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Transforming Growth Factor-Alpha: Characterization of the *Bam*HI, *Rsa*I, and *Taq*I Polymorphic Regions

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Summary

We have characterized the nature of structural alleles of the transforming growth factor-alpha (TGFα) locus by restriction-enzyme digestion with *Bam*HI, *Rsa*I, and *Taq*I. The *Bam*HI polymorphic site is located within exon VI, which codes for the 3′ untranslated region. The two *Bam*HI alleles differ by a single point mutation at the restriction site. The *Rsa*I and *Taq*I polymorphic sites are located within intron V. The two alleles differ at the restriction site, either by a point mutation (*Rsa*I) or by a 4-bp deletion (*Taq*I). This analysis permits us to devise a PCR method coupled with restriction digestions to directly identify the TGFα polymorphisms. Analysis of 99 Caucasian controls has revealed a highly significant ($P < .001$) association between the *Rsa*I and the *Bam*HI genotype. The frequency of the rare *Bam*HI allele was significantly higher ($P < .001$) in transformed cell lines (.30) than in controls (.076).

Introduction

Transforming growth factor-alpha (TGFα) is a 50-amino-acid, 5.6-kD secreted polypeptide which is cleaved from a larger integral membrane glycoprotein (Derynck et al. 1984). Structurally and functionally, TGFα resembles epidermal growth factor (EGF) and induces a mitogenic response by binding to and stimulating the tyrosine kinase activity of 170-kD cell-surface EGF receptor (Marquardt et al. 1983, 1984; Massagué 1983*b*). TGFα was originally found in culture fluids from various oncogenically transformed cells (De Larco and Todaro 1978; Todaro et al. 1980). Subsequent studies showed that TGFα expression is most prevalent in tumor-derived cell lines and in cells transformed by cellular oncogenes, retrovirus, and tumor promoters (De Larco and Todaro 1978; Todaro et al. 1980; Anzano et al. 1983; Marquardt et al. 1983; Massagué 1983*a;* Derynck et al. 1987). These findings and the transforming effect of TGFα cDNA transfected into fibroblasts and epithelial cells have suggested that TGFα might be involved in the generation or progression of some forms of neoplasia (Rosenthal et al. 1986; Watanabe et al. 1987; McGeady et al. 1989). This possibility is supported by the high incidence of mammary and liver neoplasias in transgenic mice which chronically overexpress TGFα in these tissues (Jhappan et al. 1990; Matsui et al. 1990; Sandgren et al. 1990).

It has been reported that the gene for human TGFα is 70–100 kbp (Derynck 1988) and localized by in situ hybridization on chromosome 2p13 (Tricoli et al. 1986), close to the breakpoint of the Burkitt lymphoma t(2:8) variant translocation (Brissenden et al. 1985). Murray et al. (1986) and Hayward et al. (1987) have demonstrated RFLP for the human TGFα locus, with the restriction endonucleases *Bam*HI (two alleles, 7.0 kbp and 4 kbp), *Rsa*I (two alleles, 1.5 kbp and 1.2 kbp), and *Taq*I (two alleles, 3.0 kbp and 2.7 kbp). Using RFLP to analyze the DNA, Hayward et al. (1988) found that a *Taq*I 2.7-kbp fragment was associated with melanoma cell lines. In addition, it has been reported that the *Taq*I 2.7-kbp and *Bam*HI 4-kbp fragments were associated with cleft lip and/or palate, a nonsyndromic craniofacial malformation (Ardinger et al. 1989; Chenevix-Trench et al. 1991). By restriction with *Bam*HI, we have

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Table 1

Genotypes at *Bam*HI, *Rsa*I, and *Taq*I Polymorphic Sites, for Different Human Transformed Cell Lines

Cell Line	Type	Origin	Allele Polymorphism *Bam*HI	*Rsa*I
MCF-7	Breast carcinoma	Caucasian	A'1 A'2	B1 B2
ZR-75-1	Breast carcinoma	Caucasian	A'1 A'2	B1 B2
T-47D	Breast carcinoma	Unknown	A'2 A'2	B2 B2
KHOS-240	Osteosarcoma	Caucasian	A'2 A'2	B2 B2
Saos-2	Osteogenic sarcoma	Caucasian	A'1 A'2	B1 B2
SW613-S,C13	Colon carcinoma	Caucasian	A'1 A'2	B1 B1
A-431	Epidermoic carcinoma	Unknown	A'2 A'2	B2 B2
HeLa	Epitheloid cervical carcinoma	Black	A'2 A'2	B2 B2
HT 1080	Fibrosarcoma	Caucasian	A'1 A'1	B1 B1
Raji	Burkitt lymphoma	Black	A'2 A'2	B2 B2
Frequencies			A'1 .30 A'2 .70	B1 .35 B2 .65
Observed heterozygoty			40%	30%

identified (Qian et al. 1991) two-allele polymorphisms, 10- and 7-kbp, distinct from the two-allele *Bam*HI polymorphisms described by Murray et al. (1986). However, in agreement with these authors, we showed a linkage disequilibrium between *Bam*HI and *Rsa*I polymorphisms.

We have shown elsewhere that the two-allele (10- and 7-kbp) polymorphisms are associated with bilateral sporadic cleft lip and palate (Stoll et al. 1992). These studies have revealed that TGFα RFLPs might be of interest as markers. Results presented in the present paper give the sequences of the two-allele *Bam*HI, *Taq*I, and *Rsa*I polymorphisms. This analysis allows us to introduce a PCR method coupled to restriction-enzyme digestion to directly identify these TGFα polymorphisms.

Material and Methods

Samples

Ninety-nine DNA samples obtained from Alsatian-Caucasian individuals and 10 DNA samples from transformed cell lines were analyzed for *Bam*HI, *Rsa*I, and *Taq*I RFLPs at the hTGFα locus. Nine cell lines (Saos-2, KHOS-240S, MCF-7, T-47D, ZR-75-1, A-431, HT 1080, HeLa, and Raji) were obtained from the ATCC collection. The SW613-S cell line derived from a colon carcinoma (Modjatahedi et al. 1992) was a gift from O. Brison. Ethnic origins of the individuals from whom these cell lines were derived are presented in table 1.

TGFα-specific Clones

pJF25 and pJF26 cDNA clones have already been described elsewhere (Qian et al. 1991). Map locations of these clones are given in figure 1.

A human genomic library has been screened with pTGF-C1 cDNA (Derynck et al. 1984). A 16-kbp clone

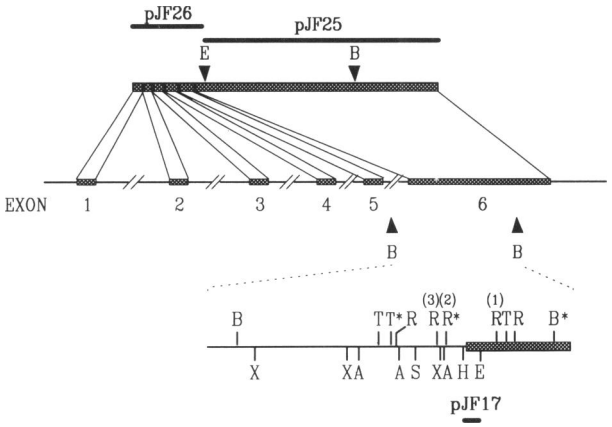

Figure 1 Schematic representation of the TGFα gene structure and the corresponding cDNA. Exons are shown as cross-hatched boxes. Positions of pJF25 and pJF26 cDNA clones are indicated at the top. The lower panel gives the partial restriction map of the 7-kbp *Bam*HI genomic fragment. Restriction sites are shown: E = *Eco*RI; B = *Bam*HI; T = *Taq*I; R = *Rsa*I; X = *Xba*I; A = *Apa*I, S = *Sac*I; and H = *Hin*dIII. The asterisks mark the polymorphic sites.

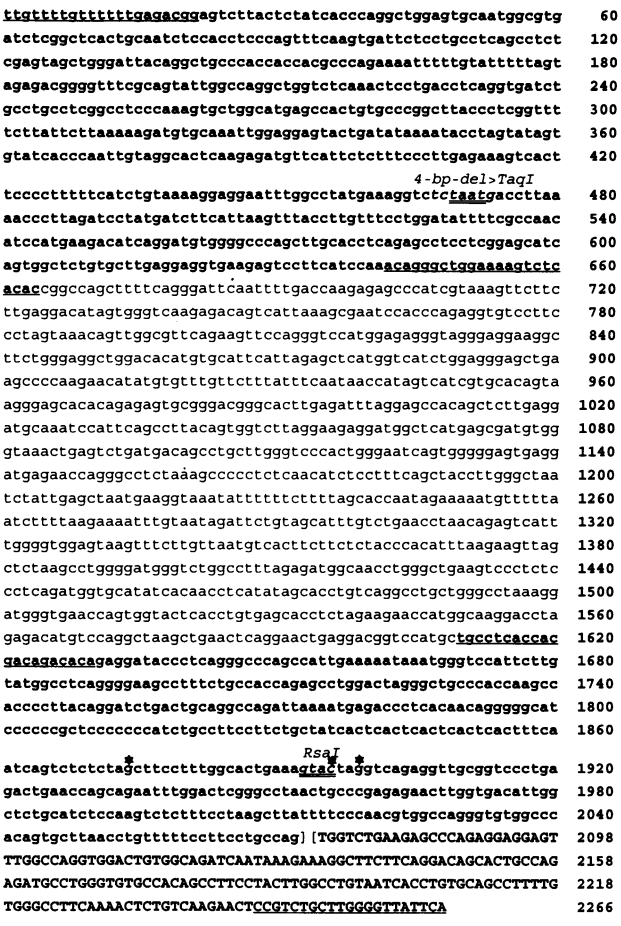

Figure 2 Nucleotide sequences of the *Rsa*I-B2/*Taq*-C1 haplotype. Amplified fragments are shown in boldface. The underlined sequences correspond to the position of primer pairs PA5-PA6 and PA7-PA8, used to amplify the *Rsa*I and the *Taq*I polymorphic regions, respectively (see table 2). The locations of the polymorphic restriction sites are shown above the sequence. The three nucleotides which are changed in the *Rsa*-B1 haplotype are indicated by asterisks. The intronic sequences are shown in lowercase letters. The intron V-exon VI boundary is denoted by "] [". This nucleotide sequence has been deposited at the EMBL Data Library (accession no. X70341).

was isolated and subcloned in Bluescript vector (Stratagene). Determination of the restriction map and partial DNA sequencing permits the identification of the intron V-exon VI junction to be identified (fig. 2). The *Hin*dIII-*Eco*RI fragment of 456 bp which encompasses this junction was subcloned in the pBS vector (Stratagene) to give the pJF17 recombinant (fig. 1).

Sequence Analysis of Genomic DNA

Overlapping clones were generated by digestion with exonuclease III and were sequenced by extension of M13 universal primer by the dideoxynucleotide chain-termination method (T7 sequencing kit; Pharmacia).

PCR of TGFα Genomic DNA

The PCR mixture contained 1 μg of genomic DNA and 25 pmol of left and right primers in a final volume of 50 μl (PCR mixture = 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.005% Tween 20, 0.005% NP-40, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 1 U of Replitherm™ thermostable DNA polymerase [Epicentre technologies]). Sequences of the three primer pairs AP3/AP4 (*Bam*HI polymorphism), AP5/AP6 (*Rsa*I polymorphism), and AP7/AP8 (*Taq*I polymorphism) are given in table 2. After an initial step of denaturation at 95°C for 5 min, 30 cycles of 92°C for 30 s, 55°C (AP3/AP4), 60°C (AP5/AP6), or 57°C (AP7/AP8) for 30 s, and 72°C for 1 min 30 s were used, followed by a final 72°C step for 15 min.

Asymmetric PCR DNA Sequencing

Asymmetric PCR methods were used to generate single-stranded DNA for sequencing. Reactions were carried out in 50 μl of PCR mixture with 5 μl of unpurified product of a previous PCR, in the presence of 25 pmol of only one primer of each pair. The PCR ran for 12 cycles at 92°C for 30 s, 55°C, 60°C, or 57°C (depending on the primer pair) for 30 s, and 72°C for 1 min 30 s, followed by a final step at 72°C for 15 min. The PCR reaction was separated from the mineral oil, extracted with phenol and $CHCl_3$, and precipitated by ethanol two times. The precipitate was collected by centrifugation and redissolved in 10 μl of 10 mM Tris-HCl pH 7.5, 1 mM EDTA. An aliquot of the resulting solution of single-stranded DNA was used directly for DNA sequencing by the dideoxynucleotide chain-termination method (T7 sequencing kit; Pharmacia).

Statistical Methods

Comparisons between allele or genotype frequencies were performed by using the χ^2 test. When necessary, Yates' correction was used.

Results

Mapping of TGFα Gene RFLP in Intron V and Exon VI

The three *Bam*HI, *Rsa*I, and *Taq*I RFLPs were detected with the two cDNA clones pJF25 and pJF26, which encompass the complete cDNA sequences (fig. 1). These two clones revealed two polymorphic bands: 10 and 7 kbp after digestion with *Bam*HI, 1.5 and 1.2 kbp after digestion with *Rsa*I, and 3 and 2.7 kbp after

Table 2

Polymorphic Sequences and Primer Pairs Used for PCR Amplification

Polymorphic Allele	Amplification Primer Pair (5′ to 3′)	Size of PCR Fragment[a] (bp)	Polymorphic Restriction Site(s)	
			Sequence[b]	Location
A′1 (10 kbp)	AP3, ACAGATGGCGGAACGAGAGGT	434	AGCATTGGCTCCCTCTGC --------A--------- *Bam*HI	Exon VI (in the 3′UTR, at 2,767 bp from the first ATG)
A′2 (7 kbp)	AP4, CTAAAGGGCAAGGAAACACAG			
B1 (1.5 kbp)	AP5, TGCCTCACCACGACAGACACA	657	ACTGAAAGTATTATGTCA ----------C--G---- *Rsa*I	Intron V (177 bp upstream of the acceptor site of exon VI)
B2 (1.2 kbp)	AP6, TGAATAACCCCAAGCAGACGG			
C1 (3 kbp)	AP7, TTGTTTTGTTTTTTGAGACGG	662	AGGTCTCTAATGACCTTA ------- ------- *Taq*I	Intron V (1,602 bp upstream of the acceptor site of exon VI)
C2 (2.7 kbp)	AP8, GTGAGACTTTTCCAGCCCTGT			

[a] According to fig. 2.
[b] Sequence gels are presented in fig. 4.

digestion with *Taq*I (Qian et al. 1991). Moreover, some additional constant bands regularly appeared with both inserts from pJF25 and pJF26. However, the clone pJF17, which maps at the junction of intron V and exon VI (fig. 1), detects only the *Bam*HI, *Rsa*I, and *Taq*I polymorphic fragments (Qian et al. 1991). The pJF17 insert was derived from a genomic clone isolated from a human library. A partial restriction map of the parental clone is also presented in figure 1. The recombinant pJF17 was constructed from the 456-bp *Hin*dIII-*Eco*RI fragment subcloned in the pBS vector. Sequence analysis indicated that this fragment includes 66 nt of intron V and 390 nt of exon VI (fig. 2). The fact that this fragment detects the three *Bam*HI, *Taq*I, and *Rsa*I RFLPs indicates that these three polymorphisms are located close to one another in a region which encompasses the intron V–exon VI junction.

Identification of the Polymorphic BamHI, RsaI, *and* TaqI *Recognition Sequences*

In an attempt to characterize the three *Bam*HI, *Rsa*I, and *Taq*I sites by PCR, the sequences surrounding the candidate restriction sites within intron V and exon VI were determined (fig. 2 and EMBL Data Library; accession nos. X70340 and X70341) and were used to design PCR primers. Table 2 summarizes the primer pairs used for amplification, the location of the polymorphic site, and the two allelic sequences for each polymorphic recognition site. In agreement with Murray et al. (1986) and Hayward et al. (1987), haplotype designations were *Rsa*I-B1 (1.5 kbp), *Rsa*I-B2 (1.2 kbp), *Taq*I-C1 (3 kbp), and *Taq*I-C2 (2.7 kbp). The *Bam*HI alleles identified in our study were designated as *Bam*HI-A′1 (10 kbp) and *Bam*HI-A′2 (7 kbp), to be distinguished from the *Bam*HI-A1 (7 kbp) and *Bam*HI-A2 (4 kbp) haplotypes reported by these authors.

The primer pair AP3 and AP4 (table 2) was chosen to amplify a 434-bp fragment which asymmetrically brackets the *Bam*HI site located on the right side of the pJF17 sequence. The amplification product of A′2 allele is cleaved by *Bam*HI, giving two fragments of 121 and 313 bp, whereas the amplified product of the A′1 allele is not cleaved (fig. 3*A*). Direct sequence analysis of PCR products revealed a single point mutation (A-to-C transversion) within exon VI, at nt 2767 (nt 1 is counted from the A of the initiation codon) of the 3′ untranslated region (UTR) of TGFα-specific mRNA. This mutation results in the loss of the *Bam*HI cleavage site. No other mutation has been detected within the 434-bp two-allele-specific PCR products (fig. 4*A* and table 2).

The two PA5 and PA6 primers were designed to flank the first *Rsa*I site located on the left side of the pJF17 insert. The 657-bp fragment obtained with the B2 allele is cleaved by *Rsa*I into two fragments of 373 and 284 bp, whereas the 657-bp amplified fragment of B1 allele is not cleaved (fig. 3*B*). Direct sequence analy-

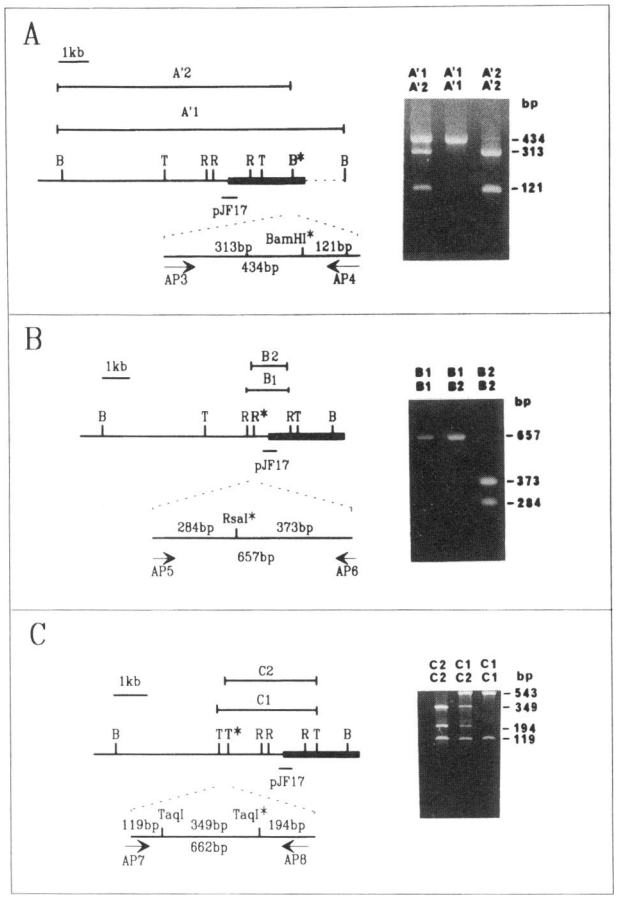

Figure 3 Mapping of TGFα gene *Bam*HI, *Rsa*I, and *Taq*I polymorphic sites by PCR/RFLP method. *A*, Position of *Bam*HI polymorphic site. *B*, Position of *Rsa*I polymorphic site. *C*, Position of *Taq*I polymorphic site. In each panel, the partial restriction map of the region which encompasses the intron V–exon VI junction is indicated, and exon VI is shown as a thick line. Asterisks mark the polymorphic recognition sites. The two lines above the restriction map give the position of the two *Bam*HI (panel *A*), *Rsa*I (panel *B*), and *Taq*I (panel C) RFLPs. For each polymorphism, a schematic representation of PCR products, with the expected fragment sizes, is indicated below the restriction map. Sequences of primer pairs AP3/AP4, AP5/AP6, and AP7/AP8 are indicated in table 1. On the right a photograph of ethidium bromide–stained 1.2% agarose gel of PCR products digested with *Bam*HI (panel *A*), *Rsa*I (panel *B*), or *Taq*I (panel C) is presented. Examples of the three phenotypes are indicated in each case.

sis of the PCR products reveals a 1-bp change in the *Rsa*I recognition sequence (C-to-T transition). In addition, both a G residue located 3 nt downstream and another G residue located 22 nt upstream were replaced by T (fig. 4*B* and table 2). No other mutation has been detected in the 657-bp two-allele-specific PCR products.

The primer pair PA7 and PA8 was chosen to amplify a 662-bp fragment which asymmetrically brackets the *Taq*I site located downstream from the pJF17 sequence. The amplified product of the C1 allele is cleaved once with *Taq*I, resulting in two fragments of 543 and 194 bp, whereas *Taq*I digestion of the amplified products from the C2 allele revealed an additional *Taq*I cleavage site (fig. 3C). Direct sequence analysis of the PCR products identified a 4-bp deletion resulting in the creation of a new *Taq*I site (fig. 4C and table 2). No other mutation has been detected in the 662-bp two-allele-specific PCR products.

Frequencies of BamHI, RsaI, *and* TaqI *Two-Allele Polymorphisms in a Population of 99 Unrelated Caucasians: Comparison with Frequencies Measured in 10 Different Human Transformed Cell Lines*

Allele frequencies estimated from 99 healthy unrelated individuals are presented in table 3. Frequencies of the *Rsa*I and *Taq*I two-allele polymorphisms correspond to the estimates published by Murray et al. (1986) and Hayward et al. (1987). The frequencies of the 10- and 7-kbp *Bam*HI alleles were .076 and .924, respectively. Moreover, there is a highly significant association between the *Rsa*I and the *Bam*HI genotypes (table 4) ($P < .001$) ($\chi^2 = 19.79$, 2 df). The value of the linkage disequilibrium (D) between A'1 and B1 is $D = .0537$. This value is very close to the maximum value of D (D_{max}) for the observed gene frequencies ($D_{max} = .0538$). There is no linkage disequilibrium between these two loci and the *Taq*I locus (data not shown).

We previously determined the sequence of a full-length TGFα cDNA clone isolated from a cDNA library derived from the human SW613-S cell line. Sequence analysis of the 3'UTR did not identify a *Bam*HI site. This result indicates that cDNA clone was derived from the *Bam*HI 10-kbp allele, suggesting that the rare A'1 allele is more frequent in tumor cell lines. To test this hypothesis, different transformed cell lines were analyzed for the presence of the rare A'1 allele. Cell-line genotypes are presented in table 1, with the ethnic origin of patients from whom the cell lines were derived. As shown in table 5, the frequency of the A'1 allele was significantly higher ($P < .001$) in transformed cells than in control cells. Moreover, results presented in table 1 show that the frequency of the A'1 allele was even higher (50% instead of 90%) when only transformed cell lines derived from Caucasian individuals were considered. Furthermore, one (HT 1080) of six cell lines derived from Caucasian individuals is homozygous for

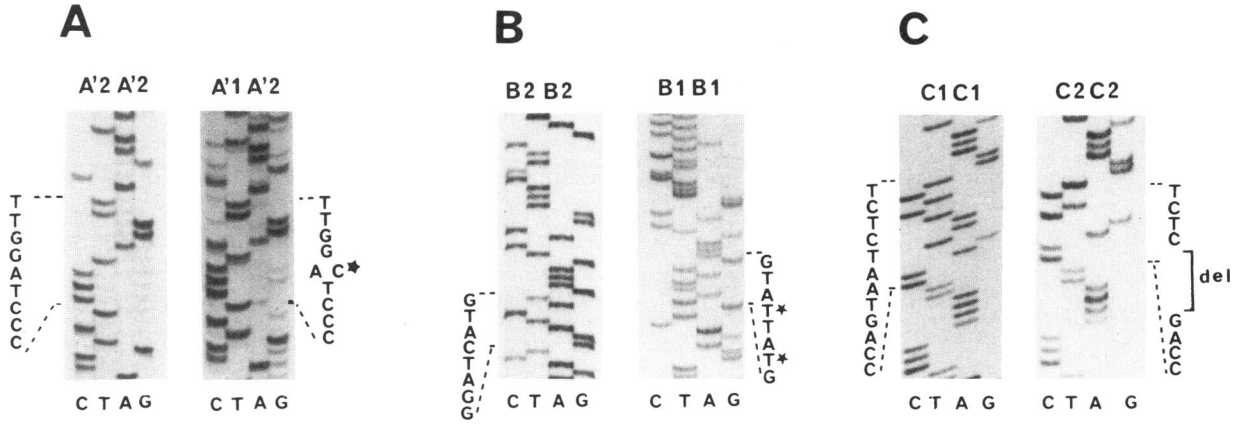

Figure 4 Identification of *Bam*HI, *Rsa*I, and *Taq*I polymorphic site mutations. Direct sequence analysis of PCR-amplified genomic DNA of a *Bam*HI 7-kbp homozygote and a 7-kbp/10-kbp heterozygote (panel *A*), a *Rsa*I 1.2-kbp/1.5-kbp homozygote (panel *B*), and a *Taq*I 3-kbp/2.7-kbp homozygote (panel *C*). Sequences of primer pairs used to generate PCR products are depicted in table 1. Sequences surrounding the polymorphic sites are indicated (read 5′ to 3′, from top to bottom). Mutated nucleotides are marked with an asterisk.

the A′1 allele, compared with none of 99 Caucasian controls (tables 1 and 4).

Discussion

This study was begun in order to investigate the nature of structural variability at the human TGFα locus. Elucidation of polymorphism at this locus may provide significant insights into potential contributions of this locus to possible associated human diseases. We have characterized the location and the nature of a structural allelism of the TGFα locus demonstrated by restriction-enzyme digestion with *Bam*HI, *Rsa*I, and *Taq*I. The *Rsa*I and *Taq*I polymorphic sites are located within intron V, whereas the *Bam*HI polymorphic site is located within the 3′UTR (exon VI) at nt 2767 (nt 1 is counted from the A of the initiation codon). Using PCR analysis, Sheffield et al. (1992) have identified another polymorphism within an upstream fragment of the 3′UTR (nt 734–1079).

Creation of a new *Taq*I site by a deletion of 4 nt within intron V leads to a 2.7-kbp fragment (C2 allele) instead of a 3-kbp fragment (C1 allele). Point mutations within and close to the *Rsa*I recognition sequence give rise to the 1.5-kbp fragment (B1 allele) instead of the 1.2-kbp fragment (B2 allele). Similarly, a single point mutation within the *Bam*HI recognition sequence leads to the emergence of the A′1 allele characterized by the 10-kbp fragment. All of these polymorphic fragments can be detected with the two cDNA probes pJF25 and pJF26, which revealed, in addition, some constant bands. However, the pJF17 probe which corresponds to a genomic DNA fragment of 456 bp mapping at the intron V–exon VI junction only revealed the polymor-

Table 3

Two-Allele Polymorphism Frequencies in Control Caucasian Individuals

Polymorphism	Alleles	Frequencies	Observed Heterozygosity
*Bam*HI	A′1 (10 kbp)	.076	.15 (15/99)
	A′2 (7 kbp)	.924	
*Rsa*I	B1 (1.5 kbp)	.293	.36 (36/99)
	B2 (1.2 kbp)	.707	
*Taq*I	C1 (3 kbp)	.930	.13 (12/92)
	C2 (2.7 kbp)	.070	

Table 4

Association between *Bam*HI/*Rsa*I RFLPs in Control Caucasian Individuals

	*Rsa*I Genotype		
*Bam*HI Genotype	B1/B1	B1/B2	B2/B2
A′1/A′2	4	11	0
A′2/A′2	7	25	52

NOTE.—No A′1/A′1 individuals were observed. $P < .001$.

Table 5

Comparison between Transformed Cell Lines and Lymphocytes from Control Individuals, for Presence of A'1 Allele

	No. (%) of Allele	
	A'1	A'2
Control individuals (n = 99)	15 (7.6)	183 (92.4)
Transformed cell lines (n = 10)	6 (30)	14 (70)

NOTE.—$\chi^2 = 8.08$; $P < .001$.

phic bands. This probe permits a better interpretation of the restriction-fragment patterns.

Murray et al. (1986) first described the polymorphism at the TGFα locus and demonstrated that *Bam*HI identifies a two-allele polymorphism of 7 and 4 kbp with frequencies of .17 and .83, respectively. In contrast, we detected two *Bam*HI polymorphic alleles of 10 and 7 kbp with frequencies of .076 and .924, respectively (Qian et al. 1991). However, the following observations strongly suggest that both studies actually identify the same *Bam*HI polymorphism: (i) We were unable to detect a 4-kbp allele, although the two pJF25 and pJF26 probes correspond to Murray's probes, according to their description. (ii) Sequence analysis of the polymorphic region did not permit us to identify another *Bam*HI site which would account for a 4-kbp fragment. (iii) Both studies revealed a linkage disequilibrium between the 10-kbp *Bam*HI locus and the 1.5-kbp *Rsa*I locus.

Whereas the 10-kbp *Bam*HI allele is detected at a very low frequency (.076) for the control population, this allele is found with a higher frequency in transformed cell lines (.30 irrespective of the ethnic origin of donor individuals, or .50 when the cell lines considered were those derived from Caucasian individuals only). In spite of the relatively low number of transformed cells analyzed, the difference in frequencies is statistically significant (P = .001), suggesting an advantage for the 10-kbp allele. As this allele is located within the 3'UTR of TGFα mRNA, it should allow the PCR-based determination of allele-specific expression within RNA samples, in addition to the detection of allele imbalance within DNA samples.

Hayward et al. (1988) have reported a statistically significant association between the *Taq*I polymorphism and cutaneous malignant melanoma (MM). The frequency of the 2.7-kbp allele in MM cell lines (.18) was significantly higher ($P < .01$) than that in lymphoblastoid cell lines derived from unaffected controls (.05). To explain the aberrant or inappropriate TGFα expression occurring frequently in melanoma, certain authors have asked whether *Taq*I polymorphism relates to an altered expression or stability of TGFα mRNA (Marquardt et al. 1983; Derynck et al. 1987; Ellem et al. 1988). The localization of the *Taq*I polymorphism site within intron V seems to exclude a direct involvement of this polymorphism in TGFα expression.

Ardinger et al. (1989) found an association between the *Taq*I and *Bam*HI RFLPs and the occurrence of cleft lip and palate, suggesting that either the TGFα gene itself or the DNA sequences in an adjacent region contribute to the development of a fraction of cases of cleft lip and palate. We have studied the occurrence of TGFα gene RFLPs both in patients with cleft lip and palate and in normal individuals (Stoll et al. 1992). Our results revealed an association between the subgroup with bilateral cleft lip and palate and the 10-kbp *Bam*HI allele of the TGFα gene. We recently confirmed this point by analyzing a larger population (results not shown). The results presented in the present paper make possible the use of a PCR-based method to extend these investigations.

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