Henry C. Lee,[1] *Ph.D.; Elaine M. Pagliaro,*[1] *M.S.; Karen M. Berka,*[2] *M.S.; Nancy L. Folk,*[2] *B.S.; Daniel T. Anderson,*[2] *M.S.; Gualberto Ruano,*[1] *M.Phil.; Tim P. Keith,*[3] *Ph.D.; Pamela Phipps,*[3] *B.S.; George L. Herrin, Jr.,*[4] *Ph.D.; Daniel D. Garner,*[4] *Ph.D.; and R. E. Gaensslen,*[2] *Ph.D.*

Genetic Markers in Human Bone: I. Deoxyribonucleic Acid (DNA) Analysis

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ABSTRACT: Deoxyribonucleic acid (DNA) was isolated from a number of spongy and compact human bone tissue specimens, and the yield was estimated on a "per milligram of starting tissue" basis. DNA was, in addition, isolated from a number of corresponding blood and bone tissue specimens. Spectrophotofluorometry and ethidium bromide visualization on minigels were used to estimate the quantity and degree of degradation of DNA. The DNA from several blood-bone pairs is shown to give concordant restriction fragment length polymorphism (RFLP) typing results by two different typing protocols with five different single-locus probes. DNA from several additional blood-bone pairs is shown to give concordant results for human leucocyte antigen (HLA)-DQα phenotypes following polymerase chain reaction (PCR) amplification and hybridization to specific allele-specific oligonucleotide (ASO) probes, and for the variable numbers of tandem repeats (VNTR) length polymorphisms 3′ to the human apolipoprotein B (APOB) gene following PCR amplification with specific primers and analysis of the products by electrophoresis and ethidium bromide visualization.

KEYWORDS: pathology and biology, deoxyribonucleic acid (DNA), genetic typing, forensic DNA typing, human bone DNA, bone DNA typing, bone genetic markers

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[1]Chief criminalist and director, supervisory criminalist, and research associate, respectively, Connecticut State Police Forensic Science Laboratory, Meriden CT. Mr. Ruano is permanently with the Department of Human Genetics, Yale University School of Medicine, New Haven, CT.

[2]Research associate, laboratory coordinator, graduate research assistant, and professor and director, respectively, Forensic Sciences Program and Laboratories, University of New Haven, West Haven, CT. Mr. Anderson is currently with the Office of Coroner/Medical Examiner, Los Angeles, CA.

[3]Manager, Human Genetics Research and Technology Development, and staff scientist, respectively, Collaborative Research, Inc., Bedford, MA.

[4]Staff scientist and laboratory director, respectively, Cellmark Diagnostics, Germantown, MD. Dr. Herrin is currently with the DNA Laboratory, Forensic Science Division, Georgia Bureau of Investigation, Decatur, GA.

Several years ago, Jeffreys described hypervariable regions of human deoxyribonucleic acid (DNA) using multilocus probes and the applicability of these DNA polymorphisms to the individualization of human blood and tissues [*1,2*]. The potential forensic applications of DNA analysis in resolving disputed parentage problems as well as in the individualization of blood and body fluids were immediately recognized [*3,4*]. The extensive polymorphism detected by this type of DNA analysis consists of variable numbers of tandem repeats (VNTR) within sequences of repetitive DNA in the human genome [*5–8*]. Numerous multilocus and single-locus probes that detect VNTR polymorphisms have been found since Wyman and White first described the single-locus probe pYNH24 [*9*]. DNA typing using probes that detect VNTR loci is widely known as restriction fragment length polymorphism (RFLP) analysis, and it has been employed extensively in human gene mapping [*10–12*]. The use of a number of single-locus DNA probes in the forensic analysis of blood and body fluid stains has recently been described [*13–17*].

Polymerase chain reaction (PCR) procedures that enable the faithful replication of millions of copies of a specific DNA sequence in vitro have also been described and refined in recent years [*18–20*]. Because many specimens submitted for forensic DNA analysis contain limited quantities of DNA, and the DNA in these specimens may be degraded, RFLP analysis is not always possible. This limitation has prompted the interest of forensic scientists in PCR typing procedures that may be applicable to small or degraded specimens. PCR techniques have been applied to the clinical diagnosis of sickle cell anemia [*21*].

The best developed PCR procedure applicable to forensic identification thus far utilizes specific primers and allele-specific oligonucleotide probes to detect genotypes at the human leucocyte antigen (HLA)-DQα locus [*22,23*]. However, PCR has also been used to amplify VNTR loci. The products have been characterized using specific, single-locus DNA probes in the case of the complex minisatellite loci described by Jeffreys et al. [*24*]. PCR products from other VNTR loci have been characterized by size following electrophoresis on agarose or polyacrylamide gels and visualization with ethidium bromide. The hypervariable locus 3′ to human apolipoprotein B has been successfully amplified and its products characterized by agarose gel electrophoresis [*25–27*]. Using denaturing polyacrylamide gel electrophoresis, Ludwig et al. detected 14 alleles at this locus in 318 unrelated people [*28*]. The *D17S30* locus detected in RFLP analysis by DNA probe pYNZ22 [*29*] and, more recently, the *D1S58* locus detected in RFLP analysis by DNA probe pCMT118 [*30*] have also recently been amplified by PCR, and the products have been characterized by gel electrophoresis [*31,32*].

As part of the continuing development of DNA typing procedures applicable in forensic sciences, some studies have been conducted on the stability of DNA and RFLP patterns obtained from postmortem tissues and organs other than blood. In death cases in which DNA analysis is appropriate, typing of a specimen from the deceased victim is typically required. DNA typing may also be used as a means of identification in cases of otherwise unidentifiable human remains, especially if parents, children, or other known relatives are available for testing. Although the DNA is expected to be virtually identical in all nucleated cells of the body, the stability of DNA might differ significantly from one tissue to another. The ability to determine RFLP patterns from postmortem tissues might in turn be influenced by the tissue chosen for DNA isolation.

Bär et al. studied DNA stability, yield, quality, and RFLP patterns from the postmortem brain, lymphatic tissue, liver, spleen, skeletal muscle, kidney, and thyroid, as well as blood [*33*]. Using the minisatellite probe 33.15 [*1,2*] to hybridize *Hinf*1-digested DNA, they found that larger restriction fragments (15 to 23 kb) gradually disappeared as autolysis progressed. No spurious bands were noted in any of the specimens. To the extent that bands were still present, the DNA RFLP patterns matched in specimens from

all the tissues studied. Swarner et al. [*34*] studied the recovery of DNA from postmortem blood, heart muscle, chest muscle, liver, spleen, hair, and bone tissues and assessed its quality on minigels containing ethidium bromide. DNA from liver tended to be the most degraded, while that from heart muscle, spleen, and bone tended to be least degraded. There was not a strict correlation between the degree of DNA degradation and the time since death. There are two recent reports showing that high-molecular-weight (high MW) DNA can be extracted from dental pulp [*35,36*]. Even in some cases where the tooth was subjected to extreme environmental conditions, RFLP patterns could be obtained from dental pulp DNA in some of the specimens. Perry et al. [*37*] looked at the degradation of human rib bone DNA under controlled conditions in experiments designed to determine whether DNA degradation could be correlated with the time since death. DNA degradation patterns were studied by hybridizing electrophoretically separated (but unrestricted) bone DNA to labeled, general human DNA probes, and then using a scanning densitometer to estimate the sizes of the DNA fragments, based on the positions of known size ladder fragments. No genetic marker analysis using RFLP or PCR techniques was done in these studies. Haglund et al. [*38*] recently reported a case in which single-locus DNA probes were used to assist in identifying the decomposed body of a female homicide victim. In this case, DNA suitable for RFLP analysis was isolated from the psoas muscle.

Pääbo [*39,40*] has reported cloning a 3.4-kb segment of human DNA from an ancient Egyptian mummy, demonstrating that DNA may be comparatively stable for very long periods of time, at least in certain human remains under certain conditions. The recently reported successful amplification by PCR of mitochondrial DNA sequences up to 600 base pairs (bp) in length from human bones 300 to over 5000 years old [*41*] further indicates the potential stability of DNA.

As part of a comprehensive study of human bone grouping, we have conducted some RFLP and PCR analyses of DNA isolated from human bone and from the corresponding blood. The results of these studies are the subject of this paper. Some earlier results on bone tissue ABO typing have appeared [*42*], and the results of more extensive studies will be reported elsewhere.

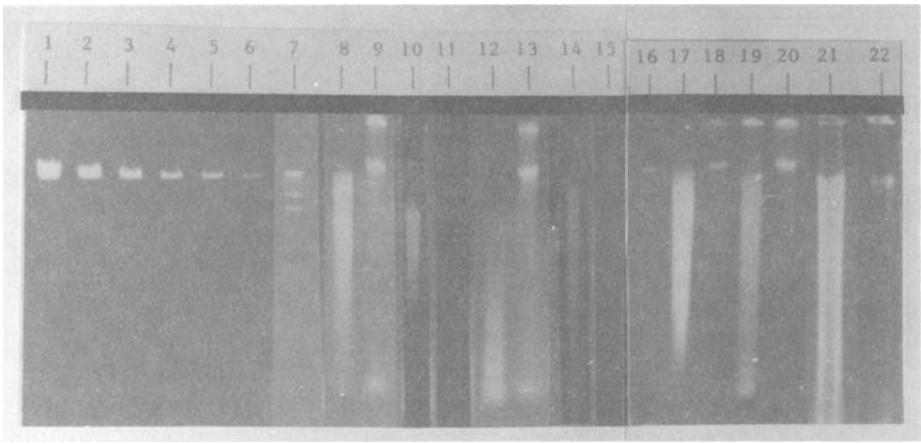

FIG. 1—*Photographic composite of minigels of blood DNA and corresponding bone DNA after isolation: Lanes 1–6—232, 116, 58, 29, 12.4, and 5.8 ng λ-DNA standards, respectively; Lane 7—*HindIII/EcoR1*-digested λ-DNA size ladder; Lanes 8–9, 10–11, 12–13, 14–15—bone-blood DNAs of Specimens 2001, 2008, 2006, 2002, respectively; Lane 16—*HindIII*-digested λ-DNA size ladder; Lanes 17–18, 19–20, 21–22—bone-blood DNAs of Specimens 2004, 2042, 2481, respectively.*

Materials and Methods

DNA was isolated from bloodstains on cotton cloth by overnight incubation of 2.5-cm^2 stain fragments at 37°C with 400 μL extraction buffer [10m*M* Tris, 10m*M* sodium ethylenediaminetetraacetate (EDTA), and 100m*M* sodium chloride (NaCl), pH 8] containing 2% sodium dodecyl sulfate (SDS) and 4.1 Sigma units of proteinase K, followed by at least two extractions with Tris/hydrochloric acid (HCl) (pH 8) equilibrated phenol and chloroform containing isoamyl alcohol (24:1 v/v). DNA was precipitated with 100% ethanol, washed with 70% ethanol, and redissolved in TE (10m*M* Tris and 1m*M* Na_2EDTA, pH 7.8) buffer.

With bone tissue, 10 to 20 mg of spongy or 75 to 100 mg of compact bone were successively immersed and agitated in cold distilled water, ethanol, and ethyl ether for 15 min each, after the adhering soft tissue had been physically removed. The specimens were then dried, immersed in liquid nitrogen for 15 to 30 s to freeze them thoroughly, and crushed to a fine powder. DNA was extracted from the resulting powder using the method described for bloodstains.

The DNA quality was assessed and the concentration was estimated by visualizing 50 to 350 ng of the specimen DNA, along with a series of λ-DNA concentration standards,

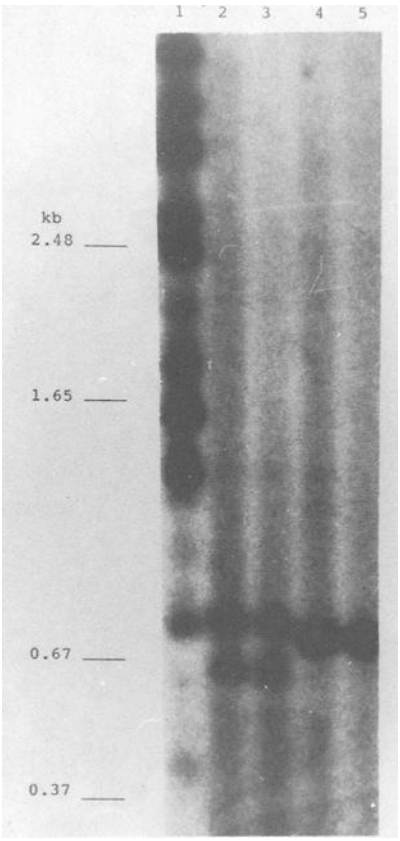

FIG. 2—*RFLP analysis of blood-bone DNAs with single-locus probe CRI-PAT-pL1077-1: Lane 1—CRI-MW-102 size ladder; Lanes 2–3 and 4–5—blood-bone DNAs of specimens 2001 and 2008, respectively.*

following submarine electrophoresis in 1X TBE (89m*M* Tris, 89m*M* boric acid, and 2m*M* EDTA, pH 8) in a 7.5 by 5-cm 0.7% agarose gel containing 10 μg ethidium bromide, on an ultraviolet (UV) transilluminator. Thirty nanograms of *Hind*III- or *Hind*III/EcoR1-digested λ-DNA was included on each of these minigels (yield gels) as a size marker. DNA was also quantitated in some experiments by spectrophotofluorometry using Hoechst 33258 dye [*43*] and a TKO 100 spectrofluorometer (Hoeffer Instruments, San Francisco, California). The DNA standards for fluorometry were prepared from pUC18 DNA (International Biotechnologies, Inc., New Haven, Connecticut).

Selected examples of human genomic DNA recovered from bloodstains and corresponding bone tissue were subjected to RFLP analysis in one of two different ways, depending on the laboratory's protocol and the probe or probes to be used. In all cases, a minigel was run on aliquots of restriction enzyme-digested genomic DNA to ensure that digestion was complete before the samples were loaded into the analytical gel.

Specimens analyzed at Collaborative Research were run in 1.5% agarose analytical gels after digestion with *Hae*III, transferred to nylon membranes by the procedure of Southern [*44*] prior to hybridization with the single-locus probe CRI-PAT-pL1077-1, which detects polymorphism at VNTR locus *D6S22* [*45*]. Standard Collaborative Research hybridization, washing, and autoradiography protocols were followed [*45,46*]. Specimens analyzed at Cellmark Diagnostics were digested with *Hinf*I before being loaded into analytical gels and subjected to electrophoresis, Southern transfer, and hybridization with a cocktail of single-locus probes MS1, MS31, MS43, and g3 [*16,47*]. Standard Cellmark Diagnostics protocols were followed.

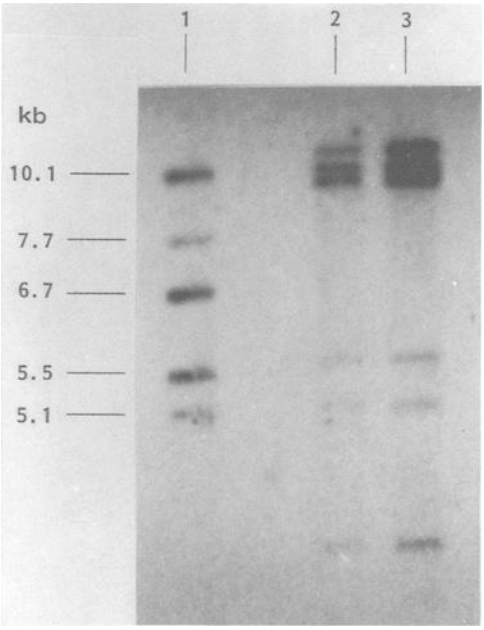

FIG. 3—*RFLP analysis of blood-bone DNA with Jeffreys' single-locus probes MS1, MS31, MS43, and g5: Lane 1—known control DNA "CRN" from which fragment sizes were estimated, based on multiple sizing estimates of this DNA specimen on other blots; Lanes 2–3—bone-blood DNA of Specimen 2060.*

Genomic DNA, in amounts of 50 to 300 ng, from specimens to be analyzed for HLA-DQα phenotypes was incubated with specific biotinylated primers obtained from Cetus Corp. (Emeryville, California) [*22*] and amplified in a Perkin-Elmer thermal cycler following the recommended protocol. PCR products were analyzed using reverse dot-blot nylon membrane strips supplied by Cetus Corp., following their recommended hybridization and detection protocols. These strips have allele-specific oligonucleotide probes for HLA-DQα type and subtype sequences covalently attached in a specific order [*22,23*] to allow convenient interpretation of the results.

Genomic DNA, in amounts of 200 to 500 ng, from specimens to be analyzed for the apolipoprotein B 3′ hypervariable region by PCR was incubated with the specific primers 5′-ATGGAAACGGAGAAATTATG-3′ and 5′-CCTTCTCACTTGGCAAATAC-3′ [*27*] at a final concentration of 0.1μ*M* in a reaction mixture consisting of 2 units of Amplitaq (Cetus Corp.) [*20*], 50m*M* potassium chloride (KCl), and 200μ*M* each of dNTP, 10m*M* Tris-HCl (pH 8.3), 1.5m*M* $MgCl_2$, and 0.01% gelatin in a final volume of 100 μL [*48*]. Each reaction mixture was overlaid with 50 μL of mineral oil.

The thermal cycling program was 94°C for 1 min, 54°C for 2 min, and 72°C for 2 min for 30 cycles. Between 15 and 30% of the PCR product was analyzed by submarine electrophoresis on 20 by 20-cm 2.5% agarose gels made from 1.5% DNA grade agarose and 1% NuSieve (FMC Corp., Rockland, Maine). The gels were immersed in 0.5 μg/mL ethidium bromide in 1X TBE solution to allow visualization of fragments on a UV transilluminator. DNA from K562 cells was routinely included as a control, and *Hae*III-digested ϕX174 DNA (Bethesda Research Laboratories, Life Technologies, Gaithersburg, Maryland) was used as a size marker.

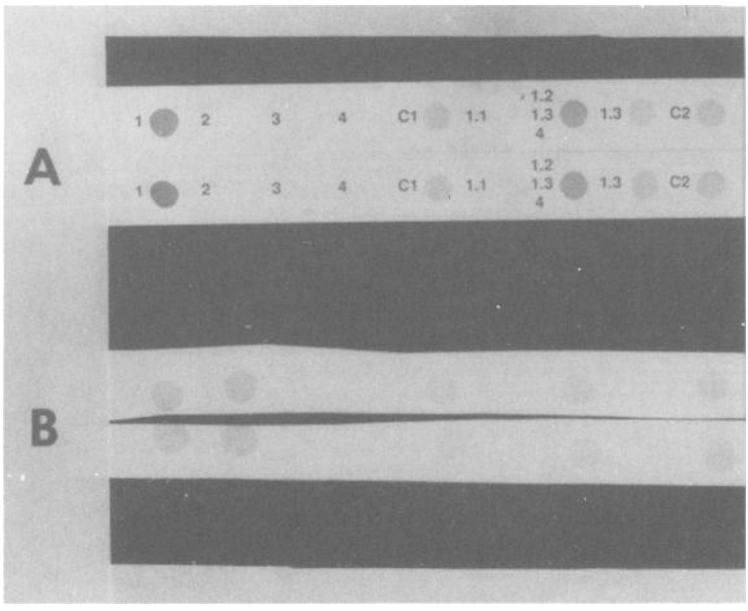

FIG. 4—*HLA-DQα locus analysis of blood-bone DNAs following PCR with specific biotinylated primers and reverse dot blot analysis on membranes containing allele-specific oligonucleotide probes (Cetus): A—blood* (above) *and bone* (below) *DNA of Specimen 2042 (pattern corresponds to Type 1.2, 1.3); B—blood* (above) *and bone* (below) *DNA of Specimen 2281 (pattern corresponds to Type 1.2, 2).*

Results and Discussion

DNA yields from bone tissue vary according to the type of bone and, to a lesser extent, from preparation to preparation. Since spectrophotofluorometric estimates of DNA concentration measure all the DNA in a specimen including degraded DNA, these measurements routinely give overestimates of the quantity of high-MW DNA in specimens that contain any significant amount of degraded DNA. Estimation of the high-MW DNA content of a specimen using ethidium-bromide-containing minigels as a guide to its suitability for subsequent RFLP analysis was found to be more accurate than fluorometry.

DNA yields were routinely 10 to 20 times higher in spongy tissue than in compact bone on a "per milligram of starting tissue" basis. DNA yields for 10 to 100 mg of bone varied from 1.5 to 10 μg/mg of spongy bone and from 0 to 500 ng/mg of compact bone when estimated by spectrofluorometry. In some experiments, no DNA was obtained from compact bone when we started with 10 or 25 mg of tissue.

Detectable quantities of DNA were always obtained from specimens of 50 mg or more. Many bone tissue specimens yield significant quantities of degraded DNA that is readily apparent on ethidium-bromide-stained minigels (Fig. 1). Average yields of high-MW DNA from bone tissue, estimated from minigels, can be 5 to 10% of those obtained by spectrofluorometric measurement. These variations must be considered in determining the size of the bone tissue specimen to be used for isolation of DNA, especially for RFLP analysis, where larger quantities of high-MW molecules are required for successful results.

Results of RFLP analysis of *Hae*III-digested DNA from two blood-bone pairs with the single-locus probe CRI-PAT-pL1077-1 are shown in Fig. 2. Restriction fragments in the 500 to 700-bp range were obtained. As expected, the autoradiogram shows that the patterns obtained from bone DNA visually match those obtained from the corresponding bloodstains. One of the bone DNA specimens that yielded successful typing results on this blot showed significant degradation when judged by the minigel (Fig. 1, Lane 8).

Results of RFLP analysis of *Hinf*I-digested DNA from a blood-bone pair with a cocktail of single-locus probes MS1, MS31, MS43, and g5 are shown in Fig. 3. Here too, the blood and bone DNA patterns visually matched one another, as expected.

Figure 4 shows the results of typing two blood-bone pair DNAs for HLA-DQα on Cetus membrane strips following PCR amplification. The blood DNA gave the same phenotype in both cases as the corresponding bone DNA, as expected. Both bone DNA specimens typed for HLA-DQα showed significant degradation when visualized on a minigel (Fig. 1, Lanes 19 and 21). It is of some interest that one of these bone DNAs (Fig. 1, Lane 21) failed to give RFLP results with the single-locus probe pYNH24 in tests using the FBI protocol [*49*].

The results of testing four blood-bone pair DNAs for the 3′APOB polymorphism by PCR, and ethidium-bromide visualization of the resulting fragments on an agarose gel following electrophoresis, are shown in Fig. 5. In all cases, the bone DNA patterns corresponded exactly to those of the corresponding blood DNAs. Two of the bone specimen DNAs that yielded successful 3′APOB results showed extensive degradation when visualized on a minigel (Fig. 1, Lanes 12 and 14), and one (Fig. 1, Lane 12) appeared to lack high-MW DNA. One of the bone specimens (Fig. 1, Lane 14) failed to give RFLP results with the single-locus probe pYNH24 in tests using the FBI protocol [*49*]. Some of the repeat sequence PCR products observed in these four specimens have been observed elsewhere [*27,28*] and in blood DNA from several individuals tested subsequently in our laboratory.

The results of these studies show that DNA suitable for both RFLP and PCR analysis can be obtained from bone tissue, and that spongy bone usually yields significant quantities of DNA. The data fully support the expectation that blood and bone DNAs from the same individual yield comparable results, regardless of the method of analysis em-

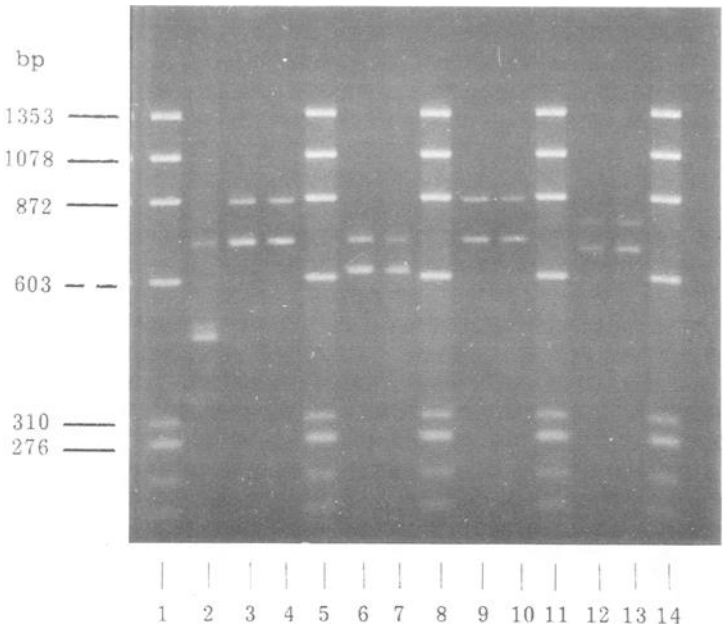

FIG. 5—*Analysis of the VNTR polymorphism 3' to apolipoprotein B following PCR with specific primers, agarose gel electrophoresis, and ethidium bromide visualization: Lanes 1, 5, 8, 11, and 14—500 ng each of* HaeIII*—digested ϕX174 DNA; Lane 2—control K562 cell line DNA (fragments correspond to 39 and 21 repeats); Lanes 3–4—blood-bone DNAs of Specimen 2001 (fragments correspond to 49 and 39 repeats); Lanes 6–7—blood-bone DNAs of Specimen 2006 (fragments correspond to 39 and 33 repeats); Lanes 9–10—blood-bone DNAs of Specimen 2004 (fragments correspond to 49 and 39 repeats); Lanes 12–13—blood-bone DNAs of Specimen 2002 (fragments correspond to 43 and 36 repeats).*

ployed. Two specimens from which RFLP results could not be obtained gave very satisfactory typing results when using PCR techniques. Further studies on DNA from bone tissue that has been subjected to aging under various environmental conditions are continuing in our laboratory.

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Address requests for reprints or additional information to
Henry C. Lee, Ph.D.
Chief Criminalist and Director
Connecticut State Police
Forensic Science Laboratory
294 Colony St.
Meriden, CT 06450