of 330 bp was protected (fig. 4*A*). Probe hybridization to the a1 clone, in contrast, resulted in the protection of a 388-bp fragment. Two other clones lacking the exon 1 deletion, pEJ and pEC, also yielded a 388-bp fragment. Several trial hybridizations with cloned DNA indicated that cleavage of the d1-related mismatch was 70%–90% efficient (fig. 4*A*, lane 2). Besides demon-

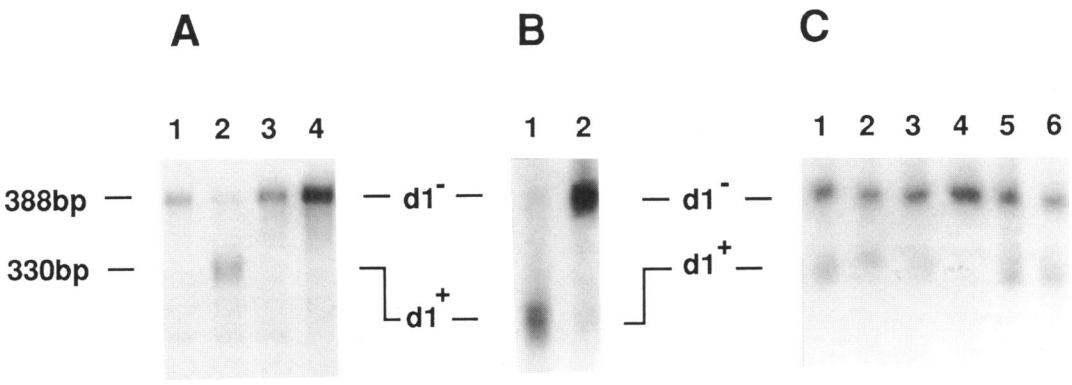

Figure 4 Probe protection assay for the d1 marker. Uniformly labeled RNA probe was hybridized to various DNAs and then processed as described in Methods. *A*, Hybridization to cloned DNAs, pGDa1 (lane 1), pGDa2.1 (lane 2), pEJ (lane 3), and pEC (lane 4). *B*, Genomic DNAs. Lane 1, The unaffected relative, an a2/a2.1 heterozygote; lane 2, An a1/a1 homozygote. *C*, Genomic DNAs. Lanes 1–6, six unrelated a1/a2 heterozygotes; lane 4, One of the two samples containing an a2 allele (from a total of 31) which lacked the d1+ marker.

strating the feasibility of this assay method, the results with cloned DNA ruled out sequencing artifact as the basis for the exon 1 deletion.

We then tested genomic DNA from an unaffected member of the melanoma kindred who was an a2/a2.1 heterozygote. This DNA possessed only the 330-bp protected fragment, indicating that this relative was a d1+/d1+ homozygote (fig. 4*B*, lane 1; compare with the a1/a1 DNA in lane 2). Therefore, the exon 1 deletion was specific neither to the patient from which the clones were obtained nor to the a2.1 allele itself. To determine whether other common VTR alleles were d1+, we analyzed genotypes from a total of 89 DNAs (table 1). All eight alleles from four a2/a2 homozygotes were d1+. All 34 alleles from 17 a1/a1 homozygotes were d1−. This disequilibrium proved absolute in our extended sample: all a3/a3 and a4/a4 homozygotes tested, 14 alleles in all, were d1− (table 1). Furthermore, no heterozygote genome that lacked a2 was d1+. Conversely, of 27 samples which were a1/a2 heterozygotes, 25 demonstrated one, and only one, d1+ allele (fig. 4*C*). Overall, therefore, 29 of 31 genotypes possessing the common a2 allele demonstrated the requisite number of d1+ alleles (one for a2 heterozygotes or two for homozygotes). None of 58 genotypes lacking a2 was d1+.

Table 1

Association of the d1 Marker with Common HRAS1 VTR alleles

Common VTR Alelle and Source of Genotype	No. of Alleles Tested[a]	No. of d1+
a1:		
a1/a1	34 (17)	0
a2:		
a2/a2	8 (4)	8
a1/a2	27 (27)	25
a3:		
a3/a3	8 (4)	0
ax[b]/a3	16 (16)	0
a4:		
a4/a4	6 (3)	0
ax[b]/a4	18 (18)	0
Total no. of a2-containing genotypes tested	31	29
total no. of other genotypes tested	58	0

[a] Numbers in parentheses are number of genotypes (DNA samples)/category.

[b] ax ≠ a2.

Table 2

Transforming Activity of Native and Hybrid HRAS1 Clones

HRAS1 Clone	Exon 1 Origin	Codon 12	Transforming Activity
pEJ	pEJ	Mutant	1424 ± 304 foci/μg[a]
pGDa2.1	pGDa2.1	Wild type	0
pCH3	pGDa2.1	Mutant	1205 ± 120[b]

[a] Based on an average of 89 foci/6.25 ng DNA/5 × 10^5 cells in triplicate 60-mm tissue culture dishes.

[b] Based on an average of 75.3 foci/6.25 ng DNA/5 × 10^5 cells in triplicate 60-mm tissue culture dishes.

Functional Integrity of the a2.1 Exon 1

To determine whether the exon 1 deletion adversely affected HRAS1 gene function, we constructed a chimeric HRAS1 gene consisting of the coding exons and polyadenylation signal of the mutant HRAS1 gene from pEJ and the promotor, exon 1, and downstream VTR from the a2.1 clone. The HRAS1 gene of pEJ, possessing a codon 12 mutation in exon 2, transformed NIH3T3 cells following transfection by plasmid DNA. The a2.1 HRAS1 gene lacked this or any other coding sequence mutations and did not transform NIH3T3s (table 2). The hybrid gene in pCH3 transformed NIH3T3 cells as well as pEJ (table 2). This assay indicated that the promotor and exon 1 region of the a2.1 allele were functional. Furthermore the deletion did not "activate" the HRAS1 gene in the traditional sense, since the a2.1 clone itself was not transforming. Northern blot analysis of RNA from NIH3T3 cells bearing an a2.1 HRAS1 gene (not shown) revealed the usual 1.2-kb mRNA species, which could not be distinguished from the pEJ transcript.

Population Analysis of the Common a2 Allele

While the NIH3T3 transfection assay indicated that the exon 1 deletion did not interrupt the expression of the HRAS1 gene, subtler manifestations of d1-induced dysfunction (e.g., developmental or tissue-specific effects) could not be detected in this fashion. Since at least 95% of a2 alleles contain the exon 1 deletion, it follows that most a2 homozygotes could potentially possess two damaged HRAS1 genes. Pathogenetic effects of a2 alleles might, therefore, be evident either in an unexpectedly low frequency of a2 homozygotes in the population or in the appearance of some disease phenotype in individuals with this genotype. As part of our population genetics analysis of the HRAS1 locus, we

Table 3

Numbers of Genotypes Containing an a2 Allele

Genotype	Observed	Expected
a2/a2	12	12.0
a1/a2	125	125.7
a2/a3	25	22.0
a2/a4	17	16.
a2/rare	13	18.7

NOTE.—A total of 1,774 alleles were typed, 896 from cancer cases and 878 from controls. Allelic frequencies for the four common alleles were .611 (a1), .116 (a2), .107 (a3), and .078 (a4). The "rare" category represented a total of 26 rare alleles; individual frequencies ranged from .024 to .0006, with an aggregate frequency of .091. Genotypic frequencies for the cancer and control subgroups also showed no significant differences between observed and expected numbers (data not shown).

have typed a large number of cancer cases and normal controls (table 3). The mean age of both groups was approximately 60 years; the sample sizes were also nearly identical (448 vs. 439). Of 1,774 total alleles, 206 were a2, yielding an allelic frequency of .116. The observed number of a2 homozygotes, 12, was identical to the number predicted from the Hardy-Weinberg relation, 12.0. Four of the 12 a2 homozygotes were cancer patients, a number not significantly different ($P > .1$) from that of controls. The a2 homozygotes, as a group, possessed no noteworthy clinical history. No phenotypically adverse couplings with other HRAS1 alleles were noted either. It is interesting that a reduced number of a2/rare genotypes were observed. However, this departure from expectation was not statistically significant.

Discussion

We have described a short HRAS1 gene deletion which was in complete linkage disequilibrium with the common a2 allele of the HRAS1 VTR. Alleles bearing this deletion were apparently functional, since a chimeric HRAS1 gene with the d1 marker and a codon 12 mutation remained capable of transforming NIH3T3 cells. At the present time we have not excluded the possibility that the deletion might influence mRNA half-life or tissue-specific expression of the HRAS1 gene. We did note, however, that no significant alterations in expected genotypic frequencies involving a2 occurred in a population of late-middle-aged Caucasians, nor were any differences in a2-containing genotypes evident between cancer cases and controls.

The d1 deletion is the second genetic marker found in linkage disequilibrium with common HRAS1 alleles. The *Xho*I polymorphism upstream of the HRAS1 promotor (fig. 2) shows the following pattern: all a1 and a3 VTR alleles are *Xho*I+, while a2 and a4 alleles are uniformly *Xho*I− (Chandler et al. 1987). The a2.1 allele, in addition to sharing the d1+ marker with a2, is also *Xho*I− (data not shown). Thus, we have strong evidence that this unique allele originated from an a2 parent. It is likely that the upstream haplotypes of common VTR alleles, derived from the above-described markers, will prove useful in determining the ancestors of other rare VTR alleles. Our most recent work (A. Kasperczyk and T. G. Krontiris, unpublished data) suggests that at least one other polymorphism in this region can be detected by the probe-protection methodology. Also in disequilibrium with VTR alleles, it differentiates a3 and a4 collectively from a1 and a2. Combining all these markers has allowed us to create a unique haplotype for each common allele, thereby facilitating the genealogy of rare alleles. Preliminary studies with several other rare alleles indicate that each possesses the upstream haplotype of the common allele nearest in size.

Two of the 35 a2 alleles we typed failed to demonstrate an exon 1 deletion. It is possible that these d1− a2 alleles represent technical failures, although the result was repeatedly obtained. Alternatively, they could represent a minor a2 subclass, perhaps derived from a distinct ancestor. It is of interest, however, that both of the d1− a2 alleles shared the same *Xho*I− marker typical of the a2 class. Therefore, the variants may have arisen from double crossovers or gene conversion.

The extent of genetic heterogeneity in noncoding exons of genes other than HRAS1 is largely uncharacterized. The utility of such markers for genetic studies should be considerable, as illustrated by our allele lineage analysis. Since the markers should also be present in mature transcripts, the same probe-protection methodology can be used to determine which allele of an informative genotype is being expressed. Thus, the typing of both functional and structural allelic losses (e.g., during tumor progression) is possible. Presently, the combined use of both the polymerase chain reaction (PCR) and nuclease protection of uniformly labeled RNA probe promises to be extremely useful in the rapid analysis of sequence heterogeneity and allelism under circumstances in which the variants are phenotypically silent. The confirmation of such useful markers would result from DNA sequencing of selected, amplified alleles. Unfortunately, PCR amplification has not been

technically possible in the region including the HRAS1 exon 1, presumably because of a GC content exceeding 95% nearly throughout. Therefore, analysis of housekeeping genes, in which a similar GC content often appears in the promoter–exon 1 segment, may be handicapped. It is to be expected, however, that technical advances in this methodology will shortly overcome such difficulties, thereby permitting general applicability.

Acknowledgments

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