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Detecting Deletions, Insertions, and Single Nucleotide Substitutions in Cloned β-globin Genes and New Polymorphic Nucleotide Substitutions in β-globin Genes in a Japanese Population Using Ribonuclease Cleavage at Mismatches in RNA:DNA Duplexes

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放影研 **Radiation Effects Research Foundation**
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The Radiation Effects Research Foundation (formerly ABCC) was established in April 1975 as a private nonprofit Japanese Foundation, supported equally by the government of Japan through the Ministry of Health and Welfare, and the Government of the United States through the National Academy of Sciences under contract with the Department of Energy.

財団法人　放射線影響研究所
業　績　報　告　書

RERF TR 1-90
研究課題 7-85

リボヌクレアーゼによるRNA:DNAデュプレックス中のミスマッチ切断法を用いたクローン化βグロビン遺伝子における塩基欠失・挿入・置換の検出，及び日本人集団におけるβグロビン遺伝子の新しい多型性置換の検出§

Detecting Deletions, Insertions, and Single Nucleotide Substitutions in Cloned β-globin Genes and New Polymorphic Nucleotide Substitutions in β-globin Genes in a Japanese Population Using Ribonuclease Cleavage at Mismatches in RNA:DNA Duplexes

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要　約

ヌクレオチド変異型の頻度を測定する場合に，「リボヌクレアーゼによるRNA:DNAデュプレックス中のミスマッチ切断法(RNase cleavage method)」が応用可能であるか否かを日本人集団を用いて検討した．クローン化したヒトの正常及び3種のサラセミアβグロビン遺伝子中の異なる4領域から，種々の長さのDNA断片を切り出し，転写ベクターに挿入し，^{32}Pラベルしたセンス及びアンチセンスRNAプローブを作製した．771ヌクレオチド以下の長さのRNAプローブをクローン化DNAとハイブリダイズし，生じた2本鎖をリボヌクレアーゼAとT_1の混合物で処理したところ，生成物の長さは理論値と一致した．考えられる12種のミスマッチについて検査した．センス及びアンチセンスプローブを用いているので，RNA鎖とDNA鎖の組合せのうち一方からは，G:T及びG:Gのように切断されないミスマッチも生じたが，これらはもう一方の組合せを用いることによって切断可能なC:A及びC:Cミスマッチに変換可能であった．1(G)，4(TTCT)，5(ATTTT)，10(ATTTTATTTT)ヌクレオチドの欠失及び挿入も容易に検出された．広島の59人の末梢Bリンパ球から樹立された細胞株のDNA及び

§本報告にはこの要約以外に訳文はない．

承認1990年1月12日　　印刷1990年8月

それをpolymerase chain reaction（PCR）により増幅したDNAを用いて，βグロビンの第2介在配列の666番目（IVS2-666）のヌクレオチドのTからCへの多型性置換を検出した．IVS2-666にCをもつ対立遺伝子の頻度は0.48で，Tをもつ対立遺伝子の頻度は0.52であった．観察値はHardy-Weinbergの法則から得られる期待値と一致した．11家族から得られた結果には，メンデル式遺伝に矛盾するものはなかった．PCRで増幅した染色体DNAを用いて，cap siteから1789番目及び1945番目のヌクレオチドにおけるCからA及びAからTへの新しい多型性置換を検出した．染色体DNAにおける変異の大規模スクリーニングにおいて，PCRを用いたRNase cleavage methodは有効であると判断した．

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Technical Report Series

Detecting Deletions, Insertions, and Single Nucleotide Substitutions in Cloned β-globin Genes and New Polymorphic Nucleotide Substitutions in β-globin Genes in a Japanese Population Using Ribonuclease Cleavage at Mismatches in RNA:DNA Duplexes[§]

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Summary

The applicability of ribonuclease (RNase) cleavage at mismatches in RNA:DNA duplexes (the RNase cleavage method) for determining nucleotide variant rates was examined in a Japanese population. DNA segments of various lengths obtained from four different regions of one normal and three thalassemic cloned human β-globin genes were inserted into transcription vectors. Sense and antisense RNA probes uniformly labeled with ^{32}P were prepared. When RNA probes of 771 nucleotides (nt) or less were hybridized with cloned DNAs and the resulting duplexes were treated with a mixture of RNases A and T1, the length of products agreed with theoretical values. Twelve possible mismatches were examined. Since both sense and antisense probes were used, uncleavable mismatches such as G:T and G:G which were made from one combination of RNA and DNA strands could be converted to the cleavable C:A and C:C mismatches, respectively, by using the opposite combination. Deletions and insertions of one (G), four (TTCT), five (ATTTT), and 10 (ATTTTATTTT) nt were easily detected. A polymorphic substitution of T to C at position 666 of the second intervening sequence (IVS2-666) of the β-globin gene was detected using genomic DNAs from cell lines established from the peripheral B lymphocytes of 59 unrelated Japanese from Hiroshima or those amplified by polymerase chain reaction (PCR). The frequency of the gene with C at the IVS2-666 (allele *C*) was 0.48 and that of the gene with T (allele *T*) was 0.52. The associations of the two alleles were in agreement with Hardy-Weinberg proportions. No contradiction to Mendelian

[§]**The complete text of this report will not be available in Japanese.**

Approved 12 January 1990 **Printed August 1990**

inheritance was observed in the results obtained from 11 family studies. Two new polymorphic substitutions of C to A and A to T were detected at nucleotide positions 1789 and 1945 from the capping site, respectively, using genomic DNAs amplified by PCR. We conclude that it would be feasible to use the RNase cleavage method combined with PCR for large-scale screening of variation in chromosomal DNA.

Introduction

Evaluating any potential genetic effects of atomic bomb radiation is important not only for the welfare of the children of A-bomb survivors, but also for industrialized societies that rely on nuclear energy and medical applications of ionizing radiation. Extensive studies on the children of A-bomb survivors of Hiroshima and Nagasaki have thus far yielded no statistically significant increases in genetic effects when compared to a control population.[1] With particular reference to biochemical studies, approximately 13,000 children of proximally exposed ($\leq$2,000 m from hypocenter) survivors from Hiroshima and Nagasaki and 10,000 control children were examined for fresh mutations of 30 blood proteins using starch gel electrophoresis. Three mutations resulting in altered mobility were detected in each group after examining approximately 670,000 loci in the former group and 470,000 loci in the latter, respectively.[2,3] A subset of the children was examined for loss of activity in nine erythrocyte enzymes. One mutation was noted in the former group, but none in the latter after examining approximately 60,000 loci in each. Thus, no significant differences in mutation rates were observed between children of the exposed and the control children.

Because of the importance of exhausting all possible approaches to the genetic evaluation of these children, the Radiation Effects Research Foundation has recently endorsed exploring the feasibility of studies at the DNA level. For a study determining nucleotide (nt) mutation rates, it is necessary to choose a method which can examine as many base pairs (bp) as possible at one time. The methods of ribonuclease (RNase) A cleavage at mismatches in RNA:DNA duplexes, reported by Myers et al,[4] and denaturing gradient gel electrophoresis (DGGE) of DNA:DNA heteroduplexes, reported by Lerman and colleagues,[5–8] seem to be the most suitable among those available at present. Myers et al.[4] reported results of the RNase A cleavage of all the 12 possible types of mismatches in duplexes of 186 bp produced by hybridization of an RNA probe transcribed from a DNA fragment including the mouse β-major globin promoter region with cloned DNA fragments of the same region having single base substitutions. They noted that 1/3 of all possible single base substitutions could be detected by the RNase A method with an RNA probe homologous to one strand of the test DNA. This number could be doubled with the use of a second RNA probe, homologous to the opposite strand of the test DNA. Several examples of the RNase A cleavage at mismatches in duplexes of approximately 615 bp made from human β-globin gene fragments were also described. The method using RNase cleavage at mismatches in RNA:RNA duplexes has been successfully applied to detect mutations in human cellular *KRAS* oncogenes,[9,10] human hypoxanthine-guanine phosphori-

bosyltransferase (*HPRT*) genes,[11] mouse ornithine carbamoyltransferase genes,[12] and influenza viruses.[13] Almoguera et al[14] successfully detected mutant c-*KRAS* genes in formalin-fixed, paraffin-embedded tumor tissues after the genes were amplified by PCR. DGGE identified two new polymorphic sites within a 1.2 kilobase (kb) region of human chromosome 20.[15] A combination of DGGE and PCR permitted rapid identification and sequencing of the C to A substitution at position 397 of *HPRT* Munich in genomic DNA.[16]

We have been experimenting with the use of both methods in combination.[17–19] Here we present results obtained by using the RNase cleavage method that detected mismatches caused by single base substitutions, deletions, or insertions of one, four, five, and 10 bp in various sizes of RNA:DNA duplexes made from hybridization of RNA probes transcribed from DNA fragments of various parts of human β-globin genes with DNA fragments of cloned human β-globin genes. We also demonstrate that this method, using chromosomal DNA obtained from a Japanese population or those DNAs amplified by PCR[20,21] could detect three polymorphic nucleotide substitutions, one of which had previously been detectable only by sequence analysis and two of which have never been reported. Results obtained from DGGE of the same RNA:DNA duplexes are described elsewhere.[22]

Materials and Methods

DNA samples

Cloned DNA samples were made from five recombinant plasmids described below by digesting them with restriction enzymes which do not cleave within the sequence homologous to the RNA probe. YF204 is a plasmid pBR322 carrying the *Bgl*II fragment from the clone of a human normal β-globin gene originally isolated by Dr. T. Maniatis (Department of Biochemistry and Molecular Biology, Harvard University, MA), YF226 is a plasmid pUC9 carrying the *Bgl*II fragment of a thalassemic β-globin gene from a Thai individual that has the same TTCT deletion within codons 41 and 42, as reported previously,[23] and polymorphic nucleotide substitutions of C to T, C to G, G to T, and T to C at the third position within codon 2 and positions 16, 74, and 666 of IVS2 (IVS2-16, 74, and 666), respectively. YF227 is a plasmid pMK2004 carrying the *Bgl*II fragment of a thalassemic β-globin gene from a Chinese. A substitution of C to T occurred at position 654 of IVS2 (IVS2-654). πSVWT2 is a plasmid πSVHP carrying the *Bgl*II-*Pst*I fragment of a human normal β-globin gene. πSVβ°39 is a plasmid πSVHP carrying the *Bgl*II-*Pst*I fragment of a human thalassemic β-globin gene. A substitution of C to T occurred within codon 39. Chromosomal DNAs were isolated from cell lines established by Epstein-Barr virus transformation from peripheral B lymphocytes of 59 healthy unrelated Japanese and their 20 children using the standard procedure.[24] They were digested with appropriate restriction enzymes before hybridization with RNA probes.

RNA probes

To obtain DNA templates to synthesize RNA probes, each one of the DNA fragments of regions I, II, III, and IV of the β-globin genes (Figure 1) from the

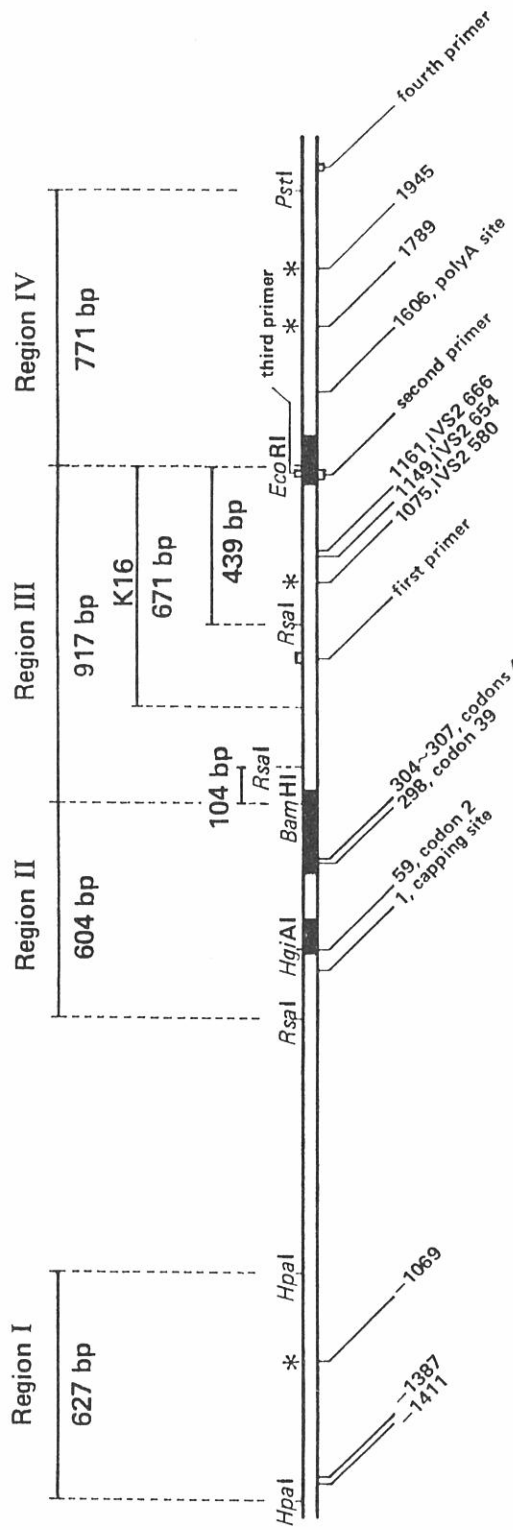

Figure 1. A schematic diagram of the human β-globin gene. Four regions (I, II, III, IV), the K16 fragment of YF226, and *Rsa*I-*Eco*RI and *Bam*HI-*Rsa*I fragments in region III of YF204 are indicated by the lengths (bp) of the sense DNA strands. Exons (solid blocks), introns (open blocks), the capping site, the polyA addition site, the sequences used as four primers for the polymerase chain reaction, and the locations of four sequence differences (*) detected in this study are also indicated. The repetitive sequence of $(ATTTT)_5$ starts at –1411 and ends at –1387. Nucleotides are numbered beginning with 1 at the capping site.

above described five plasmids or a 439 bp *Rsa*I-*Eco*RI fragment from region III of YF204 was inserted into pGEM-3 or pGEM-3 blue vector (Promega Biotec, Madison, Wis, USA). A plasmid containing a 671 bp K16 fragment (see Figure 1) was produced from a recombinant pGEM-3 plasmid containing a *Bam*HI-*Eco*RI fragment of YF226 by deletion of 246 bp from the 5′ side of the fragment with exonuclease III. The K16 fragment was longer than the *Rsa*I-*Eco*RI fragment by 232 nt on the 5′ side. In this 232 nt region, no variation was reported. The resulting recombinant plasmid was linearized by digestion with an appropriate restriction enzyme. Transcription and isolation of the RNA probe was carried out following the method of Melton et al[25] with minor modifications. RNA probes were named to show sequences of DNA templates from which they were transcribed. For example, probes transcribed from the 439 bp coding strand of region III of the YF204 clone and the coding strand of K16 were named sense YF204III-439 and sense K16, respectively. To synthesize the probe for examining cloned DNA samples, 1 μg of template DNA was treated for one hour at 40°C with 10 units of SP6-RNA or T7-RNA polymerase (Promega Biotec) in 20 μl of transcription mixture containing 40 mM tris-HCl, pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM dithiothreitol, 1 unit/μl of RNasin (Promega Biotec), 500 μM each of ATP, GTP, and UTP, 740 KBq of α-^{32}P-CTP (1.48 TBq/mmol, 25 μM), and 5 μg of transfer RNA (BRL, Gaithersburg, Md, USA). For the RNA probe with high specific activity used to examine genomic DNA samples, 3.7 MBq of α-^{32}P-CTP (14.8 TBq/mmol, 12 μM) was used. The template DNA was removed by the addition of RNase-free DNaseI (Worthington, Freehold, NJ, USA) and RNasin to the transcription mixture at the final concentration of 0.1 unit/μl and 1 unit/μl, respectively. After a 15-minute incubation at 37°C, the mixture was extracted with phenol:chloroform, and the transcript was precipitated by the addition of sodium acetate (pH 5.5) to 0.25 M and 2.5 volume of ethanol. The RNA probe was dissolved in a solution of 10 mM tris-HCl (pH 7.5), 1 mM EDTA, and 0.1% SDS to make 0.5 μl of the solution containing approximately 3×10^5 cpm for cloned DNA samples or 2×10^6 cpm for genomic DNA samples.

Hybridization and RNase treatment

Hybridization of 200 ng of cloned DNA or 4.5 μg of genomic DNA in 30 μl of hybridization buffer with labeled RNA probe in 0.5 μl of the probe solution, and RNase treatment of the duplex and extraction of the products from the reaction mixture were performed following the method of Myers et al[4] with minor modifications: the hybridization was carried out overnight for both cloned and genomic DNA samples, the concentration of RNase A (Sigma, St. Louis, Mo, USA) was decreased to 10 μg/ml or 5 μg/ml, and 250 ng/ml of RNase T1 (Sankyo, Tokyo) was added in the RNase treatment procedure.

Gel electrophoresis

Extracted products from RNase treatment were suspended in 2 μl of loading buffer containing 80% formamide, 0.05% xylene cyanol, and 1% sarcosine, and they were analyzed by denaturing polyacrylamide gel electrophoresis using 8%

Gensor gel (Fuji Photo Film, Kanagawa) developed for DNA sequence analysis. As markers, 22 kinds of template DNAs with various lengths in a mixture were transcribed by T7-RNA polymerase with 1.48 TBq/mmol α-^{32}P-CTP, extracted without DNaseI treatment, precipitated, and suspended in the loading buffer. Autoradiography was carried out at –80°C with Fuji RX X-ray film and a Dupont Gronex HI-Plus intensifying screen (Dupont, Wilmington, Del, USA).

DNA sequencing

The chain termination method[26,27] was used for sequencing recombinant pGEM-3 and pGEM-3 blue clones using SP6- or T7-promoter primer (Promega Biotec) and Klenow fragment of *E. coli* DNA polymerase I.

DNA amplification by PCR and direct sequencing of PCR products

The first and the second primers of 30 mer have a sequence between IVS2-353 and IVS2-382 in the sense DNA strand and one between positions 13 and 42 of exon 3 in the antisense strand, respectively, of the human β-globin gene. The third and the fourth primers of 20 and 30 mers, respectively, have a sequence between positions 23 and 42 of exon 3 in the sense DNA strand and one between the 2208th and 2237th nt from the capping site in the antisense DNA strand, respectively, of the human β-globin gene (Figure 1). Amplification of the target sequences was carried out following the protocol recommended by Perkin Elmer-Cetus[28] (Norwalk, Conn, USA). A reaction mixture of 100 μl contained 1 μg of genomic DNA and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase in 50 mM KCl, 10 mM tris (pH 8.3), 1.5 mM $MgCl_2$, each primer at 1 μM, each dNTP (dATP, dCTP, TTP, dGTP) at 200 μM, and gelatin at 100 μg/ml. The reaction mixture was heated at 94°C for 10 minutes without *Taq* polymerase and rapidly cooled to 4°C. After addition of the polymerase, the reaction mixture was overlaid with mineral oil and subjected to amplification in a programmable heat block (Perkin Elmer-Cetus Instruments). Conditions of thermal cycling for the first target sequence of 540 bp between IVS2-353 and position 42 of exon 3 were 94°C for one minute, 60°C for two minutes, 72°C for three minutes, and 30 cycles. For the second target sequence of 870 bp between position 23 of exon 3 and the 2237th nt from the capping site, conditions were 94°C for 20 sec, 68°C for two minutes, 72°C for one minute, and 40 cycles. In the last cycle, heating at 72°C was extended to 10 minutes. Products from PCR were precipitated with ethanol after phenol-chloroform extraction[24] and 1/20 aliquots of the products were examined using the RNase cleavage method. Direct sequencing of the first target sequence after PCR amplification was carried out[28,29] using approximately 1 pmol (1/5) of the products and 5 pmol of sequencing primer of 20 mer that has a sequence of the antisense DNA between IVS2-743 and IVS2-762 and AMV reverse transcriptase (Seikagaku Kogyo, Tokyo).

Results

In the preliminary experiments, we found that full-length transcription products could be obtained using either SP6-RNA polymerase or T7-RNA polymerase and DNA templates of approximately 2 kb inserted into a pGEM-3 plasmid which

contains both SP6-RNA and T7-RNA polymerase promoters. Nevertheless, it was difficult to get good results using RNase cleavage at mismatches in RNA:DNA duplexes of lengths longer than 1 kb, since many nonspecifically cleaved bands appeared in addition to the expected bands. Therefore, we produced RNA probes by transcription of various DNA fragments shorter than 917 bp obtained from four regions (see Figure 1) of cloned human β-globin genes of the normal and thalassemic types.

A part of the data obtained from cloned DNA samples is shown in Table 1 and brief explanations are given. Results obtained using genomic DNA samples are also described.

Table 1. Detection of mismatches, deletions, and insertions in the cloned β-globin genes using the RNase cleavage method

Regions	Probes*	Sample DNA	Mismatches, deletions, and insertions	
			Detected**	Not detected
I	Sense YF227I	YF204, πSVWT2	AAAAT del.	
		YF226	AAAAT ins.	
		πSV$\beta^0$39		G:T
	Antisense YF227I	YF204, πSVWT2	ATTTT del.	
		YF226	ATTTT ins.	
		πSV$\beta^0$39	C:A	
II	Sense YF204II	YF226	AGAA del. C:A	
		πSV$\beta^0$39	C:A	
	Antisense YF204II	YF226	TTCT del.	G:T
	Sense YF226II	YF204	AGAA ins. U:G	
	Antisense YF226II	YF204	TTCT ins.	A:C
III	Sense K16† (YF226)	YF204	C:A	
		YF227	C del. 2 × C:A‡	
	Antisense K16 (YF226)	YF204		G:T
		YF227	G del.	2 × G:T‡
	Sense YF204III-439	YF226	U:G	
		YF227	C del. C:A	
	Antisense YF204III-439	YF226	A:C	
		YF227	G del.	G:T
	Sense YF204III-104	YF226	C:C	G:A
	Antisense YF204III	YF226	C:T	G:G
	Sense YF226III-104	YF204	U:C	G:G
	Antisense YF226III	YF204	A:G C:C	
IV	Sense YF204IV	YF226	C:T	
	Antisense YF204IV	YF226	G:A	
	Sense YF226IV	YF204	A:G	
	Antisense YF226IV	YF204	U:C	
		PCR-0010-IV	U:C U:T	
	Antisense PCR-0010-IV	YF226	G:A A:A	

*The sense YF227I defines a probe which has a sequence corresponding to that of the coding strand of region I of YF227.

**Mismatches detected by both complete cleavage and partial cleavage are shown in this column.

†The sense or antisense K16 probe has a sequence corresponding to a coding or complementary strand of YF226 of 671 bp in region III shown in Figure 1.

‡Two C:A or G:T mismatches at different positions of duplexes.

Results with cloned DNA samples

Region I. A variable number of tandemly repeated sequences, (ATTTT)n, is reported to exist approximately 1400 nt 5′ to the capping site (–1411 to –1387) of the β-globin gene.[30,31] Sequence analysis revealed four repeats in YF204 and πSVWT2, five in YF227 and πSVβ°39, and six in YF226. *Hpa*I (Region I) fragments from these clones were employed as DNA samples and templates to make RNA probes. With various combinations of the samples and probes, deletions as well as insertions of 5 or 10 nucleotides were detected. Deletions were more sensitive to the RNase treatment. In addition, bands of approximately 370 nt and 250 nt appeared for πSVβ°39. Sequence analysis revealed a new G to A substitution 1069 nt 5′ to the capping site of the β-globin gene.

Region II. YF226 had a deletion of TTCT in codons 41 and 42 and a polymorphic substitution of C to T in codon 2 (loss of the *Hgi*AI site), whereas πSVβ°39 had a C to T substitution in codon 39. When YF226 was examined with a sense RNA probe produced from the region II fragment of YF204 (sense YF204II), four bands corresponding to those of 432 nt, 176 nt, 246 nt, and 186 nt were observed without a band of 608 nt that was expected to appear for a DNA sample having the β-globin gene with no variations in this region. The first two intense and rather broad bands and two remaining weak bands indicated that the RNA probe was cleaved completely at the site of four base deletions and partially at the site of the polymorphic substitution (Figure 2).

Region III. For YF226, there were substitutions of C to G, G to T, and T to C at IVS2-16, 74, and 666, respectively. For YF227, a C to T substitution at IVS2-654 was detected by Y. Fukumaki of Kyushu University (personal communication). In addition, a deletion of one of the three Gs between IVS2-579 and IVS2-581 happened to be detected in this study. When the sense K16 probe having a sequence of 671 nt corresponding to that of YF226 (Figure 3) was employed, a 237 nt band appeared for both YF204 and YF227. This band was one of the products of RNase cleavage at a C:A mismatch produced from a T to C substitution at the IVS2-666 of YF226. For YF227, in addition to the C:A mismatch described above, another C:A mismatch at IVS2-654 and a deletion of C around IVS2-580 were detected by the appearance of bands of approximately 250 nt and 320 nt, respectively. Thus, three sequence differences in the 671 nt fragment were clearly detected in one experiment. Employing the sense and antisense YF204III-439 probes, the substitutions of T to C at the IVS2-666 in YF226 and C to T at the IVS2-654 in YF227 produced mismatches of U:G, A:C, C:A, and G:T, and the former three were detected.

Region IV. The five clones were examined with sense and antisense probes produced from the *Eco*RI-*Pst*I (region IV) fragment from YF204 (called sense YF204IV and antisense YF204IV, respectively). For both of the probes, YF226 showed two new bands of approximately 390 nt and 380 nt without the band of approximately 770 nt which was observed for the other four clones. Sequence analysis revealed a C to A substitution at the 1789th nt from the capping site in YF226.

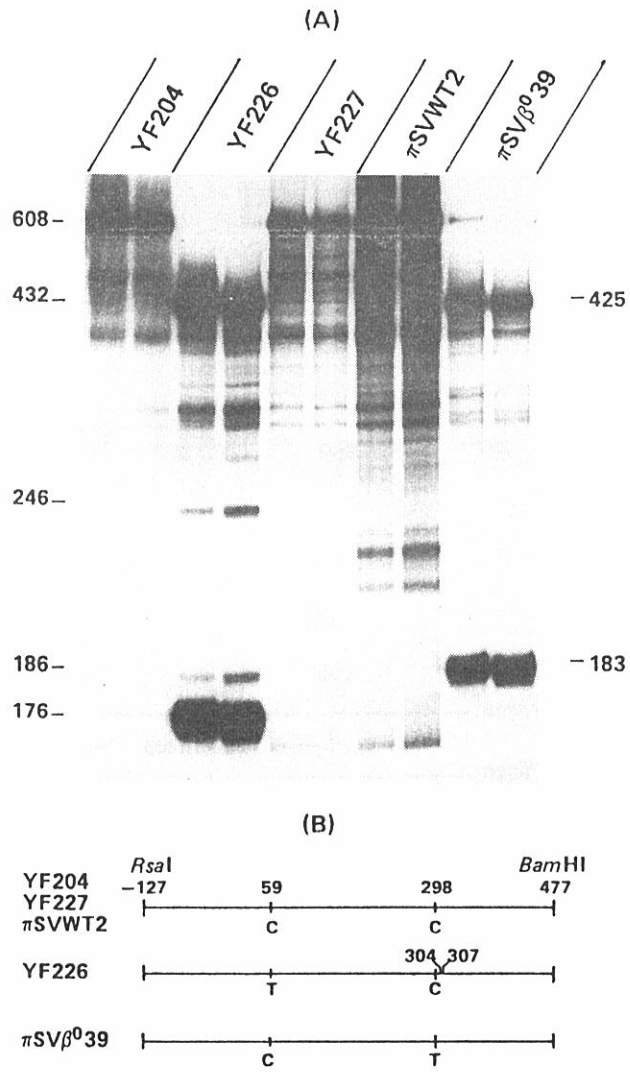

Figure 2. RNase cleavage of region II fragments of human β-globin gene clones. Each of five human β-globin gene clones was digested by *Rsa*I and *Bam*HI and was hybridized with the sense YF204II probe. The resulting duplex was treated with RNases A and T1. Concentrations of RNase A were 5 μg/ml and 10 μg/ml, respectively, for the duplexes in the left and right lanes, and that of RNase T1 was 250 ng/ml for the duplexes in both lanes. Expected sizes of bands are shown by the number of nucleotides outside the autoradiogram (A). Schematic diagrams of region II fragments of five clones are shown in (B). Positions of the nucleotide substitutions are shown by numbers, beginning with 1 at the capping site. In YF226, TTCT between positions 304 and 307 was deleted.

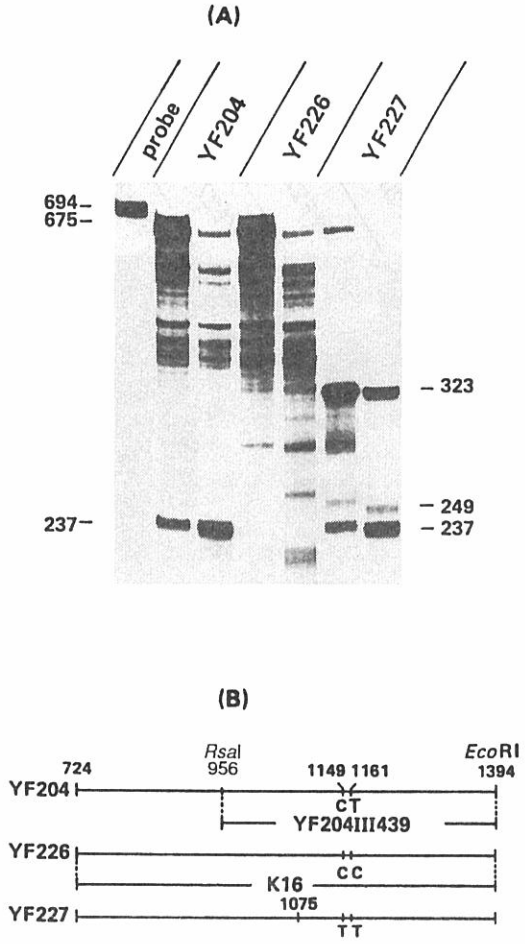

Figure 3. Region III fragments of the human β-globin gene clones. Each of the three human β-globin gene clones was digested by *Bam*HI and *Eco*RI, hybridized with the sense K16 probe, and treated with RNases A and T1. Concentrations of RNase A were 5 μg/ml and 10 μg/ml, respectively, for the duplexes in the left and right lanes, and that of RNase T1 was 250 ng/ml for the duplexes in both lanes. The untreated sense K16 probe is shown in the first lane from the left in the autoradiogram (A). Expected sizes of bands are shown by the number of nucleotides outside the autoradiograms. Schematic diagrams of region III fragments of the three human β-globin gene clones are shown in (B). Positions of nucleotide substitutions are shown by numbers, beginning with 1 at the capping site. In YF227, one of the three Gs between 1074 (IVS2-579) and 1076 (IVS2-581) was deleted.

Results with genomic DNA samples

Region III. Genomic DNA samples obtained from 59 unrelated individuals were examined with the sense K16 probe. The 237 nt band appeared for 45 individuals, showing a sequence difference at the IVS2-666 in the β-globin gene. Considering a) the four frameworks of β-globin genes which are defined by combinations of five polymorphic nucleotide substitutions within codon 2 and at the IVS2-16, 74, 81, and 666,[32] and that b) the sense K16 probe had C at the IVS2-666, the 45 individuals were therefore believed to have T at the IVS2-666 on one or two

chromosomes. However, the possibility that they had A or G was not neglected. The remaining 14 individuals who did not show any 237 nt band should have had C on both chromosomes. Since the 59 individuals included 11 husband–wife pairs, their 20 children were also examined. Examples of results are shown in Figure 4. To confirm the results described above and to clarify whether the 45 individuals had T at IVS2-666 on one or two chromosomes, amplification by PCR of a DNA fragment of 540 bp from the original 59 individuals was carried out with the first and second primers (Figure 1). After digestion with *Rsa*I, a fragment of 432 bp was examined with the sense K16 and the sense YF204III-439 probes, respectively. Examination with the sense K16 probe confirmed the observations described above. Using the sense YF204III-439 probe, the 14 individuals who showed no cleaved bands for the sense K16 probe and 29 among the 45 individuals who showed the 237 nt band showed two bands corresponding to those of 226 nt and 206 nt, whereas the remaining 16 among the 45 individuals showed neither band. Therefore, it was concluded that the 16 and the 14 individuals had T and C, respectively, on both chromosomes 11, whereas the 29 probably had T and C, respectively, on each chromosome 11. The results are shown in Table 2. No contradiction to Mendelian inheritance was observed in the 11 family studies. The identical conclusion was obtained by DGGE, and precise family study data are shown in Takahashi et al.[22] One of the family studies is shown in Figure 5. Nucleotides at the IVS2-666 of the husband, wife, and their son were determined to be T/T, C/C, and T/C, respectively. Direct sequencing of the PCR products confirmed these findings.

Region IV. To determine the genetic nature of the C to A substitution at the 1789th nt from the capping site detected in YF226 and to determine whether it was a new polymorphic substitution or a rare phenomenon, PCR-amplified region IV fragments obtained from members of three families were examined. Among six unrelated individuals (three husbands and three wives who were included among the 59 individuals examined for region III), two exhibited a very intense band of approximately 380 nt and a weak band of approximately 390 nt for the antisense YF226IV probe as shown in Figure 6(A), but showed none for the antisense YF204IV probe. Three individuals exhibited two bands for the latter, but none for the former, whereas the remaining person showed two bands for both probes. The observations indicated that the C to A substitution at the 1789th nt from the capping site of the β-globin gene may not be a rare Japanese phenomenon, and that the first two and the second three individuals should have had C and A, respectively, on both chromosomes 11, whereas the last individual most probably had C on one chromosome 11 and A on the other. The results obtained from examining their children conformed to Mendelian expectation.

In the course of the examinations, three individuals (the husband and wife of the second family and the husband of the third family) exhibited a band of approximately 220 nt for both probes, suggesting the existence of another sequence abnormality in the region IV fragment. Results obtained by the antisense YF226IV probe are shown in Figure 6(A). The region IV fragment of the

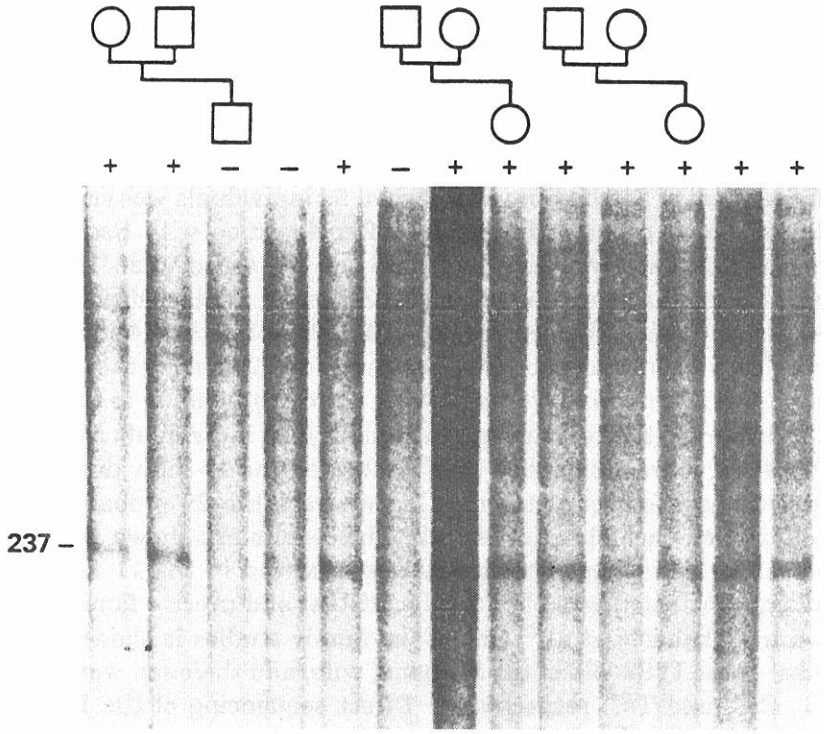

Figure 4. Examination of region III of the human *beta*-globin genes in genomic DNAs using the RNase cleavage method. *Bam*HI and *Eco*RI digest of genomic DNA was hybridized with the sense K16 probe and treated with RNase A (10 μg/ml) and RNase T1 (250 ng/ml). The autoradiogram shows results obtained from 13 individuals, who were members of three families plus four unrelated individuals. (+) indicates the presence of the 237 nucleotide band.

Table 2. Examination of PCR-amplified genomic DNA fragments including position 666 of IVS2 of the β-globin gene obtained from 59 unrelated Japanese by using the sense K16 probe and the sense YF204III-439 probe

Number of individuals	Cleavage of probes in 432 base pair duplexes		Nucleotides at IVS2-666
	Sense K16*	Sense YF204III-439**	
16	+	–	T/T
29	+	+	T/C
14	–	+	C/C

*The sense K16 probe has C at IVS2-666.

**The sense YF204III-439 probe has T at IVS2-666.

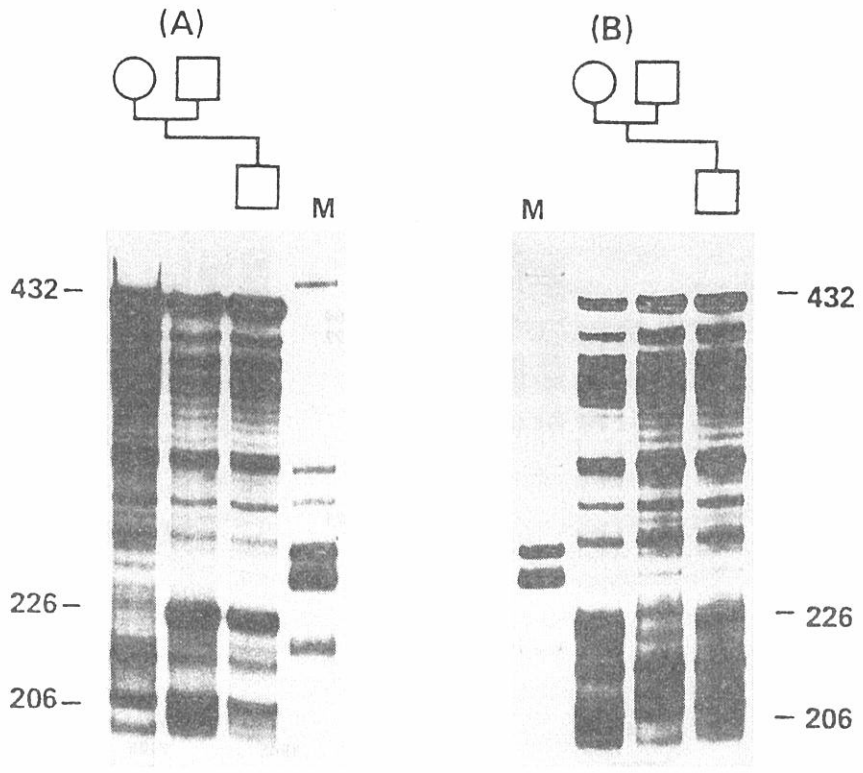

Figure 5. Examination of 432 bp fragments including the IVS2-666 of the human β-globin gene from members of one family. The fragments in genomic DNAs from a husband, wife, and son of one family were amplified by polymerase chain reaction and examined with the sense K16 probe (A) and the sense YF204III-439 probe (B), respectively. Expected sizes of bands are shown by the number of nucleotides outside the autoradiogram with DNA markers (M).

PCR product from the wife of the second family (identification number [ID No.] 0010) was ligated to the pGEM-3 blue plasmid, and the clone was named PCR-0010-IV. Sense and antisense RNA probes were produced from it. The region IV fragments of the PCR products from the three individuals were examined using the new antisense PCR-0010-IV probe. The individual having ID No. 0010 did not show the band of approximately 220 nt as shown in Figure 6(B), whereas the other two individuals exhibited it. Sequence analysis of the region IV fragment of the plasmid PCR-0010-IV revealed a substitution of A to T at the 551th nt from the 5′ side in the fragment corresponding to the 1945th nt from the capping site. Thus, the individual with ID No. 0010 should have T at the 1945th nt on both chromosomes 11, whereas the two individuals most probably have T and A, respectively, on each chromosome 11. The remaining three individuals who did not show the band for the YF204IV probe or for the YF226IV probe were also examined with the antisense PCR-0010-IV probe, and all of them showed the band. Thus, they were inferred to have A at position 1945 on both chromosomes 11, as was subsequently confirmed by sequence analyses.

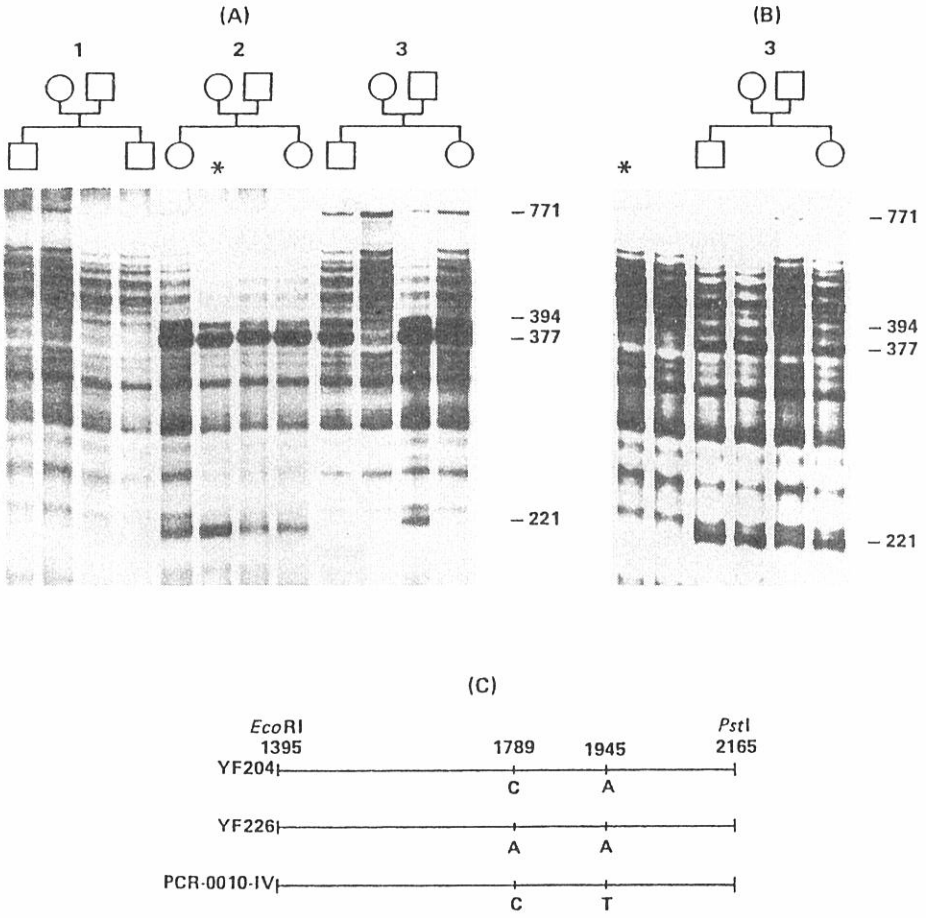

Figure 6. Region IV fragments of human β-globin genes. The 870 base pair fragments containing the sequence between position 23 of exon 3 and the 2237th nucleotide from the capping site from members of three families were amplified by polymerase chain reaction, digested with *Eco*RI and *Pst*I, and examined with the antisense YF226IV probe (A). The members of the third family and the wife (*) of the second family (ID No. 0010) were examined with the antisense PCR-0010IV probe in (B). Expected sizes of bands are shown by the number of nucleotides outside the autoradiograms. Schematic diagrams of region IV of the three human β-globin gene clones are shown in (C).

Discussion

All 12 possible types of mismatches were examined. The G:T and the G:G mismatches were not cleaved, though RNase T1 was added in addition to RNase A in the cleavage reaction. Since both sense and antisense probes were used, uncleaved mismatches resulting from one combination of RNA and DNA strands were converted to cleavable mismatches using the opposite strand combination. Deletions and insertions of one, four, five, and 10 nucleotides were easily detected. Three unreported sequence differences, namely, nucleotide substitutions of G to A

and C to A and a G deletion in clones $\pi SV\beta^{o}39$, YF226, and YF227, respectively, were detected by this method and were confirmed by sequence analysis. The T to C and the C to A substitutions at the IVS2-666 and at the 1789th nt from the capping site, respectively, originally detected in the human β-globin gene clone YF226 obtained from a Thai individual and a new A to T substitution at the 1945th nt were detected in genomic DNAs from Japanese using this method after PCR amplification at the polymorphic proportion. Though the A to T substitution at the 1945th nt occurs in a *Hin*fI site, it had never been reported heretofore. These results, too, were substantiated by sequence analysis. The length of the cleaved product signaled the position at which the variation occurred in the DNA fragment. We confirmed that this technique was very useful in detecting sequence differences in cloned DNAs or PCR-amplified genomic DNAs.

DGGE was applied to examine the RNA:DNA duplexes which were made from genomic DNAs without amplification. Following the method that Lerman and his colleagues had developed for DNA:DNA heteroduplexes, the difference in the mobilities of RNA:DNA duplexes with and without a mismatch was clearly observed for the nucleotide substitutions at IVS2-666.[19,22] Moreover, homozygosity and heterozygosity for the substitution were clearly shown by the appearance of a single band and double bands, respectively. The results obtained using DGGE of the RNA:DNA duplexes made from genomic DNAs were identical to those obtained using RNase cleavage of the RNA:DNA duplexes made from PCR-amplified genomic DNAs from the 59 unrelated individuals shown in Table 2 and their 20 children. These observations are described in detail elsewhere.[22]

YF227 is a β-globin gene clone obtained from DNA of a thalassemic patient and a C to T substitution at the IVS2-654 was detected (Y. Fukumaki, private communication). By using the RNase cleavage method, a deletion of one G from GGG between IVS2-579 and IVS2-581 was detected, as well as the reported substitution. A genomic DNA from which YF227 was cloned (also obtained from Y. Fukumaki) was examined using the RNase cleavage method after PCR amplification. Using sense K16 and sense YF204III-439 probes, no bands corresponding to the G deletion appeared. Direct sequencing of the PCR product confirmed that there was no deletion of G in the original genomic DNA sample. Accordingly, the deletion of G in YF227 was probably an artifact which occurred during gene cloning. Other examples of cloning artifacts and their correction by PCR were recently reported. Another possible explanation is that a somatically mutated gene happened to be cloned in YF227.

The observations described above indicated that the techniques which can directly detect variations in genomic DNAs are necessary for mutation screening. For our purpose, that is, mutation detection in DNAs from many individuals, DGGE of RNA:DNA duplexes made from genomic DNAs and the RNase cleavage of RNA:DNA duplexes made from PCR-amplified genomic DNAs are presently the best approaches. Unfortunately, both approaches have imperfections. The RNases cannot always cleave all types of mismatches, and DGGE cannot detect

variants which have sequence differences in the highest temperature melting domains.[34,35] Moreover, when DGGE detects variants, it does not reveal the nature or location of the sequence differences. Therefore, we feel these two approaches should be employed in an efficient combination. In the first screening, DGGE, which can directly detect variation in genomic DNAs, should be used. Secondly, a combination of PCR and the RNase cleavage method will not only permit rapid identification and sequence analysis of variants, but will also detect deletions, insertions, and 2/3 of mismatches not detectable by DGGE in the highest melting domain of DNA molecules.

Saiki et al[28] observed a 0.25% cumulative misincorporation rate by *Taq* polymerase after the 30 PCR cycles, and the actual rate per nucleotide per cycle was estimated to be 2×10^{-4}. Goodenow et al[36] reported an approximately 10-fold lower frequency. In our study, we sequenced 13 separate clones, each with 771 bp of the amplified region IV fragment of the genomic β-globin gene, obtained from products of 40 PCR cycles, and we detected only one misincorporation. Thus, the cumulative misincorporation rate was less than 1/25 of that reported by Saiki et al. Because misincorporation will be randomly distributed over many sequences, misincorporated nucleotides will not interfere with interpretation of results obtained using the RNase cleavage method, since it directly examines the accumulated PCR products without cloning. In addition to this misincorporation in PCR, the possibility of cloned expansion of a somatic mutant in the established cell lines was considered, because we used genomic DNA samples extracted from established B lymphocyte cell lines. Since no contradiction of the Mendelian inheritence was observed in any one of the families examined in our study, we assume that such clonal expansion did not occur in our cell lines or occurred at too low a level to interfere with the results. However, we keep intact lymphocytes and mononuclear cells in liquid nitrogen in order to examine their DNA whenever somatic mutation is suspected.

Recently, Sheffield et al[37] described DGGE which uses PCR-amplified DNA fragments with a GC-rich sequence attachment, and single base substitutions in genomic DNAs are reported to be detected by ethidium bromide staining. We are independently accumulating data on DGGE of PCR-amplified DNA fragments, and we think that the new method will markedly enhance screening procedures.

Acknowledgments

We are grateful to Dr. J. V. Neel, University of Michigan, for critical reading of this manuscript; Dr. Y. Fukumaki, Kyushu University, for providing cloned human β-globin genes YF204, YF226, and YF227; and Dr. R. M. Myers, University of California, San Francisco, for providing πSVWT2 and πSVβ^{o}39. We also thank Dr. J. Asakawa for synthesizing PCR primers, and Messrs. H. Omine, N. Kosaka, Y. Kimura, and Mrs. J. Kaneko and A. Miura for their excellent technical assistance.

References

1. Neel JV, Schull WJ, Awa AA, Satoh C, Otake M, Kato H, Yoshimoto Y: Implications of the Hiroshima-Nagasaki genetic studies for the estimation of the human "doubling dose" of radiation. Genome 31:853–9, 1989

2. Satoh C, Neel JV: Biochemical mutations in the children of atomic bomb survivors. In *Genetics of Human Tumors in Japan* (Gann Monograph on Cancer Research No. 35). Tokyo, Japan Scientific Societies Press, and London, Philadelphia, Taylor & Francis, 1988. pp 191–208

3. Neel JV, Satoh C, Goriki K, Asakawa J, Fujita M, Takahashi N, Kageoka T, Hazama R: Search for mutations altering protein charge and/or function in children of atomic bomb survivors: Final report. Am J Hum Genet 42:663–76, 1988

4. Myers RM, Larin Z, Maniatis T: Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 230:1242–6, 1985

5. Fischer SG, Lerman LS: DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: Correspondence with melting theory. Proc Natl Acad Sci USA 80:1579–83, 1983

6. Myers RM, Lumelsky N, Lerman LS, Maniatis T: Detection of single base substitutions in total genomic DNA. Nature 313:495–8, 1985

7. Lerman LS, Silverstein K, Grinfeld E: Searching for gene defects by denaturing gradient gel electrophoresis. Cold Spring Harb Symp Quant Biol 51:285–97, 1986

8. Myers RM, Maniatis T, Lerman LS: Detection and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol 155:501–27, 1987

9. Winter E, Yamamoto F, Almoguera C, Perucho M: A method to detect and characterize point mutations in transcribed genes: Amplification and overexpression of the mutant c-Ki-*ras* allele in human tumor cells. Proc Natl Acad Sci USA 82:7575–9, 1985

10. Forrester K, Almoguera C, Han K, Grizzle WE, Perucho M: Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature 327:298–303, 1987

11. Gibbs RA, Caskey CT: Identification and localization of mutations at the Lesch-Nyhan locus by ribonuclease A cleavage. Science 236:303–5, 987

12. Veres G, Gibbs RA, Scherer SE, Caskey CT: The molecular basis of the sparse fur mouse mutation. Science 237:415–7, 1987

13. Lopez-Galindez C, Lopez JA, Melero JA, Fuente L, Martinez C, Ortin J, Perucho M: Analysis of genetic variability and mapping of point mutations in influenza virus by the RNase A mismatch cleavage method. Proc Natl Acad Sci USA 85:3522–6, 1988

14. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-*ras* genes. Cell 53:549–54, 1988

15. Noll WW, Collins M: Detection of human DNA polymorphisms with a simplified denaturing gradient gel electrophoresis technique. Proc Natl Acad Sci USA 84:3339–43, 1987

16. Cariello NF, Scott JK, Kat AG, Thilly WG, Keohavong P: Resolution of a missense mutant in human genomic DNA by denaturing gradient gel electrophoresis and direct sequencing using in vitro DNA amplification: HPRT Munich. Am J Hum Genet 42:726–34, 1988

17. Hiyama K, Kodaira M, Satoh C: Detection of mismatches in RNA:DNA duplexes. Jpn J Hum Genet 33:234, 1988

18. Satoh C, Hiyama K, Takahashi N, Kodaira M: Detection of new variations in cloned β-globin genes and a DNA polymorphism in a Japanese population with ribonuclease cleavage at mismatches in RNA:DNA duplexes. Genome 30(suppl 1):369, 1988

19. Takahashi N, Hiyama K, Kodaira M, Omine H, Satoh C: Detection of mismatches in RNA:DNA duplexes. Report 3. Jpn J Hum Genet 34:77, 1989

20. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N: Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–4, 1985

21. Mullis KB, Faloona FA: Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335–50, 1987

22. Takahashi N, Hiyama K, Kodaira M, Satoh C: An improved method for the detection of genetic variations in DNA with denaturing gradient gel electrophoresis. Mutat Res 234:61–70, 1990

23. Kimura A, Matsunaga E, Takihara Y, Nakamura T, Takagi Y, Lin S, Lee H: Structural analysis of a β-thalassemia gene found in Taiwan. J Biol Chem 258:2748–9, 1983

24. Maniatis T, Fritsch EF, Sambrook J: Molecular cloning: A laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1982

25. Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR: Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res 12:7035–56, 1984

26. Sanger F, Coulson AR, Barrell BG, Smith AJH, Roe BA: Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol 143:161–78, 1980

27. Hattori M, Sakaki Y: Dideoxy sequencing method using denatured plasmid templates. Anal Biochem 152:232–8, 1986

28. Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487–91, 1988

29. Wong C, Dowling CE, Saiki RK, Higuchi RG, Erlich HA, Kazazian HA Jr: Characterization of β-thalassaemia mutations using direct genomic sequencing of amplified single copy DNA. Nature 330:384–6, 1987

30. Spritz RA: Duplication/deletion polymorphism 5′- to the human globin gene. Nucleic Acids Res 9:5037–47, 1981

31. Moschonas N, Boer E, Flavell RA: The DNA sequence of the 5′ flanking region of the human β-globin gene: evolutionary conservation and polymorphic differences. Nucleic Acids Res 10:2109–20, 1982

32. Orkin SH, Kazazian HH Jr, Antonarakis SE, Goff SC, Boehm CD, Sexton JP, Waber PG, Giardina PJV: Linkage of β-thalassaemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. Nature 296:627–31, 1982

33. Paabo S, Wilson AC: Polymerase chain reaction reveals cloning artifacts. Nature 334:387–8, 1988

34. Myers RM, Fischer SG, Maniatis T, Lerman LS: Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. Nucleic Acids Res 13:3111–30, 1985

35. Myers RM, Fischer SG, Lerman LS, Maniatis T: Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. Nucleic Acids Res 13:3131–46, 1985

36. Goodenow M, Huet T, Saurin W, Kwok S, Sninsky J, Wain-Hobson S: HIV-1 isolates are rapidly evolving quasispecies: Evidence for viral mixtures and preferred nucleotide substitutions. J Acquired Immune Deficiency Syndromes 2:344–52, 1989

37. Sheffield VC, Cox DR, Lerman LS, Myers RM: Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA 86:232–6, 1989