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Ribosomal Ribonucleic Acid (rRNA) Gene Typing for Species Identification

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ABSTRACT: Deoxyribonucleic acid (DNA) typing of ribosomal ribonucleic acid (rRNA) genes was performed with a polymerase chain reaction (PCR) assay for species identification. A variable region of the 28S ribosomal RNA gene was amplified with primers complementary to flanking sequences phylogenetically well conserved. The products of twelve animal DNAs (human, Japanese monkey, dog, cattle, pig, cat, rabbit, mouse, rat, chicken, frog, and fish) were separated by polyacrylamide gel electrophoresis, each revealing a few bands ranging from 150 to 100 base pairs. The band patterns obtained from each DNA sample differed in number and size, which indicates the applicability of the method to species identification. Samples containing either as little as 1 pg of DNA or degraded DNA of 0.2 to 0.5 kb in length were able to give detectable bands. Postmorten human tissue DNAs were tested as an example. They showed a pattern identical to the human control one, which was distinct from those of the other animals examined.

KEYWORDS: pathology and biology, species identification, polymerase chain reaction (PCR), ribosomal RNA gene (rDNA)

Animal genomes contain several hundred copies of ribosomal ribonucleic acid (rRNA) genes, which are usually clustered on several chromosomes [*1*]. Numerous sequences of rRNA genes have been obtained by isolating and sequencing individual cloned genes [*2*]. Comparative studies of the nucleotide sequences provide a means of analyzing phylogenetic relationships over a wide range of taxonomic levels. Most regions of the large-subunit (28S) rRNA sequence evolve slowly and constitute conserved regions, whereas certain parts of 28S rDNA evolve rapidly and vary among species within a genus accordingly. Such variable regions may provide a probe for detecting genetic differences between species.

The polymerase chain reaction (PCR) method, which is an in vitro deoxyribonucleic acid (DNA) amplification technique [*3*], has been developed and its capacity has made it an invaluable tool in forensic science as well as in molecular biology. In ascertaining the genetic individuality of human beings, Higuchi et al. [*4*] first used PCR to amplify discrete DNA regions covering the multiallelic human leucocyte antigen (HLA) gene and analyzed polymorphism of the resulting fragments at the locus. As described above, a certain rRNA gene region has a variable loop region flanked with conserved sequences

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on both sides [2]. We synthesized PCR primers of the conserved sequences to amplify the variable region and questioned whether the products reveal genetic differences between vertebrate species or not.

This paper presents a simple PCR method for typing rRNA genes of animal genomes. Using this technique, the rRNA gene types of twelve animal species, including the human, were identified.

Materials and Methods

DNA Isolation

Genomic DNAs were extracted from tissues or blood of twelve species (human, Japanese monkey, dog, cattle, pig, cat, rabbit, mouse, rat, chicken, frog, and fish) by a standard method [5]. Tissues and leukocytes from blood were suspended in TNE buffer [consisting of 10m*M* tris(hydroxymethyl)aminomethane (tris)/hydrochloric acid (HCl) at pH 7.5, 0.1*M* sodium chloride (NaCl), and 1m*M* disodium edetate (EDTA), containing 0.5% sodium dodecylsulfate (SDS) and digested with 0.2 mg/mL of proteinase K (Merck) at 55°C overnight. The DNA was extracted twice with phenol/chloroform and precipitated with ethanol. The DNA pellet was dissolved in TE buffer (10m*M* Tris-HCl at pH 7.5 and 1m*M* EDTA).

DNA Amplification

Two pairs of primers, 5′-ATCTAGTAGCTGGTTCCCTC-3′ (Primer A) and 5′-CCTCTAATCATTCGCTTTAC-3′ (Primer B), and 5′-AAACTCTGGTGGAGGTCCGT-3′ (Primer C) and 5′-CTTACCAAAAGTGGCCCACTA-3′ (Primer D) were synthesized by the phosphoramidite method on a synthesizer (Appled Biosystems 380B DNA). An amount between 0.1 and 10 ng of sample DNA was mixed with 30 pmol of each primer and 1 unit of Taq polymerase (Perkin-Elmer Cetus) in 30 μL of PCR buffer (10m*M* Tris-HCl at pH 8.4, 50m*M* potassium chloride (KCl), 2.5m*M* magnesium chloride ($MgCl_2$), and 200 μ*M* amounts of each deoxy nucleotide triphosphates [dNTP]), and the solution was covered with 20 μL of mineral oil (Aldrich Chemical). DNA amplification was carried out in a temperature controller with 35 cycles of reaction at 92, 57, and 72°C for 2, 1.5, and 1.5 min, respectively. After 35 cycles, the mixture was incubated an additional 10 min at 72°C. The PCR product was extracted with phenol/chloroform, precipitated with ethanol, and then dissolved in 10 μL of TE. An aliquot (5 μL) was electrophoresed on a 12% polyacrylamide gel. Electrophoresis was performed through a 40-cm-long gel at 280 V for about 24 h and visualized with ethidium bromide staining.

Results

The synthetic oligonucleotides for amplifying 28S rRNA gene (rDNA) regions are presented in Fig. 1. The sequence of Primer A is complementary to 20 nucleotides in the coding strand at the 5′ terminus of human 28S rRNA (Position 1626 to 1645). Primer B is complementary to 20 nucleotides in the noncoding strand at the 3′ terminus of human 28S rRNA (Position 1714 to 1733). The DNA sequence between the Primer A and B regions is known in several vertebrates and the size varies among species, whereas these primer sequences are well conserved among higher vertebrates [2].

DNA was prepared from twelve species, mixed with a pair of the primers and subjected to a PCR assay. The cycling reaction consisted of denaturation at 92°C for 2.0 min, annealing at 57°C for 1.5 min, and extention at 72°C for 1.5 min. Thirty-five cycles of amplification were performed. The PCR products were separated by 12% polyacrylamide

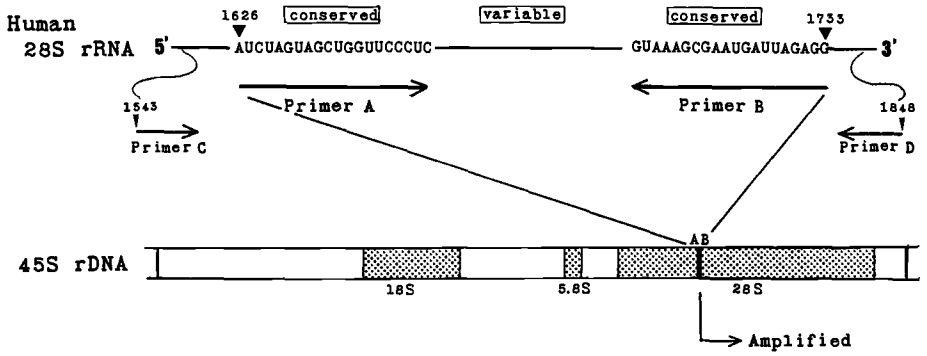

FIG. 1—*A strategy of DNA amplification for species identification. Two pairs of primers—Primers A and B and Primers C and D—were used to amplify the rDNA regions that contained a variable sequence in the 28S ribosomal RNA gene. The sizes of the products with Primers A and B and Primers C and D for human DNA are 108 and 306 bp, respectively. Primer A, 5'-ATCTAGTAGCTGGTTCCCTC-3'; Primer B, 5'-CCTCTAATCATTCGCTTTAC-3'; Primer C, 5'-AAACTCTGGTGGAGGTCCGT-3'; and Primer D, 5'-CTTACCAAAAGTGGCCCACTA-3'.*

gel electrophoresis and visualized by staining with ethidium bromide (Fig. 2). The human rRNA gene fragments amplified consisted of four bands, the sizes of which were 108, 104, 101, and 99 bp from the top to the bottom. Twelve samples from human individuals showed an identical pattern (Fig. 2*a*), which implies that the rRNA type for humans is monomorphic. The appearance of the four different fragments probably reflects the presence of rRNA gene clusters on five different chromosomes [*1*].

Products of the other species tested contained one to four bands (Fig. 2*b*). The sizes of some of the bands conformed to those estimated from the available sequence data [*2*] as far as the data were available. The band pattern of each species differed from that of humans in the number and position of the bands. These results show that the rRNA gene typing (rDNA typing) performed here is useful for identification of sample DNA as being of human origin.

It has been, however, suggested that PCR products may not be amplified with high fidelity because of base-pair mismatching in the primer binding regions. To confirm the bands detected, amplification of the twelve samples was carried out in two stages. The first stage amplified the longer 28S rDNA region, covering the variable region (Position 1543 to 1848) by using Primers C and D, shown in Fig. 1. The sequences of Primers C and D are also well conserved [*2*]. These amplification products were diluted to 10^{-4}, and an aliquot (1 μL) was used in a second stage of amplification with Primers A and B. The products were then analyzed as described above. As shown in Fig. 2*c*, the pattern of each rDNA type was almost identical to that obtained by the direct amplification shown in Fig. 2*b*, although the intensity of bands varied in some cases. This result indicates that amplification products with Primers A and B are derived from the variable 28S RNA region, shown in Fig. 1. The change in band intensity may result from preferential amplification due to differences in their sizes and base compositions. A human rDNA clone exhibited a single band with this assay (data not shown). These results indicate that amplification using Primers A and B has enough fidelity to provide rDNA typing for species identification.

We examined the amount of sample DNA required for this assay. Figure 3 shows that as little as 1 pg of DNA was able to provide clear bands. One picogram of DNA is equivalent to ⅙ of the DNA contained in a single human cell [*6*]. The result is, however, not surprising because the human genome contains several hundred copies of rRNA genes [*1*].

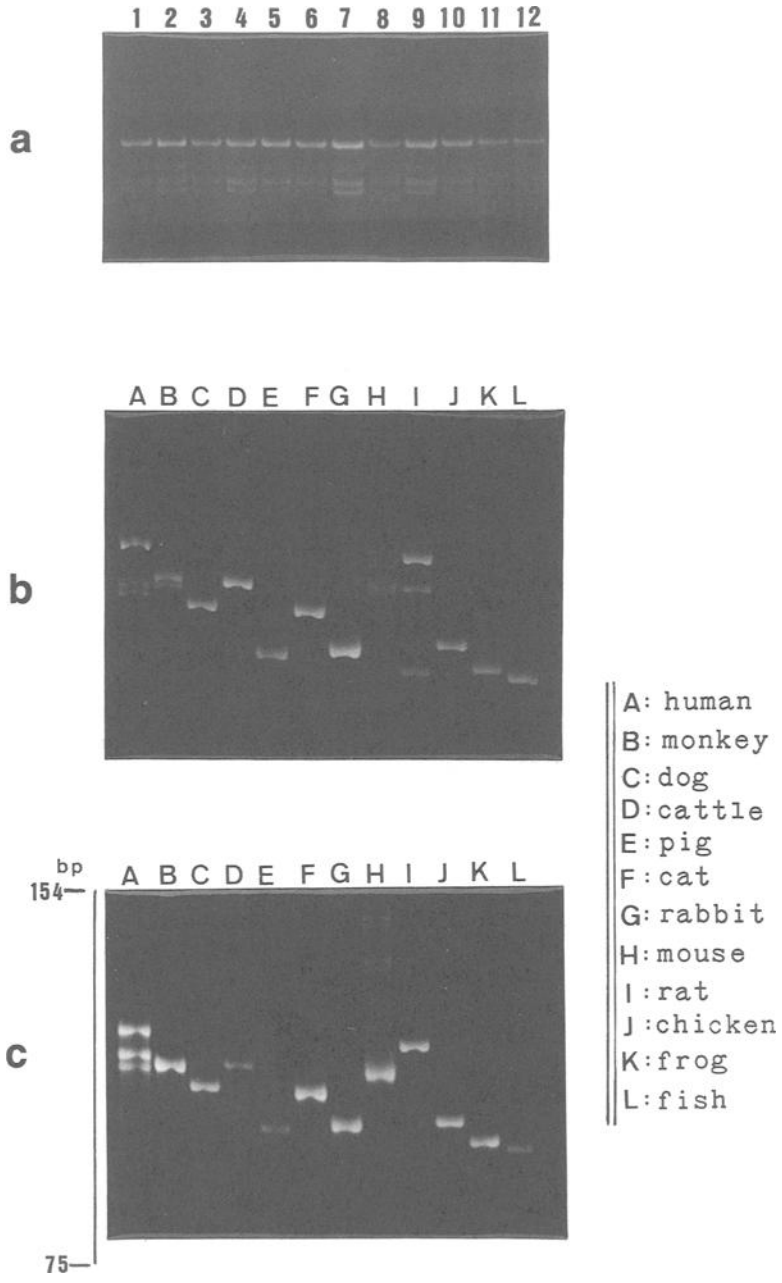

FIG. 2—*Typing of human and animal DNAs using rDNA.* (a) *Twelve human DNA samples (six male and six female) from blood were used for the PCR assay with Primers A and B, indicated in Fig. 1. The products were separated by 12% polyacrylamide gel and stained with ethidium bromide. All the samples showed an identical pattern consisting of four bands.* (b) *Genomic DNAs of twelve animal species (A through L) were amplified. One to four bands could be obtained from each animal DNA.* (c) *Dual amplification of the twelve DNA samples was performed. Primers C and D were used in the first stage, and Primers A and B in the second stage. The size of the fragments was estimated with* Hinf*I-digested pBR322 DNA as size markers.*

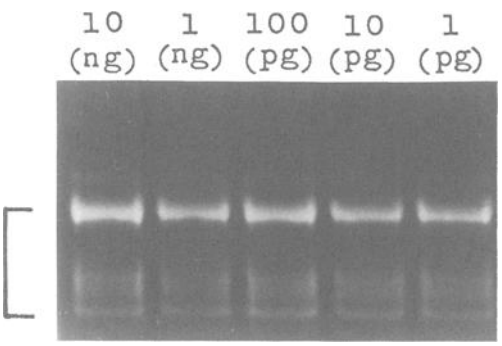

FIG. 3—*The small amount of DNA required for the assay. Different amounts of human DNA indicated in the top of the figure were used for the PCR assay. The products were analyzed as described in the Materials and Methods section.*

DNA samples are often degraded before DNA preparation and are of short length. The degradation can be a cause of band loss, which leads to mistaking the loss for a true genetic difference between samples of human and other species. We therefore tested the effect of fragment size on the PCR products. Human DNA was cleaved into smaller fragments by sonication. Two samples ranging from 7.5 to 2.5 kb and from 0.5 to 0.2 kb were obtained and subjected to the PCR assay. Figure 4 shows that the degraded samples also gave a clear band pattern almost identical to that of the unsonicated DNA, indicating that DNA samples consisting of short fragments can serve as a template for the assay of rRNA gene typing.

Figure 5 shows an example of application of this method to the postmorten tissues of an infant. He was smothered right after birth and left in a kitchen for 6 days. DNA was extracted from the blood, thymus, spleen, skin, and liver, and its size was determined by agarose gel electrophoresis (Fig. 5*a*). All the tissues contained high-molecular-weight DNA, and the blood and skin samples, especially, were almost intact. As expected, they provided clear bands of PCR products, all of which exhibited a human rDNA type (Fig. 5*b*).

Discussion

Species identification of bloodstains and tissues is important in forensic science. Several immunological methods have been developed and present considerable potential [*7–9*]. However, DNA is more stable than most protein markers and DNA typing should offer the promise of absolute identification. Analysis at the DNA level is therefore expected to provide considerable information for individual and species identification. Common methods of DNA analysis based on DNA hybridization have also been applied to species identification [*10–12*]. We have developed a novel PCR method for rDNA typing which detects genetic differences between man and the common animals (at least in Japan). With this assay, rDNA types of twelve vertebrates were determined.

An rRNA gene region spans a variable region in length and in the flanking conserved sequences. Primers comprising conserved sequences among higher vertebrates have been designed and used to amplify the variable region. When the products are separated by acrylamide gel electrophoresis, the result provides an rDNA type consisting of various bands. The band pattern of human DNA is distinct from that of the eleven animal samples examined. It is worth noting that the origin of samples can be judged by a band pattern with this method and not require the presence or absence of hybridization signals on a filter, which are used in other methods [*10–12*].

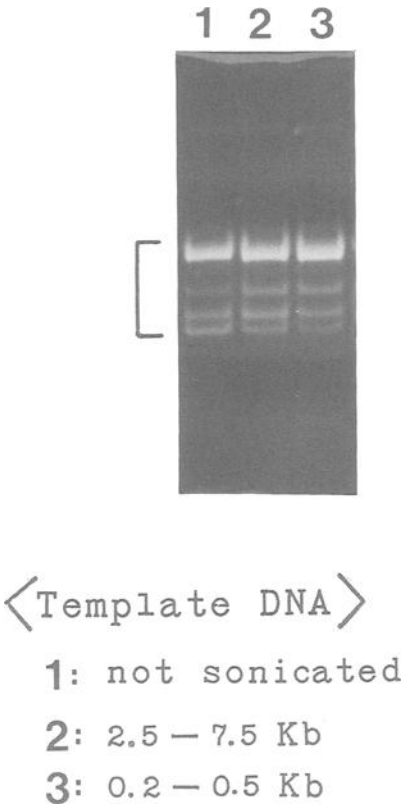

FIG. 4—*The effect of DNA fragment sizes on the amplification. Genomic DNA from one person was sonicated and used as a template for PCR. Sample 2 was obtained by sonication for 5 min and Sample 3 for 30 min. The size of the template DNA is shown in the top of the figure.*

Two features of the method are of obvious benefit in the analysis of biological evidence. First, amplification is possible from very small amounts of DNA. Only 1 pg of DNA from tissues was sufficient for rDNA typing (Fig. 3). In contrast, the dot blot hybridization method requires more than 40 ng of DNA to produce visible signals [*13*]. The rDNA typing method allows genetic information to be obtained from evidence samples that contain insufficient cellular material for other genetic typing approaches. Second, this method is applicable to DNA of short length, which is often recovered from environmentally compromised samples. The DNA in a significant proportion of biological evidence is degraded. Yokoi and Sagisaka demonstrated the rapid degradation observed in liver, pancreas, and spleen tissues, which may depend on the conditions of the environment surrounding the bodies [*14*].

It is unlikely that bacterial and yeast contamination in evidence samples affects rDNA typing, because the sequences used for the primers are not conserved in the genomes of bacteria and lower eukaryotes [*2*]. The primers cannot hybridize to those rDNA sequences and hence fail to amplify the sequences. Indeed, a mixture of human, yeast, and *Escherichia coli* DNAs were prepared and analyzed by the PCR method. It provided an rDNA type identical to that of an uncontaminated human DNA (data not shown).

In conclusion, the experimental evidence presented here proves that rDNA typing using ribosomal gene primers is useful for species identification in human and animal tissue DNAs.

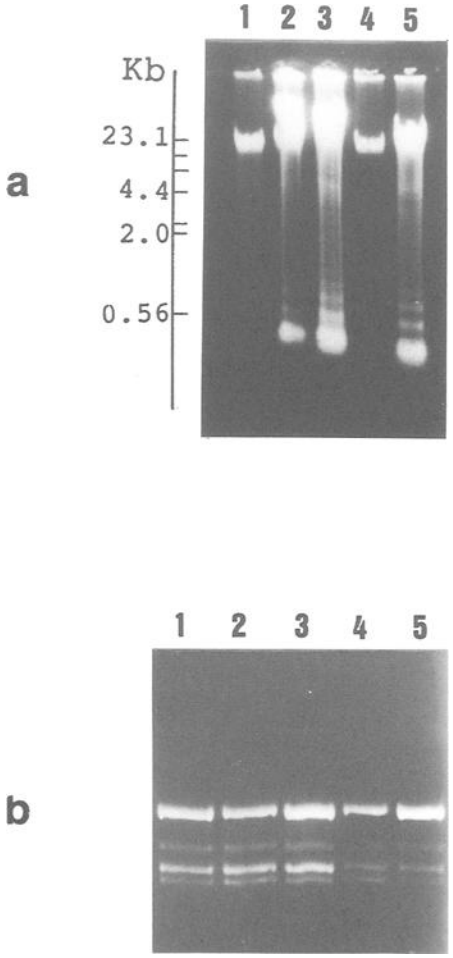

FIG. 5—*Application to postmorten tissue DNAs.* (a) *Five DNA samples were obtained from the postmorten tissues of an autopsy case, and their sizes were determined using 1.0% agarose gel electrophoresis; these samples were (1) blood, (2) thymus, (3) spleen, (4) skin, and (5) liver.* (b) *The DNA samples Fig. 5a were analyzed by PCR assay, followed by acrylamide gel electrophoresis.*

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