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Research Article

A form of thyroxine-binding globulin (TBG) with reduced affinity for hormone and increased susceptibility to heat and acid denaturation has been identified in Australian Aborigines (TBG-A). Results of heat denaturation of TBG established that the TBGA allele is X linked and has a frequency of 50.9% in Western Australian Aborigines. The sequence of an isolated TBGA allele differed at two positions from that of the normal TBG allele (TBGC). One substitution was in codon 191, ACA (threonine) rather than GCA (alanine), and the other was in codon 283, TTT (phenylalanine) instead of TTG (leucine). These nucleotide substitutions resulted in the loss of sites for the enzymes Bgl 1 and Tth 111 II, respectively. The nucleotide substitutions in the TBG-A allele was confirmed by digestion of genomic DNA segments amplified using the polymerase chain reaction. The Bgl 1 and Tth 111 II sites were absent in the genes of two Aboriginal men expressing TBG-A and were present in those of three Aboriginal and six Caucasian males expressing TBG-C. The TBG gene of a seventh Caucasian male possessed the Bgl 1 site but had lost the Tth 111 II site; sequencing of this allele revealed only the substitution in codon 283 identical to that in the TBGA allele. As the biochemical properties of TBGPhe-283 expressed by this individual were indistinguishable from normal [...]

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Sequence of the Variant Thyroxine-binding Globulin of Australian Aborigines

Only One of Two Amino Acid Replacements Is Responsible for Its Altered Properties

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Abstract

A form of thyroxine-binding globulin (TBG) with reduced affinity for hormone and increased susceptibility to heat and acid denaturation has been identified in Australian Aborigines (TBG-A). Results of heat denaturation of TBG established that the TBG_A allele is X linked and has a frequency of 50.9% in Western Australian Aborigines.

The sequence of an isolated TBG_A allele differed at two positions from that of the normal TBG allele (TBG_C). One substitution was in codon 191, ACA (threonine) rather than GCA (alanine), and the other was in codon 283, TTT (phenylalanine) instead of TTG (leucine). These nucleotide substitutions resulted in the loss of sites for the enzymes Bgl I and Tth 111 II, respectively. The nucleotide substitutions in the TBG-A allele were confirmed by digestion of genomic DNA segments amplified using the polymerase chain reaction. The Bgl I and Tth 111 II sites were absent in the genes of two Aboriginal men expressing TBG-A and were present in those of three Aboriginal and six Caucasian males expressing TBG-C. The TBG gene of a seventh Caucasian male possessed the Bgl I site but had lost the Tth 111 II site; sequencing of this allele revealed only the substitution in codon 283 identical to that in the TBG_A allele. As the biochemical properties of TBG^{Phe-283} expressed by this individual were indistinguishable from normal TBG^{Leu-283}, we believe that the abnormal properties of TBG-A are due to substitution of alanine for threonine at residue 191.

Introduction

Thyroxine-binding globulin (TBG)¹ is the major thyroid hormone carrier protein in human serum (1, 2). It is synthesized

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1. Abbreviations used in this paper: IEF, isoelectric focusing; PCR, polymerase chain reaction; TBG, thyroxine-binding globulin; TBG-A, Aboriginal TBG; TBG-C, common type TBG; T₃, triiodothyronine; T₄, thyroxine.

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by liver parenchymal cells and secreted as a 54-kD glycoprotein (3, 4). Although TBG is not necessary for thyroid hormone action, alterations in its concentration produce proportional changes in the level of circulating thyroxine (T₄) and triiodothyronine (T₃) (1, 2).

In 1980, two groups of investigators (5, 6) noted low total T₄ concentration in ~40% of healthy Australian Aborigines. Dick and Watson attributed this finding to the presence of a variant TBG (7). Subsequently, we confirmed their hypothesis by showing that, compared with the common type TBG (TBG-C), this variant TBG (TBG-A) has decreased affinity for T₄ and T₃ and increased susceptibility to denaturation by heat and acid but normal immunoreactivity and microheterogeneity on isoelectric focusing (IEF) (8). The inheritance of TBG-A was X-linked (9) as expected from the recent demonstration that a single TBG gene is located at Xq 21–22 (10).

We now present the amino acid sequence of TBG-A deduced by sequencing of this variant TBG gene. Nucleotide substitutions were found in the codons for amino acids 191 and 283 resulting in replacement of alanine for threonine and leucine for phenylalanine, respectively. Both substitutions were present in the TBG genes of two unrelated Aboriginal men expressing TBG-A but were absent in the TBG genes of three Aboriginal men expressing TBG-C. Interestingly, an identical nucleotide substitution was found in the codon for amino acid 283 in the TBG gene of a Caucasian man, expressing a protein with properties indistinguishable from TBG-C. It thus is likely that the threonine substitution at amino acid 191 is responsible for the altered properties of TBG-A.

Methods

Subjects. Serum samples were obtained from 68 unrelated euthyroid Aborigines, 34 men and 34 women, from Western Australia. Their mean ages \pm SD were 34.9 ± 13 yr (range: 16–60) and 35.5 ± 17 yr (range: 13–78), respectively. These samples were used to determine the prevalence of TBG-A in this population. In addition, serum samples and whole blood collected in EDTA, were obtained from five Aboriginal men, two known to have TBG-A (subjects 1 and 2) and three expressing the common protein, TBG_C (subjects 3, 4, and 5). Sera and whole blood were also obtained from seven Caucasian men having TBG-C. Sera were kept frozen at -20°C and whole blood was kept frozen at -80°C until analysis of TBG and DNA extraction, respectively, except during the 48-h period of shipment in dry ice from Western Australia to Chicago.

Tests of thyroid function. The serum concentrations of total T₄, total T₃, and thyrotropin (TSH) were measured in serum by standard RIAs. TBG concentration in serum was determined by a specific RIA measuring native TBG as previously described (11).

Typing of TBG. The presence of TBG-A, TBG-C, or both TBG-A and TBG-C in heterozygous subjects, was determined by their rate of inactivation at 56°C as previously described (12). Briefly, residual na-

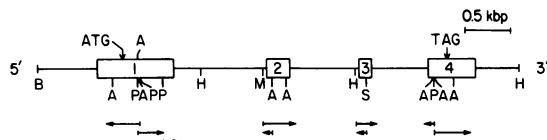


Figure 1. Schematic representation of the TBG gene and strategy of sequencing. Exons 1–4 are denoted by open boxes. Translational initiation (ATG) and termination (TAG) codons are indicated. Only those restriction endonuclease sites relevant to subcloning into M13 phage for sequencing are shown (A, Alu I; B, Bam HI; H, Hind III; M, Msp I; P, Pst I; S, Sph I). Arrows indicate the regions sequenced.

tive (nondenatured) TBG was measured by RIA in diluted serum samples before and 20, 40, and 60 min after exposure to 56°C (11, 12). The $t_{1/2}$ of TBG denaturation was determined by the method of least-squares applied to the best fitting straight line of the logarithm of residual native TBG concentrations and the corresponding times of incubation analyzed. Data were expressed as percent of control to correct for intraassay variation and small (0.2°C) but significant temperature variations during batch analysis. Sera from five Caucasians containing TBG-C were included in each analysis. All samples were submitted to IEF and the pattern of TBG microheterogeneity was determined as previously described (12).

Isolation and sequencing of TBG genes. Unless otherwise specified, recombinant manipulations were carried out using standard protocols (13). Restriction enzymes were purchased from either Bethesda Research Laboratories, Inc. (Gaithersburg, MD) or New England Nuclear (Boston, MA) and used as suggested by the suppliers.

DNA was isolated from the nuclei of peripheral blood lymphocytes as previously described (14) and was digested with Eco RI. Resultant fragments of 10–20 kb, which included the 14-kb fragment containing the TBG gene, were isolated by electrophoresis in a 0.8% low melting-temperature agarose gel and then ligated into the Eco RI arms of λ EMBL4. After packaging and infection of *Escherichia coli* strain LE 392 (Stratagene Corp., San Diego, CA), recombinant phages containing the TBG gene were identified by hybridization with a human liver TBG cDNA probe (λ cTBG-8, reference 15) labeled with 32 P by nick translation (kit; Amersham Corp., Arlington Heights, IL). Appropriate restriction fragments of the TBG gene (Fig. 1), were subcloned into M13mp18 or M13mp19 and sequenced by the dideoxynucleotide chain termination method (16).

Amplification of specific gene segments and their analysis. Exons 2 and 3 of the TBG gene containing the mutation sites were amplified by the polymerase chain reaction (PCR) method. Oligonucleotides A, B, C, and D (Table I and Fig. 2), serving as primers, were synthesized on a DNA Synthesizer (model 308 B; Applied Biosystems, Foster City, CA) by the β -cyanoethyl chemistry.

PCR was carried out by a modification of the method described by Saiki et al. (17). 1.5 μ g of undigested genomic DNA served as the initial template for each pair of primers, 100 pmol of which were added

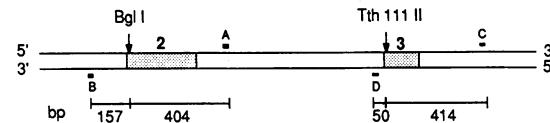


Figure 2. Strategy of amplification of exons 2 and 3 by PCR and analysis using restriction endonucleases. Exons 2 and 3 are indicated. A, B, C, and D are primers used for PCR (Table I) positioned at binding sites. Bars with numbers represent fragment sizes generated by digestion of the amplified TBG-C DNA segments with the indicated restriction endonucleases, whose sites of cleavage are shown by arrows.

together with 2.5 U *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT). The 100- μ l reaction mixture also contained 1.25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and TTP), and 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, and 0.01% gelatin, pH 8.3. The reaction mixture was covered with 100 μ l mineral oil (Sigma Chemical Co., St. Louis, MO) and submitted to amplification using a DNA thermalcycler (Perkin-Elmer Cetus Corp.). The initial template denaturation was at 94°C for 1.5 min. Afterwards, a typical cycle profile was 2.5 min at 55°C for annealing, 3.5 min at 65°C for extension, and 1 min at 94°C for denaturation. After 30 such cycles, the reaction mixtures containing the amplified DNA segments (Fig. 2) were extracted with chloroform to remove the mineral oil and 10 μ l of each was digested separately with either Bgl I and Tth 111 II. To remove DNA binding proteins present in the Tth 111 II preparation and which interfere with the subsequent electrophoretic separation, it was necessary to extract these samples twice with an equal volume of buffer (50 mM Tris-HCl, 1 mM EDTA, and 0.1% β mercaptoethanol, pH 8.0) saturated phenol and once with an equal volume of chloroform/isoamyl alcohol (24:1) and then precipitate with 100% ethanol. DNA fragments were resolved by electrophoresis in a 2% GTG agarose gel (NuSieve; FMC BioProducts, Rockland, ME). The separated DNA fragments were visualized with ultraviolet light after staining with ethidium bromide.

Results

The prevalence of TBG-A. The rates of TBG denaturation in serum samples from the 68 unrelated Australian Aborigines are shown in Fig. 3. Those of the 34 Aboriginal men segregated into either of two distinct groups, one ranging from 94 to 125%

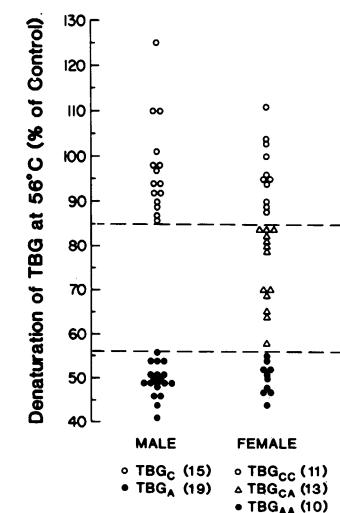


Figure 3. Typing of TBG in serum by the rate of its denaturation at 56°C. Results are expressed as percent of the mean $t_{1/2}$ of denaturation of TBG-C from Caucasians analyzed in the same assay. In parentheses are the number of individuals of the indicated phenotype according to sex.

Table I. Sequences and Locations of Oligonucleotide Primers

Primer	Sequence	Location
A	5'-CTTGGCATATTCTAGTGATC-3'	136 nucleotides 3' from exon 2
B	5'-CCTTGATCCTCAGTCACCTG-3'	151 nucleotides 5' from exon 2
C	5'-TCAGAACTGCATCTCACCA-3'	268 nucleotides 3' from exon 3
D	5'-AAGCTTGATATGGTGATTGC-3'	48 nucleotides 5' from exon 3

Table II. Prevalence of TBG-A and TBG-C and Allele Frequency in Aborigines

	Phenotype (No.)			Allele (No.)	
	A	AC	C	A	C
Men	19	0	15	19	15
Women	10	13	11	33	35
Total	29	13	26	52	50
(%)	42.6	19.1	38.3	50.9	49.1
Expected (%)*	38.4	25.0	36.6		

* Calculated by the formula of Hardy-Weinberg, assuming a TBG_A allele frequency of 50.9% and X-linked inheritance.

of mean Caucasian controls and the other from 56 to 41% of control values. By contrast, the rates of heat denaturation of TBG in samples from Aboriginal women distributed as a continuum spanning the extreme values observed in men (43 to 111% of control). The lowest value in males with normal rates of TBG denaturation (56% of control) and the highest value in males with rapid rates of TBG denaturation (85% of control) served as cut-off points to identify heterozygous women expressing both TBG-A and TBG-C.

Based on these results, the prevalence of TBG-A was determined (Table II). The prevalence of hemizygous men with TBG_A was 55.9% and that for men with TBG-C was 44.1%. 29.4% of women were homozygous for TBG-A, 32.4% were homozygous for TBG-C, and the remaining 38.2% were heterozygotes. This distribution is in good agreement with that expected based on Hardy-Weinberg equilibrium, the TBG_A allele frequency being 50.9%.

The IEF pattern (not shown) of TBG was determined in all samples, and as previously shown (8), no differences were observed between TBG-A and TBG-C.

Sequence of TBG-A. The sequences of the coding regions and adjacent introns of a TBG-A allele from subject 1 (Table III) were compared with those of an allele isolated from an Aboriginal man expressing a TBG with the properties of TBG-C (subject 3 in Table III). The nucleotide sequence of this latter allele was identical to that of three alleles isolated from Caucasian men expressing TBG-C. The sequence of the

TBG_A allele differed at sites in exons 2 and 3 and were verified by sequencing both strands: a G-to-A transition in the codon for amino acid 191 of the mature protein in exon 2 resulted in the substitution of alanine (GCA) with threonine (ACA), and a G-to-T transversion in the codon for amino acid 283 in exon 3 replaced leucine (TTG) with phenylalanine (TTT). These nucleotide substitutions result in the loss of the recognition sites for Bgl I [GCC(N)₅GGC→GCC(N)₅GAC] and Tth 111 II (TGP₃TTG→TTP₃TTG) in exons 2 and 3, respectively (Fig. 4). The mutation in exon 2 was 9 nucleotides downstream from the splice junction with exon 1 (Ala-188, G|CC) and that in exon 3, 13 nucleotides downstream from the splice junction with exon 2 (Gly-279, GG|A).

Identification of TBG-A type substitutions by exon amplification and digestion with restriction endonucleases. To determine whether one or both substitutions are responsible for the observed changes in the properties of TBG-A, we exploited the loss of the recognition sites for Bgl I in exon 2 and Tth 111 II in exon 3 to screen available DNA samples. Exons 2 and 3 from five Aboriginal men (two with TBG-A and three with TBG-C, Table III) and seven Caucasian men with TBG-C were amplified by the PCR (Fig. 2) and digested with Bgl I and Tth 111 II, respectively. As shown in Fig. 5, the Bgl I and Tth 111 II sites were absent in the amplified DNA samples containing exon 2 (561 bp) and exon 3 (464 bp), respectively, generated from two Aboriginal men expressing TBG-A. In contrast, amplified segments of DNA from the three Aboriginal men expressing TBG-C were digested as predicted yielding DNA segments of 404 and 157 bp with Bgl I and a 414-bp segment with Tth 111 II. The 50-bp segment resulting from the latter digestion was not visualized on this agarose gel; furthermore, because digestion with this endonuclease was incomplete, both the original 464-bp segment and the 414-bp fragment of the Tth 111 II digested samples are evident; both fragments hybridized with a TBG-C DNA probe (not shown). The TBG typing of the five Aboriginal men using this procedure was in complete agreement with results of typing based on both heat inactivation and serum T₄ and T₃ concentrations (Table III). The amplified DNA segments of the Aboriginal men expressing TBG-A (subject 2 in Table III) and that of another Aboriginal man expressing TBG-C (subject 4 in Table III) whose TBG genes were not cloned, were sequenced, confirming the results from specific endonucleases digestion.

Table III. Typing of Serum TBG from Men Whose DNA Was Analyzed

Origin	TBG Type	Subject No.	T ₄	T ₃	TSH	TBG					
						μg/dl	ng/dl	μU/ml	min	% of control	mg/dl
Aborigine	TBG-A	1	2.2	31	0.7	23			51		0.76
Aborigine	TBG-A	2	2.0	34	4.0	22			49		0.73
Aborigine	TBG-C	3	10.3	145	4.4	41			90		1.72
Aborigine	TBG-C	4	6.5	91	3.7	51			113		1.10
Aborigine	TBG-C	5	7.2	77	3.1	46			102		1.16
Caucasian [‡]	TBG-C	6	7.8	118	2.4	54			120		1.60
Caucasian	TBG-C	7	6.2	103	1.8	40			89		1.10

* Heat denaturation at 56°C. [‡] Data from five additional Caucasian men with TBG-C are not shown.

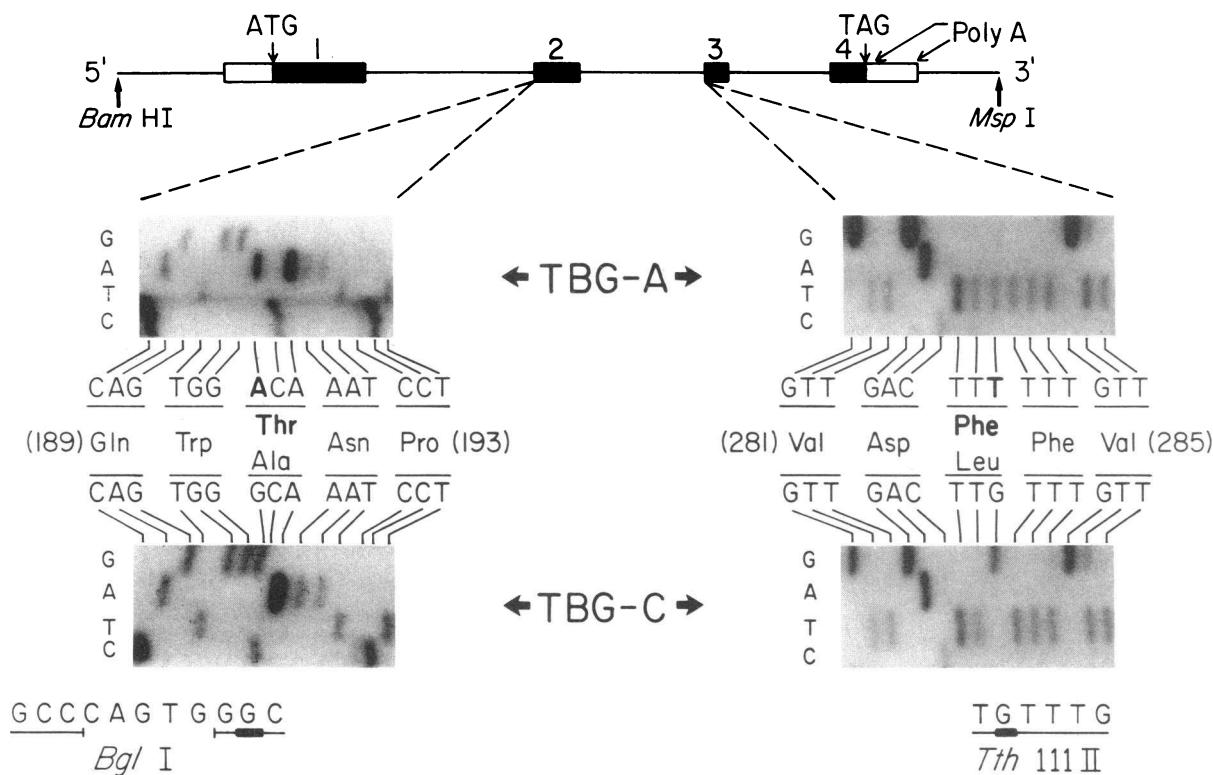


Figure 4. Structure of the TBG gene and sections of sequencing gels showing the substitutions in TBG-A. On top of the figure is the schematic representation of the gene with translated sequences (■), untranslated exon sequences (□), intervening and flanking sequences (—). Initiation (ATG) and termination (TAG) codons and the alternative polyadenylation sites (Poly A) are indicated. Amino acid residues numbered consecutively from the NH₂-terminus of the mature protein are shown in brackets. G in codon 191 of TBG-A is replaced by A in TBG-C and G in codon 283 by T. The resulting amino acid substitutions are indicated. The losses of restriction endonucleases recognition sites resulting from the two point mutations are also shown.

More revealing were the results obtained on the analysis of seven Caucasian men expressing TBG-C. Whereas exon 2 of each could be digested with Bgl I, one of the seven samples resisted digestion with Tth 111 II (Fig. 5, subject 7). The sequence of exon 3 of this subject revealed the same nucleotide substitution in the codon for residue 283 as that found in the Aboriginal men with TBG-A. However, the TBG synthesized by this individual had properties indistinguishable from those of TBG-C, including rate of heat denaturation and IEF pattern. Moreover, his serum T₄ and T₃ concentrations were within the normal range (Table III, subject 7).

Discussion

Measurement of the rate of TBG denaturation at 56°C provides a means of distinguishing not only TBG-A from TBG-C but also of identifying heterozygotes having both TBG-A and TBG-C (9). The demonstration of the TBG_{AC} phenotype in 13 of 34 Aboriginal women and its absence in 34 Aboriginal men, is consistent with X-linked mode of inheritance of this gene. An earlier report suggesting an autosomal inheritance (18) was due to the inability to identify heterozygotes combined with the high frequency of the TBG_A gene in Australian Aborigines. In the larger sample presented here, the frequencies of the TBG_A and TBG_C alleles are almost equal, 50.9 and 49.1%, respectively. Because men have a single TBG gene and women have two alleles, in the mixed population 29 (42.7%) had in

serum only TBG-A, 26 (38.2%) had only TBG-C, and 13 (19.1%) had both TBG-A and TBG-C.

Of the two amino acid replacements in TBG-A, the substitution of phenylalanine for leucine-283 represents a relatively conservative replacement and thus is unlikely to be responsible for the abnormal properties of TBG-A. Moreover, we also observed the same substitution in one of seven Caucasian men examined, in whom it was not associated with detectable changes in the properties of the TBG molecule. Thus, it seems likely that the Leu/Phe-283 represents a common polymorphism that antedates the mutation at amino acid 191. The latter, present in both Aboriginal men with TBG-A examined, results in the substitution of a polar threonine for the nonpolar alanine-191.

The data strongly suggest that the threonine-191 substitution is responsible for both abnormal binding affinity and stability of TBG-A. What remains unclear is whether it is solely responsible for the observed alterations or whether the phenylalanine-283 is also required. As the threonine-191 mutation likely occurred on a chromosome having phenylalanine at residue 283, it is unlikely that the analysis of a larger group of Aboriginal men with TBG-A will resolve this quandary. A more direct approach would require the in vitro expression in cultured cells of variants having one and both substitutions.

Finally, we would propose changing the eponym, TBG-A, for TBG^{Thr-191, Phe-283} and to designate the TBG with properties indistinguishable from TBG-C, TBG^{Phe-283}.

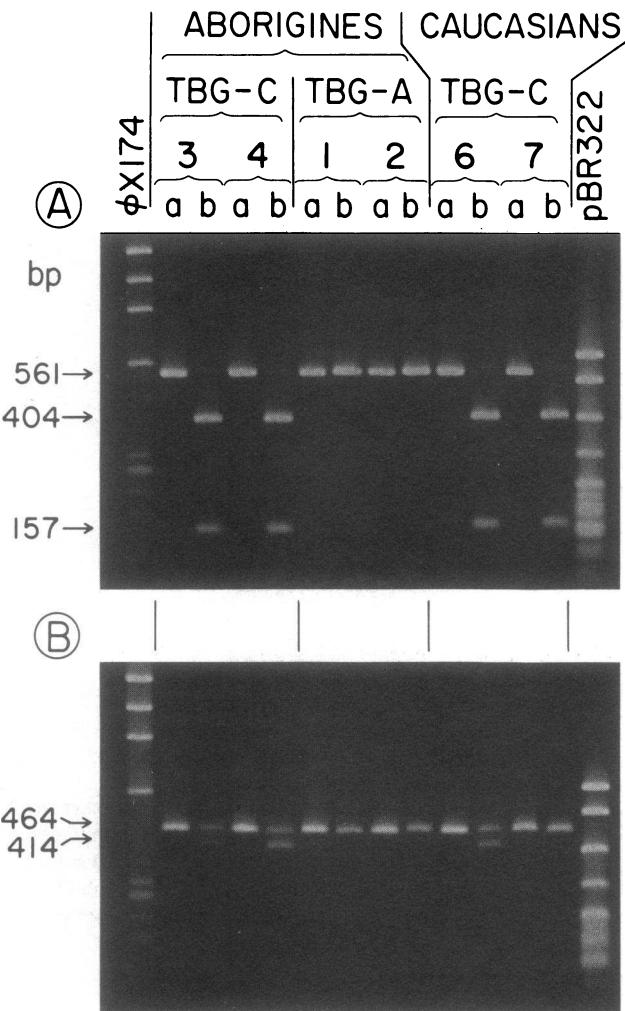


Figure 5. Detection of nucleotide substitutions by restriction endonuclease digestion of amplified DNA sequences. Numbers identifying lanes correspond to the subjects described in Table III. *a* and *b* denote undigested and digested samples, respectively. (A) Digestion of the amplified DNA segments containing exon 2 (561 bp) with Bgl I generated two fragments of 404 and 157 bp in all DNA samples, except those from Aboriginal men expressing TBG-A. (B) Digestion of the amplified DNA segments containing exon 3 (464 bp) with Tth 111 II should generate two fragments of 414 and 50 bp in TBG-C. The 50-bp fragment was not visualized and incomplete digestion (see Methods) resulted in two fragments, the undigested fragment of 464 bp and a 414-bp fragment. The three DNA samples from Aboriginal men with TBG-C (only two shown) and seven of eight samples from Caucasian men with TBG-C (only one is shown, subject 6) were digested by the enzyme. DNA samples from the Aboriginal men with TBG-A and from a Caucasian man with TBG-C (subject 7) were not digested.

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