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Radiation Effects Research Foundation
A Cooperative Japan–United States Research Organization

RERF Technical Report Series

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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 3-90
研究課題　7-85

Denaturing gradient gel electrophoresisを用いてDNA中の遺伝的変異を検出するための改良法

An Improved Method for Detecting Genetic Variation in DNA using Denaturing Gradient Gel Electrophoresis

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要　約

我々は，RNA：DNAデュプレックスのDenaturing gradient gel electrophoresis（DGGE）が，染色体及びクローン化DNA中の変異を検索する実効的方法であるかを検討した．その結果は，多数のサンプル中の変異を検出するためには，このDGGEがLermanらにより最初に開発されたDNA：DNAヘテロデュプレックスを用いたものより，より実用的であることを証明した．なぜならば，RNAプローブはDNAプローブより簡単に合成されるからである．3種類の^{32}Pで標識されたプローブが作製された．我々の方法においては，染色体及びクローン化DNAは，制限酵素で切断された後，標識されたRNAプローブとハイブリダイズされ，得られたRNA：DNAデュプレックスはDGGEにより検査された．ミスマッチの存在は，ゲル上におけるバンドの移動度の差として検出された．実験条件は，1名の正常人及び3名のサラセミア患者のヒトβ-グロビン遺伝子から得られたクローン化DNA断片を用いて決定された．クローン化DNAの実験結果は，RNA：DNAのDGGEはDNA中の塩基置換及び欠損を検出することを示唆した．この研究の過程において，染色体DNA試料中のヒトβ-グロビン遺伝子のIVS2の666残基（IVS2-666）における1塩基置換による多型性が直接同定された．広島在住の血縁関係にない59名の日本人について検査を行ったところ，IVS2-666がCである対立遺伝子の頻度は0.48であり，また，Tである対立遺伝子頻度は0.52であった．この方法は，既知の制限酵素部位外に存在する遺伝的変異を検出するためには非常に有効であり，また，DNA中に新しく起こった突然変異を検出するための強力な技法となるに違いないと思われる．

§本報告にはこの要約以外に訳文はない．

承認1990年1月18日

印刷1990年5月

RERF TR 3-90
Research Project 7-85

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An Improved Method for Detecting Genetic Variation in DNA using Denaturing Gradient Gel Electrophoresis§

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Summary

We have examined the feasibility of denaturing gradient gel electrophoresis (DGGE) of RNA:DNA duplexes to detect variations in genomic and cloned DNAs. The result has demonstrated that use of RNA:DNA duplexes makes DGGE much more practical for screening a large number of samples than use of DNA:DNA heteroduplexes, because preparation of RNA probes is easier than that of DNA probes. Three different ^{32}P-labeled RNA probes were produced. Genomic or cloned DNAs were digested with restriction enzymes and hybridized to labeled RNA probes, and resulting RNA:DNA duplexes were examined by DGGE. The presence of a mismatch(es) was detected as a difference in the mobility of bands on the gel. The experimental conditions were determined using DNA segments from cloned normal and three thalassemic human β-globin genes. The results from experiments on the cloned DNAs suggest that DGGE of RNA:DNA duplexes will detect nucleotide substitutions and deletions in DNA. In the course of these studies, a polymorphism due to a single-base substitution at position 666 of IVS2 (IVS2-666) of the human β-globin gene was directly identified using genomic DNA samples. A study of 59 unrelated Japanese from Hiroshima was undertaken in which the frequency of the allele with C at IVS2-666 was 0.48 and that of the allele with T was 0.52.

This approach was found to be very effective for detecting heritable variation and should be a powerful tool for detecting fresh mutations in DNA, which occur outside the known restriction sites.

Introduction

A study to detect mutations at the protein level to elucidate the genetic effects of atomic bomb radiation was started at RERF in 1976. The final report[1,2] of the study showed no demonstrable genetic effects of parental exposure. Four fresh

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

Approved 18 January 1990 **Printed May 1990**

mutations were detected in the exposed group and three in the control group. The mutation rates per locus per generation of 0.60×10^{-5} and 0.64×10^{-5} in the two groups, respectively, did not differ significantly. It was emphasized at the same time, however, that a still greater number of gene loci must be examined before this negative conclusion can be ascertained with confidence. Recently, this laboratory has undertaken pilot studies to detect mutations at the DNA level, in which not only exon regions coding the proteins but also noncoding regions are examined.

Many methods have been developed for detecting sequence heterogeneities in DNA molecules. Each has provided important information for gene linkage studies and for diagnosis of genetic diseases. Two recently developed methods have significantly increased the number of nucleotides that can be examined at one time for single-base substitutions or more complex sequence differences. One is based on the melting behavior of DNA and uses denaturing gradient gel electrophoresis (DGGE).[3–7] In this procedure, a single-stranded, radioactive DNA probe is hybridized to cloned or genomic DNA, and the resulting DNA:DNA duplex can be analyzed using DGGE. DGGE can also be employed in detecting variations in RNA using RNA:RNA duplexes or DNA:RNA duplexes.[8] The other, so-called "ribonuclease (RNase) A cleavage" method, is based on the susceptibility of single-base mismatches in RNA:DNA duplexes to RNase A cleavage.[9,10] Myers et al[9,11] reported that these two methods could independently detect known single-base mutations in β-globin genes. We have examined whether these methods are suitable for the large-scale screening of DNA samples necessary for the projected new effort to study the genetic effects of the A-bombs. Preliminary reports have been presented.[12–16] The results obtained from the "RNase cleavage" method will be described elsewhere.[17]

The disadvantage of DGGE of DNA:DNA duplexes is that many steps are involved in the preparation of labeled, single-stranded DNA probes, including digestion of primer extension products with an appropriate restriction enzyme, separation of single-stranded probes from other extension products, and the DNA segments used as templates by electrophoresis on an appropriate type of gel, recovery of the probes by electroelution, and purification of the probes by Sephadex G-50 (Pharmacia, Uppsala, Sweden) spin column. These procedures are tedious and the probes prepared by primer extension are not necessarily separated from the templates.[18] The labeled RNA probes, on the other hand, can be easily prepared from DNA templates by means of the bacteriophage transcription system. This procedure is relatively simple in comparison with the DNA procedure and consistently provides pure probes. Moreover, we had already produced various RNA:DNA duplexes from RNA probes transcribed from the pGEM-3 and pGEM-3 blue vectors containing various fragments of human β-globin genes with either cloned or genomic β-globin genes to examine the practicality of the RNase cleavage method in screening many samples.[17]

In this report, we demonstrate the feasibility of using DGGE with RNA probes, and we show that this method can detect a polymorphic substitution at IVS2-666 of the β-globin gene both in cloned and genomic DNA samples which cannot be directly observed using the restriction fragment length polymorphism (RFLP).[19] This method can also be applied to the detection of nucleotide variations such as insertions and deletions.

Materials and Methods

High molecular weight DNA samples were extracted from cell lines established from Epstein–Barr virus-transformed B lymphocytes obtained from 59 unrelated Japanese from Hiroshima using a modification of the techniques described by Maniatis et al.[20] The 59 individuals included 11 husband-wife pairs, whose 20 children were also examined in the family study. The genomic DNAs were digested with *Rsa*I and *Eco*RI and were hybridized with RNA probes.

A plasmid containing the normal β-globin gene (YF204) and plasmids (YF226,[21] 15H-3 [Fukumaki, personal communication, 1986] and YF227[17]) containing three thalassemia genes were obtained from Dr. Y. Fukumaki of the Research Laboratory for Genetic Information, Kyushu University. Cloned DNA fragments were obtained by digestion of YF204, YF226, 15H-3, and YF227 with *Eco*RI and *Rsa*I. The known variations in these fragments are as follows (Figure 1): Thymine (T) at position 1161 relative to the mRNA cap site which corresponds to IVS2-666 in YF204 is replaced by cytosine (C) in YF226. There is a C to T substitution in 15H-3 at position 1149 (IVS2-654). YF227 has a deletion of one of the three Gs between position 1074 and 1976 (IVS2-579 to IVS2-581) in addition to the substitution noted in 15H-3.[17] Therefore, three sequence differences are involved between YF226 and YF227, namely, two base substitutions and one base deletion.

Restriction enzymes were obtained from Takara Shuzo Co. (Kyoto) or Toyobo Co. (Osaka), SP6-polymerase, RNasin, pGEM-3 and pGEM-3 blue plasmids from Promega (Madison, Wisc, USA), [α-^{32}P]-CTP (800 Ci/mmol, 20 mCi/ml; 1 Ci = 37 GBq) from Amersham (Amersham, UK), RNase-free DNase I from Worthington (Freehold, NJ, USA), proteinase K from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany), RNase A from Sigma (St. Louis, Mo, USA), and RNase T1 from Sankyo Co. (Tokyo). Water used in this study was treated with diethylpyrocarbonate and was autoclaved.

Preparation of RNA probes and RNA:DNA duplexes

Preparation of uniformly labeled, single-stranded RNA probes and RNA:DNA duplexes were carried out as described by Hiyama et al.[17] In brief, in order to obtain DNA templates for synthesizing RNA probes, three types of plasmid were prepared by recloning each of three types of fragments (Figure 1) into pGEM-3 or pGEM-3 blue. These plasmids were linearized by *Eco*RI and were used as templates for preparation of probes using SP6-polymerase. The resulting probes were designated as sense YF204-III-917, sense YF204-III-439, and sense

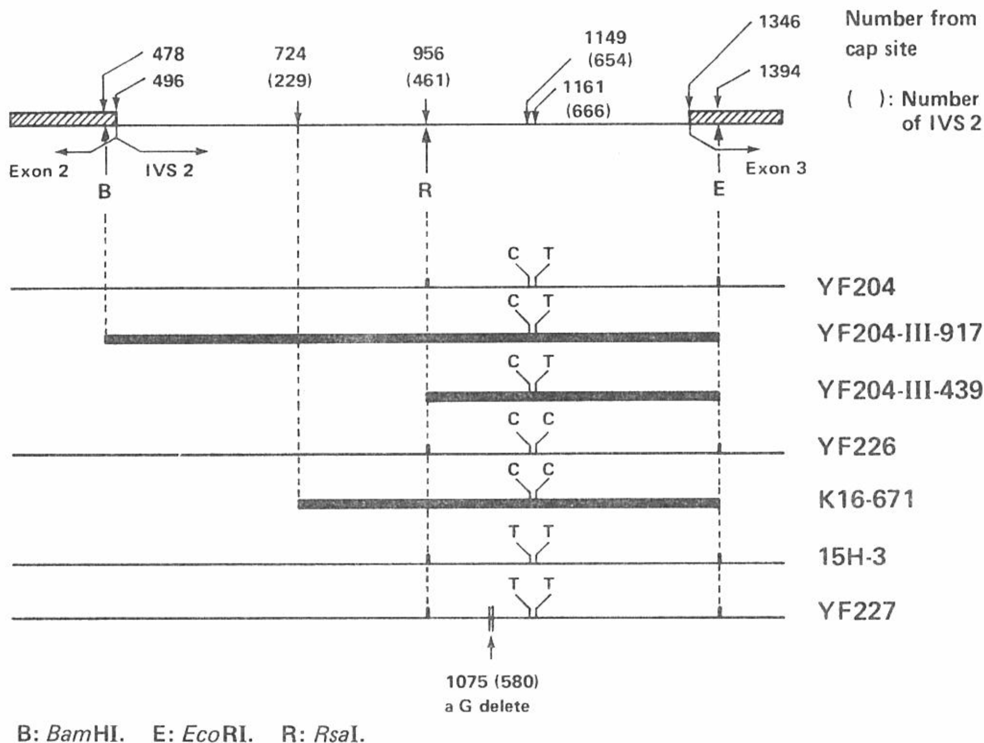

Figure 1. Schematic diagram showing the location of sequence differences in DNA fragments cloned from the human β-globin gene used in this study. Thick lines show the fragments which were ligated into a transcription vector and the resulting plasmids were used for preparing the RNA probes.

K16, respectively, since all probes used in the present study are designated "sense probes" which have the same nucleotide sequence (except uracil [U] to T) of the sense strands of the DNA fragments. Both sense YF204-III-917 and sense YF204-III-439 have U at position 1161, whereas sense K16 has C at the same position. To synthesize the probe for examining cloned DNA samples, 1 μg of template was treated in the transcription mixture containing 500 μM each of ATP, GTP, and UTP, 20 μCi of [α-^{32}P]-CTP (40 Ci/mmol, 25 μM). For the RNA probe with high specific activity to examine genomic DNA samples, 100 μCi of [α-^{32}P]-CTP (400 Ci/mmol, 12 μM) were used. After transcription, the template DNA was digested with DNase I, and the single-stranded RNA probe was resuspended in the appropriate amount of buffer containing 10 mM tris [hydroxymethyl] aminomethane (Tris)-HCl (pH 7.5), 1 mM ethylenediamine-tetraacetic acid (EDTA), and 0.1% sodium dodecyl sulfate.

Restriction enzyme-treated cloned DNAs (200 ng) or genomic DNAs (4.5 μg) were hybridized with the probe (1×10^5 to 5×10^5 cpm of probes made for cloned DNA samples, 1×10^6 to 2.5×10^6 cpm for genomic DNA samples) according to the

method of Myers et al.[9] The length of sense YF204-III-917, sense YF204-III-439, and sense K16 were 960 nucleotides (nt), 489 nt and 694 nt, respectively, since each probe included the transcript of the polylinker part. All of these probes are longer than the *Rsa*I-*Eco*RI fragments (439 base pairs [bp]) of examined DNA (Figure 1). After the hybridization reaction, the single-stranded "overhang" part of the probe and the unhybridized RNA probe were digested with RNase A and RNase T1. The quantities of RNase A and T1 were critical. The quantities had to be minimized to a level that was enough for completing the above-mentioned reaction because too large an amount of RNase causes the cleavage of the probe at mismatches and the resulting products may disturb the pattern of DGGE. After preliminary experiments, we determined the conditions as follows: RNase solution of 300 μl should contain 0.2 μg/ml of RNase A and 5 ng/ml of RNase T1 for examination of cloned DNAs and 5 μg/ml of A and 250 ng/ml of T1 for duplexes derived from genomic DNAs.

Electrophoresis

In this study, we used two types of gels. One is termed "parallel gradient gel," since the gradient of concentration of the denaturant in a polyacrylamide gel is parallel to the direction of electrophoresis. The other is the "perpendicular gradient gel" in which the gradient is perpendicular to the direction of electrophoresis.[22]

Parallel gradient gel electrophoresis was performed using the apparatus (Model SE-520, Vertical Flat Gel) purchased from Hoefer Scientific Instruments (San Francisco, Calif, USA). The gel with dimensions of 14 cm wide $\times$ 31 cm high $\times$ 0.75 mm thick was prepared by the method of Fischer and Lerman[23] with the following modifications: A 6.5% polyacrylamide gel in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, and adjusted by acetic acid to pH 7.4) was prepared 2 cm depth from the bottom of the gel to prevent leakage of the denaturant from the gel into the electrode solution during electrophoresis because our equipment had no bottom trough. The gels were composed of a fixed acrylamide concentration (acrylamide in gel = 6.5% [wt/vol]; bisacrylamide in total acrylamide = 2.6% [wt/wt]) in TAE buffer with a linearly increasing concentration gradient of the denaturant. In most of the experiments, gels having a denaturant gradient of 0–100% were used (100% denaturant = 7 M urea and 40% formamide). Depending on the situation, gels with gradients of 0–50% or 0–25% were also used. Gradient gels were formed by mixing two solutions containing no denaturant and denaturant of the appropriate concentration in a two-chamber gradient maker (92-90-1886, LKB, Bromma, Sweden) and poured into the gel cassette from the top. These gels had a short stacking gel containing no denaturant. Perpendicular gradient gel electrophoresis was performed with an equipment made according to Fischer and Lerman.[23] Gel solution with 0–25% gradient was poured into the gel cassette and was rotated 90°. The sample was applied as a band across the top of the gel. In both types of electrophoresis, the gel plates were fixed onto an apparatus which had a cathodal chamber at the top, and the whole assembly was completely

submerged in a buffer-filled tank that functioned as the anode. A peristaltic pump circulated buffer between the two chambers. The electrophoresis chambers were maintained at 60°C with a circulating heater. The parallel gel was run at 200 V for 17 hours and the perpendicular gel at 150 V for 10 hours. After electrophoresis, the gels were dried on a Gel Drying Processor AE-370 (Atto, Tokyo). Autoradiography was performed at −80°C with Fuji RX X-ray film (Kanagawa) and a Du Pont Cronex HI-Plus intensifying screen (Wilmington, Del, USA).

Results

Detection of sequence differences in cloned DNA fragments

In order to establish the optimum conditions for detecting base mismatches, we used four types of duplexes such as sense K16/YF204, sense K16/YF226, sense K16/15H-3, and sense K16/YF227. Sense K16/YF204 denotes a duplex produced from the ^{32}P-labeled sense K16 probe and a complementary strand of DNA fragment between *Eco*RI and *Rsa*I of YF204.

The general melting behavior of these duplexes was examined in the perpendicular gradient gel. The pattern on the gel containing the gradient of 0–25% denaturant is shown in Figure 2. The sense K16/YF227 separated from three other duplexes in the gel where the denaturant concentration was less than 9%. The remaining three duplexes separated from each other between denaturant concentrations of 9% and 16%. We, therefore, examined their separation by electrophoresis on three types of parallel gels containing 0–25%, 0–50%, and 0–100% denaturant, respectively, for 17 hours at 200 V. Under our conditions, the gel containing the gradient of 0–100% denaturant provided the best separation. The duplexes were retarded between the denaturant concentration of 10% and 20%.

We conducted an experiment to establish optimum electrophoresis time to separate three types of duplexes (sense K16/YF204, sense K16/YF226, and sense K16/YF227) on a parallel gel. At 11 hours, the band of sense K16/YF227 was distinguishable from those of sense K16/YF204 and sense K16/YF226, but the latter two were still indistinguishable. After 15–19 hours, these latter two bands were clearly separated. When electrophoresis was continued for 27 hours, these three bands migrated to higher denaturant concentrations, but no remarkable change was observed in the differences between them. The mobility of the bands decreased rapidly as they migrated to higher concentrations of denaturant.

Based on these results, we employed the following electrophoretic conditions: acrylamide concentration = 6.5%, denaturant gradient = 0–100%, electrophoresis time = 17 hours, and temperature = 60°C. Typical patterns obtained under these conditions are shown in Figure 3. That is, sense K16/YF226, which contained no

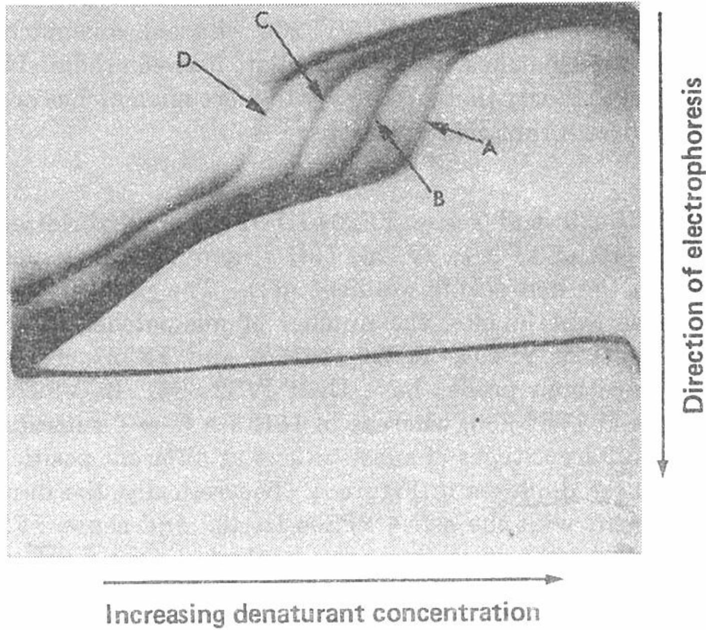

Figure 2. Melting profiles of four types of RNA:DNA duplexes. DNAs used for making duplexes with the labeled sense K16 probe are as follows: A. YF226, B. YF204, C. 15H-3, and D. YF227. Electrophoresis was carried out on acrylamide gel containing a denaturant (0–25%) gradient perpendicular to the electric field.

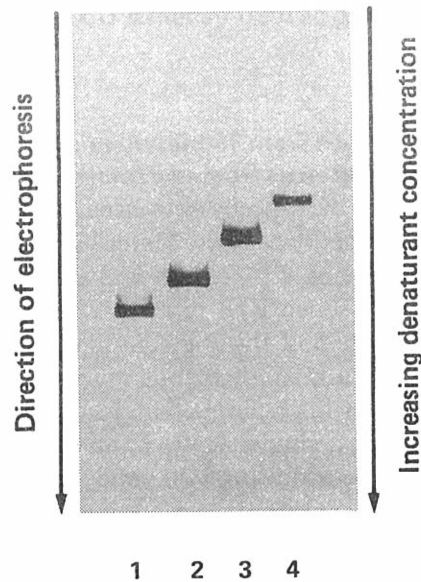

Figure 3. The typical pattern of DGGE of duplexes between sense K16 and the cloned DNAs. DNAs used for making duplexes are as follows: lane 1, YF226; lane 2, YF204; lane 3, 15H-3; and lane 4, YF227. Electrophoresis was carried out in a denaturant (0–100%) gradient gel.

mismatch, migrated farthest. Sense K16/YF227 with three types of mismatches showed the slowest mobility. Sense K16/YF204 with one mismatch and sense K16/15H-3 with two mismatches were intermediate between them. These results are consistent with the theory that duplexes with more mismatches are less stable and melt at lower denaturant concentrations.[11]

Sense YF204-III-439 and sense YF204-III-917 were hybridized with the *Rsa*I-*Eco*RI fragments of YF204, YF226, 15H-3, and YF227, respectively, and electrophoresed on the denaturing gradient gels. The patterns are shown in Figure 4. In these experiments, the number of mismatches in the duplexes made from fragments of YF204, YF226, 15H-3, and YF227 are 0, 1, 1, and 2, respectively, since both probes have U at IVS2-666. In YF226, a T $\rightarrow$ C substitution occurs at IVS2-666, whereas in 15H-3 a C $\rightarrow$ T substitution occurs at IVS2-654. The different types of substitutions at different positions reflected the mobilities of these duplexes in Figure 4. Theoretically, the duplexes made from a DNA fragment with the sense YF204-III-439 and sense YF204-III-917, respectively, were the same when they were applied to a gradient gel since the overhang part of the probes had been cleaved. However, in Figure 4, bands obtained from a shorter probe (sense YF204-III-439) were broader than those from longer probe (sense YF204-III-917). Since the band of sense YF204-III-439/YF204 (lane 1 of Figure 4) was not clearly separated from that of sense YF204-III-439/YF226 (lane 2 of Figure 4), the duplexes made of genomic DNA from an individual exhibiting two bands will be likely to be observed as a single broad band. From this reason, the sense YF204-III-917 was used in addition to the sense K16 in the screening of total genomic DNAs to be described below.

Screening of genomic DNA

Total genomic DNAs obtained from 79 individuals (including 20 children) were examined by DGGE. DGGE patterns from one family using two probes are shown in Figure 5. When the sense K16 probe was used, two clearly identified bands were observed (Figure 5B). Mobilities of the faster band and the slower band were identical with those of the sense K16/YF226 and the sense K16/YF204 (Figure 5A), respectively. Since the sense K16 probes, YF226, and YF204 have C, C, and T, respectively, at IVS2-666 of the β-globin gene, the former duplex has no mismatch but the latter has a mismatch that makes its migration slower. The genotype of an individual showing a single faster band was termed C/C, whereas that of an individual showing a single slower band was termed T/T. Accordingly, the genotype of an individual exhibiting two bands was C/T.

Screening of the same population was also conducted using sense YF204-III-917 which has U at IVS2-666. Two clearly identified bands were observed (Figure 5D). In this case, duplexes made of genomic DNAs from individuals with genotype C/C migrated more slowly than those made of genomic DNAs obtained from individuals with genotype T/T. Thus, the genotype of each individual was

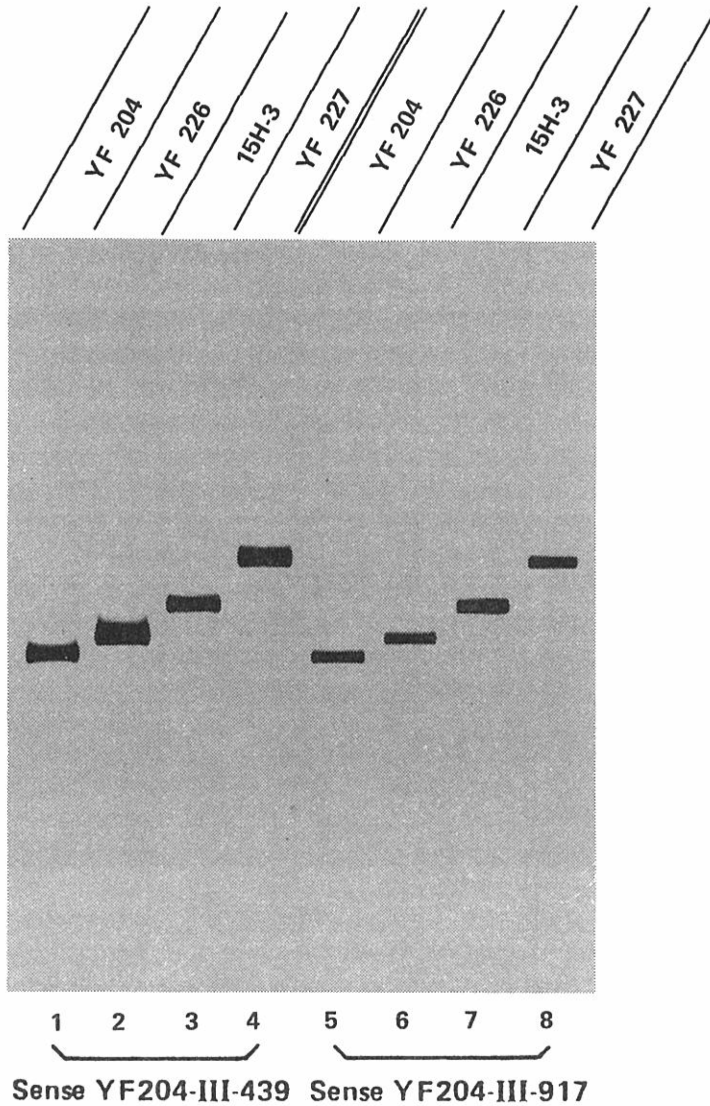

Figure 4. The typical pattern of DGGE of duplexes between either sense YF204-III-439 (lanes 1–4) or sense YF204-III-917 (lanes 5–8) and cloned DNAs. DNAs used for making duplexes are shown above the photograph. Electrophoresis was carried out in a denaturant (0–100%) gradient gel.

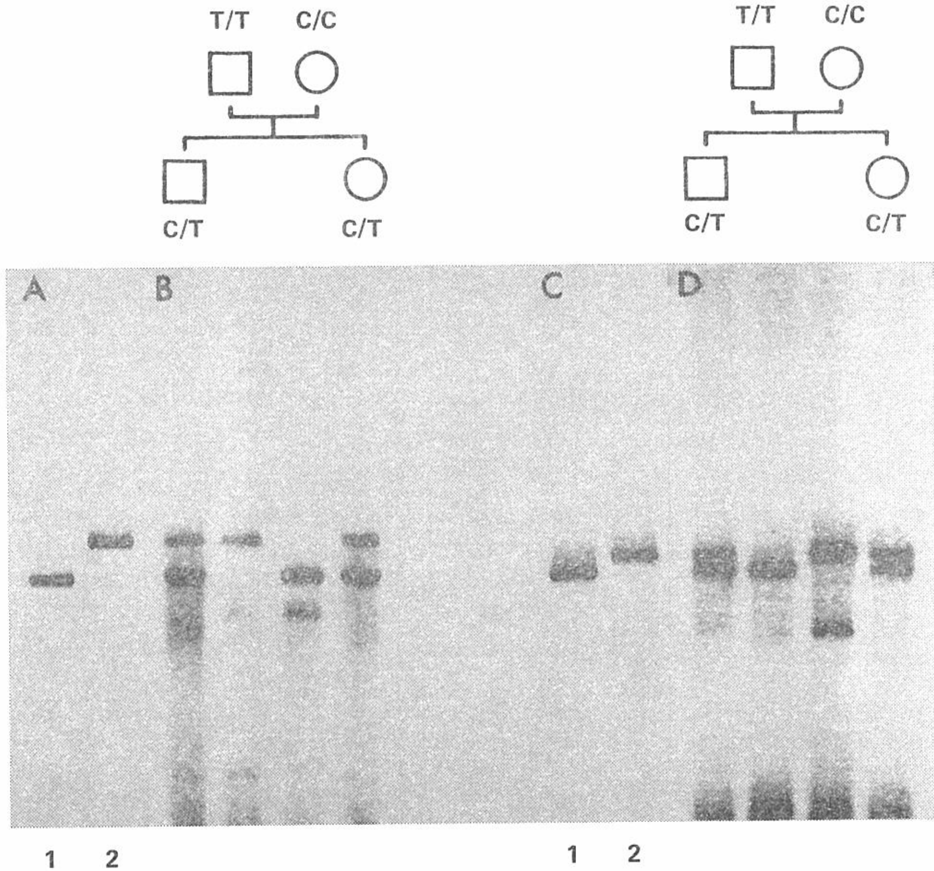

Figure 5. DGGE patterns of duplexes from human genomic DNAs, together with those from cloned DNAs as comparison. A) The duplexes with sense K16 and the cloned DNAs. Lane 1: sense K16/YF226. (YF226 has C at IVS2-666.) Lane 2: sense K16/YF204. (YF204 has T at IVS2-666.) B) The duplexes with sense K16 and genomic DNAs from one nuclear family. The genotypes are shown with the pedigree. The mobilities of faster bands and slower bands are identical with those of sense K16/YF226 (lane 1 of A) and sense K16/YF204 (lane 2 of A), respectively. C) The duplexes with sense YF204-III-917 and the cloned DNAs. Lane 1: sense YF204-III-917/YF204. Lane 2: sense YF204-III-917/YF226. D) The duplexes with sense YF204-III-917 and the genomic DNAs from the same family shown in B. The mobilities of faster bands and slower bands are identical with those of sense YF204-III-917/YF204 (lane 1 of C) and sense YF204-III-917/YF226 (lane 2 of C), respectively.

independently determined using these two probes, and the results were in complete agreement. Table 1 presents the distribution of the genotypes and allele frequencies among 59 unrelated individuals obtained from these experiments. The frequencies of the alleles C and T were 0.48 and 0.52, respectively. The distribution of the observed genotypes is in good agreement with the expectation predicted from the allele frequencies on the assumption of the Hardy-Weinberg equilibrium. The distribution of genotypes in children from 11 matings is shown in Table 2. There was no exception to the genotypes among 20 children of the 11 matings expected on the basis of a two-allele system.

Table 1. Distribution of genotypes and their frequencies from 59 unrelated Japanese

Genotypes	Number observed	Number expected	Frequencies
C/C	14	13.77	
			C = 0.4831
C/T	29	29.47	
			T = 0.5169
T/T	16	15.76	
			$\chi^2 = 0.0150$
			$0.950 > P > 0.900$
Total	59	59.00	(df = 1)

Table 2. Distribution of genotypes at IVS2-666 of the β-globin gene in 11 families

Genotype of parents	Number of matings	Number of children	Expected genotypes of children	Observed genotype of children
T/T × C/T	1	2	C/T	2 × C/T
			T/T	
C/T × C/T	4	8	T/T	1 × T/T
			C/T	4 × C/T
			C/C	3 × C/C
C/C × T/T	1	2	C/T	2 × C/T
C/C × C/T	4	6	C/T	5 × C/T
			C/C	1 × C/C
C/C × C/C	1	2	C/C	2 × C/C

Discussion

The results obtained by DGGE showed that we detected the polymorphism based on a nucleotide substitution at IVS2-666 (see Results section). The samples of the same population were also screened in our laboratory by the RNase cleavage method.[17] The results obtained using this method, in which the location of the mismatch can be estimated from the size of the newly produced fragments from RNase A cleavage, also demonstrated that the polymorphic substitution occurred at IVS2-666. In addition, results of family studies employing these two methods demonstrated that the sequence difference that produced the polymorphism is inherited in a Mendelian fashion (Table 2).[17] This indicates that the alteration of melting behavior observed in this study was not caused from artifacts due to base methylation or partial digestion of examined DNAs with restriction enzymes.

Several methods have been developed for detecting mutations in genomic DNA. RFLP is the method that was first introduced and has been used most widely.[24,25] Unfortunately, this approach has one significant limitation in that variations occurring outside the restriction enzyme recognition sites cannot be detected, and even large panels of restriction enzymes can recognize only a fraction of the existing sequence variations. In this method, per diploid genome, each combination of a unique probe and a six-cutter restriction enzyme examines 36 bp consisting of 24 bp for loss of a cutting site and on average 12 bp for the gain of a cutting site.[26]

Base substitutions which do not alter the restriction enzyme cleavage sites can be detected by the differential hybridization of the synthetic oligonucleotide probe, if the location and nature of the mutation and the surrounding nucleotides are known.[27,28] This method, therefore, is applicable only to genes that are well characterized before examination. Only 40 bp per diploid genome can be examined at one examination, since, for practical purpose, the maximum length of each probe should be less than 20 nt. For these reasons, the above-mentioned two methods are not efficient in screening for new mutations.

The RNase A cleavage method has been developed to detect single-base substitutions in cloned and genomic DNA.[9] Since several hundreds to 1,000 bp can be examined at a time with one probe, the efficiency of this method is greater than the two methods described above. Moreover, the advantage of this method is that it not only detects mutations but also localizes them. The limitation of this method, however, is that all types of mismatches are not always cleaved by RNase A. Myers et al[9,10] reported that approximately 1/3 of all possible single-base substitutions were not detectable, even if two types of probes, one of which was homologous to one strand of the test DNA and the other homologous to the opposite strand of the test DNA, were used. Based on the results of our study described elsewhere[12] employing the RNase cleavage method, we are confident that this is a very effective method to detect variations in cloned DNAs or DNAs amplified by polymerase chain reaction (PCR)[29] although it has this limitation.

DGGE of DNA:DNA heteroduplexes has been reported to be a very useful tool for identifying base substitutions in human genomic DNA.[10,11] Using this method, approximately 600 bp can be analyzed with one probe.[30] The limitations of this method, however, were described earlier and until they are resolved, our calculation based on our preliminary experiments showed that the efficiency of this method will be similar to that of the RNase cleavage method. In contrast to this, pure single-stranded RNA probes may be consistently obtained very easily employing the in vitro transcription system in which DNA fragments inserted into transcription vectors are used as templates.[9,31] Smith et al[8] demonstrated that single-base mutations in the *NS* gene of the influenza virus were detectable using DGGE of RNA:RNA duplexes or DNA:RNA duplexes which had been made by hybridization between virion RNA and RNA probes prepared using this method or using DNA probes.

In the present study, we demonstrated that sequence differences present not only in cloned DNA but also in genomic DNA are detectable by DGGE of duplexes made by hybridization of RNA probes with DNAs. Because of reduced time for preparing RNA probes, the utility of this method for screening is remarkably increased. Hybridization can be carried out in a solution containing two or more different probes selected so that the bands of resulting different duplexes will be distributed broadly over the gradient at the end of the run. For example, let us assume that 500 bp per haploid genome will be able to be effectively examined by one probe, and five probes will be used in one examination to produce five duplexes which can be separated on one lane. Based on this assumption, we estimate that this method is at least 5–10 times more efficient than the DGGE of DNA:DNA heteroduplexes.

The detection of mutations should be done by directly comparing DNA sequences of appropriate genes isolated from each individual of the study group. However, it is very questionable whether present sequencing techniques have the necessary accuracy for screening for mutations. The error level may be still of the order of 10^{-4}, even after improvement in accuracy is attained by using a better optical processor and a powerful computer.[32] At present, moreover, the efficiency of DNA sequencing analysis is too low to detect mutations.

However, we could demonstrate the usefulness of DGGE of RNA:DNA duplexes in this model experiment during which sequence differences in the genes of individuals were efficiently detected.

The sensitivity of DGGE of RNA:DNA duplexes could be dramatically increased by amplifying the genomic DNA by PCR.[29] From our preliminary experiences (Takahashi et al, in preparation), the intensity of the bands of duplexes made from genomic DNAs amplified by PCR was the same as that of the band of duplexes from cloned DNAs. However, even if this improvement is incorporated, the DGGE alone will not allow the detection of all possible single-base mutations, since our preliminary data showed that some single-base

mismatches would not lead to a shift in the mobility of the duplex in DGGE. If the RNase cleavage method is employed in combination with DGGE, there is a high probability of detecting mutations which cannot be detected using DGGE alone. Thus, a very large fraction of all possible mutations should be detected by combining the two methods, although there is some overlap in the mutations that can be detected by the two methods.

The sequence differences caused by the deletion of large fragments will be detectable using DGGE, if the following technique is incorporated. The use of either electronic autofluorography[33] or a two-dimensional beta-scanner to impart a greater dynamic range to quantification of ^{32}P-labeled bands should be especially effective for detection of the bands containing half of the expected diploid amount of DNA. Introduction of these techniques could be powerful in studies of radiation mutagenesis, where deletion mutations appear to be predominant.

At present, we think that combining DGGE and the RNase cleavage method is effective and practicable for screening of mutations. Use of these methods will also be conducive to detecting a great number of polymorphisms and will make it possible to prepare linkage maps in great detail.

Acknowledgments

We are grateful to Messers N. Kosaka, H. Omine, and Y. Kimura for their excellent technique in DGGE. We also thank Ms. J. Kaneko for her technical work throughout this study. We are much indebted to Dr. J. V. Neel for his assistance in the preparation of this manuscript.

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