R. E. Gaensslen,[1] Ph.D.; Karen M. Berka,[1] M.S.;
Dina A. Grosso,[1] M.S.; Gualberto Ruano,[2] M. Phil.;
Elaine M. Pagliaro,[2] M.S.; Deborah Messina,[2] M.S.; and
Henry C. Lee,[2] Ph.D.

A Polymerase Chain Reaction (PCR) Method for Sex and Species Determination with Novel Controls for Deoxyribonucleic Acid (DNA) Template Length

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ABSTRACT: Human X and Y chromosome α-satellite sequences lying within higher order repeats were amplified by the polymerase chain reaction (PCR) in genomic deoxyribonucleic acid (DNA) isolated from blood, bone, and several other tissues and specimens of potential forensic science interest. X and Y sequences could be coamplified under some of the PCR conditions employed. Monomorphic sequences in the 3′-apolipoprotein B gene (designated "H") and in an α-satellite higher order repeat on Chromosome 17 (p17H8, D17Z1) were likewise amplified in the specimens. X and Y sequence amplification can provide information about the sex of origin. Amplification of the X, H, and D17Z1 sequences was found to be primate-specific among the common animals tested and can thus provide species of origin information about a specimen. The authors suggest that amplification of X and D17Z1 or H sequences might provide "relaxed" and "stringent" controls for appropriate PCR amplification tests on forensic science specimens. Testing was carried out using PCR protocols that employed *Thermophilus aquaticus* (Taq) and *Thermus flavis* (Replinase) thermostable DNA polymerases.

KEYWORDS: forensic science, polymerase chain reaction (PCR), deoxyribonucleic acid (DNA), human identification, species identification, bone DNA, tissue DNA, sexing, species, X chromosome, Y chromosome, amplification, monomorphic sequences, D17Z1, p17H8, apolipoprotein B gene

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[1]Professor and director, research associate, and graduate research assistant, respectively, Forensic Sciences Program and Laboratories, University of New Haven, West Haven, CT.

[2]Research associate, supervisory criminalist, senior criminalist, and chief criminalist and director, respectively, Connecticut State Police Forensic Science Laboratory, Meriden, CT. The permanent address for G. Ruano is the Department of Human Genetics, Yale Medical School, New Haven, CT.

Deoxyribonucleic acid (DNA) analysis of biological evidence can be conducted by one of two principal methods: the restriction fragment length polymorphism (RFLP) of genomic DNA [*1–3*] method and length polymorphism or sequence variation analysis of DNA amplified by the polymerase chain reaction (PCR), sometimes called amplification [*4*]. PCR analysis has been explored as a method for analyzing biological evidence in several ways, including (1) typing of the HLA-DQα locus [*5–8*], (2) typing of group-specific component (GC) variants [*9*], (3) typing of repeat sequence polymorphisms [*10–12*], and (4) amplification of Y sequences [*12,13*].

Determination of the sex of origin can be an important element in the analysis of biological evidence submitted to forensic science laboratories. Older cytological procedures and methods based on determining sex hormones in biological evidence were never in very wide use, at least not in the United States. Determination of the sex of origin by the direct detection of X- and Y-chromosome-specific DNA sequences appears to offer better prospects as a routine forensic laboratory method.

There are a number of reports on the determination of male specimens using DNA analysis techniques. Y chromosome sequences have been identified on ethidium-bromide-stained gels following electrophoretic separation of restriction-endonuclease-digested genomic DNA [*14*], on yield gels *in situ* [*15*], and on Southern [*16*] and dot [*17*] blots, following hybridization of restricted and unrestricted genomic DNA with specific Y probes. Y chromosome sequences have been amplified by PCR in DNA from blood, bloodstains, saliva specimens, single hair roots, semen specimens, "male" and "female" fractions of differentially extracted semen-containing vaginal swabs [*13*], and human teeth [*14*] for forensic science testing purposes. Y chromosome sequences have also been detected in trophoblast biopsy specimens from male fetuses following hybridization of Y probes to dot blots [*18*], and in fetal nucleated erythrocyte DNA from peripheral maternal blood following PCR amplification, for diagnosing fetal sex during pregnancy [*19*].

Using centromeric alphoid repeat sequence data from both the X [*20*] and Y chromosomes [*21*], Witt and Erickson constructed primers that could be used to amplify sequences from both chromosomes in DNA from bloodstains [*22,23*].

We have used certain of their primer sets (X1,X2 and Y1,Y2) and others of modified design (X3,X4 and Y3,Y4) to amplify X and Y sequences, both separately and simultaneously, in DNA isolated from various types of biological materials of forensic science interest. In addition, we have designed primers for the amplification of other human monomorphic genomic sequences to serve as controls for X and Y sequences and, potentially, for other PCR reactions. Testing of DNA from a number of animal and infra-human species with X, Y, and control primers was conducted as well. The results, which are part of our continuing studies on genetic markers in human bone and tissue [*24–26*], are reported in this paper.

Materials and Methods

DNA was isolated from blood cells, bloodstains, hair roots, buccal cells, sperm-containing postcoital vaginal swabs, and bone tissue by the proteinase K digestion and phenol-chloroform extraction methods, described previously [*24,27*]. The DNA-containing aqueous layer from the last organic solvent extraction was dialyzed, concentrated, and recovered in 10m*M* Tris/hydrochloric acid (HCl), 1m*M* sodium (Na_2) ethylenediaminetetraacetate (EDTA), pH 7.8 (TE), or in sterile neutral distilled water (glass-distilled water, adjusted to pH 7 and steam sterilized) using Centricon 100 miniconcentrators (Amicon, Beverly, Massachusetts). The DNA quantity and quality were assessed by ultraviolet visualization of ethidium-bromide-stained agarose minigels following submarine electrophoresis, as previously described [*24*]. The DNA quantity was also estimated by a spectrophotofluorometric method [*28*].

Variable quantities of target genomic DNA were amplified by PCR using three different protocols.

In Protocol A, the PCR was performed in 10m*M* Tris-HCl, pH 8.4, 50m*M* potassium chloride (KCl), 200μ*M* each dNTP, 2m*M* magnesium chloride ($MgCl_2$), 0.01% gelatin, 2 units (U) of Amplitaq (Perkin-Elmer/Cetus, Norwalk, Connecticut), and 0.1μ*M* primers as follows: X1, 5′-AATCTGCAAATGGAGATTTG; X2, 5′-GTTCAGCTCTGTGAGTGAAA; Y1, 5′-ATGATAGAACGGAAATATG; and Y2, 5′-AGTAGAATGCAAAGGGCTCC. The reactions were conducted in a final volume of 100 μL. These X primers yield a 130 base pair (bp) product, and the Y primers yield a 172 bp product. X and Y amplification reactions were carried out in separate tubes in this protocol. The thermal cycle profile was 3 cycles of 94°C for 4 min, 51°C for 1 min, and 72°C for 1 min, followed by 27 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min. Amplification of the H and D17Z1 sequences (see below) was also performed with Protocol A.

In Protocol B, the PCR was performed in 50m*M* Tris-HCl, pH 9.0, 20m*M* ammonium sulfate, 2m*M* $MgCl_2$, 200μ*M* each dNTP, 2 U Replinase (DuPont Biotechnologies, Boston, Massachusetts) [*29,30*], and either 0.08μ*M* X and Y primers, or 0.1μ*M* H or D17Z1 primers as follows: X3, 5′-TATTTGGACTCTCTCTGAGGA; X4, 5′-TTCTACTACAAGGGTGTTGCA; Y3, 5′-GTGTATTCACCTCCGGGAG; Y4, 5′-ACAAAAGGTTCAATTCTGTGAG; H1, 5′-TGAAGATGACGACTTTTCTAAAT; H2, 5′-GTGAGTTTTTTATCTGGAGAGG; D17Z1-1, 5′-AAACGGGATAATTTCAGCTGA; and D17Z1-2, 5′-AATATCCACTTGCAGATCCTA. The reactions were conducted in a 50-μL final volume. The X and Y amplifications were carried out in separate tubes, as well as simultaneously in the same reaction tube. The H and D17Z1 amplifications were carried out in separate tubes. These X primers amplify a 157-bp product; the Y primers amplify a 200-bp product. The primers designated "H" flank the last intron in the apolipoprotein B gene [*31*] and yield a 1005-bp product. The primers designated "D17Z1" flank a 949-bp sequence in monomers 1 through 6 of a 2712-bp α-satellite higher order repeat on Chromosome 17 (p17H8) [*32*] and yield a 991-bp PCR product. The thermal cycle profile was 3 cycles of 94°C for 4 min, 57°C for 2 min, and 72°C for 2 min, followed by 30 cycles of 94°C for 2 min, 57°C for 2 min, and 72°C for 2 min.

Protocol C was identical to Protocol A, except that the final volume was 50 μL, the $MgCl_2$ concentration was 2.5m*M*, and the PCR reaction mixtures contained 3.5% formamide [*33*]. The X and Y sequences were amplified separately and simultaneously with 0.1μ*M* X3,X4 and Y3,Y4 primers; the D17Z1 sequence was amplified with 0.1μ*M* D17Z1 primers; and the thermal cycle profile was 3 cycles of 94°C for 4 min, 60°C for 2 min, and 72°C for 2 min, followed by 30 cycles of 94°C for 2 min, 60°C for 2 min, and 72°C for 2 min.

In species-specificity testing of various nonhuman DNAs with X, H, and D17Z1 primers, a 123-bp DNA homeobox sequence [*34*] was amplified for every specimen. Homeobox sequences are highly conserved phylogenetically [*35*], and we thus expected this homeobox sequence to be amplifiable will all the nonhuman specimens. Since we did not expect X, H, and D17Z1 sequences to amplify with many of the nonhuman specimens, the homeobox sequences were amplified to obtain positive amplification results with those specimens that did not amplify with X, H, and D17Z1 primers. These PCR reactions were carried out following Protocol A, except that the $MgCl_2$ concentration was 3m*M*, and the 0.1μ*M* primers were as follows: BOX50+, 5′-CGCTGGAGCTGGAGAAGGA; BOX150−, 5′-TGCGCCGGTTCTGAAACCA. Homeobox sequence amplification was also successful using the thermal profile given for Protocol B or with the following profile: 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, for 30 cycles.

A PCR reaction control was run using λ-DNA in every experiment. In this control, 1 ng of λ-DNA was amplified with Protocol A or Protocol B, as appropriate, using

0.1μ*M* primers as follows: λ-1, 5′-GATGAGTTCGTGTCCGTACA; λ-2, 5′-GGTTATCGAAATCAGCCACAG. These λ primers yield a 500-bp product.

PCR reaction products were analyzed by ultraviolet (UV) visualization of ethidium-bromide-stained 4% gels made from 2.5% conventional agarose and 1.5% NuSieve agarose (FMC Bioproducts, Rockland, Maine) after electrophoresis in 89m*M* Tris-HCl, 89m*M* boric acid, and 2m*M* EDTA, pH 8.0 (TBE). From 150 to 250 ng of *Hae*III-digested ϕX-174 DNA (Bethesda Research Labs, Gaithersburg, Maryland) was routinely used as a size marker on gels. In some experiments, a GelMarker ladder (Research Genetics, Huntsville, Alabama), having eight bands (of 1000, 700, 500, 400, 300, 200, 100, and 50 bp) was used.

We routinely loaded 20 μL of PCR product into the gels.

Results and Discussion

Results of amplifications following Protocol B (Replinase with X3,X4 and Y3,Y4 primers) are shown in Figs. 1 through 4, and those following Protocol A (Taq with X1,X2 and Y1,Y2 primers) in Figs. 5 through 8. Results with Protocol C (Taq with X3,X4 and

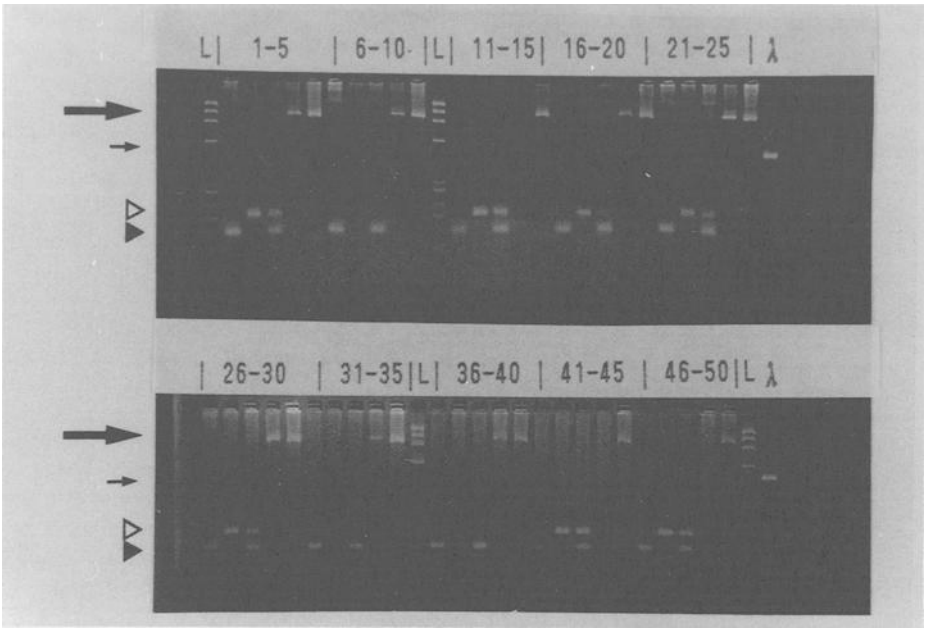

FIG. 1—*Amplification of DNA from a series of specimens of forensic science interest using Protocol B: Lanes 1, 6, 11, 16, 21, 26, 31, 36, 41, and 46, amplification products with X primers; Lanes 2, 7, 12, 17, 22, 27, 32, 37, 42, and 47, with Y primers; Lanes 3, 8, 13, 18, 23, 28, 33, 38, 43, and 48, with X and Y primers together; Lanes 4, 9, 14, 19, 24, 29, 34, 39, 44, and 49, with H primers; and Lanes 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50, with D17Z1 primers. Lanes 1–5 contain target DNA from male blood: Lanes 6–12, from female blood; Lanes 11–15, from hair root cells (male); Lanes 16–20, from buccal cells (male); Lanes 21–25, from the "female" fraction of a postcoital vaginal swab extract; Lanes 26–30, from the "male" fraction of a postcoital vaginal swab extract; Lanes 31–35, from buccal cells (female); Lanes 36–40, from bone (female); Lanes 41–45, from bone (male); and Lanes 46–50, from thymus gland (male). Lanes labeled L contain* Hae*III-digested ϕX-174 DNA; Lanes labeled λ contain the 500-bp product of amplification of control λ-DNA. The closed arrowhead indicates the position of the 157-bp X product; the open arrowhead, of the 200-bp Y product; the small arrow, of the 500-bp λ product; and the large arrow, of the 1005-bp H and the 991-bp D17Z1 products.*

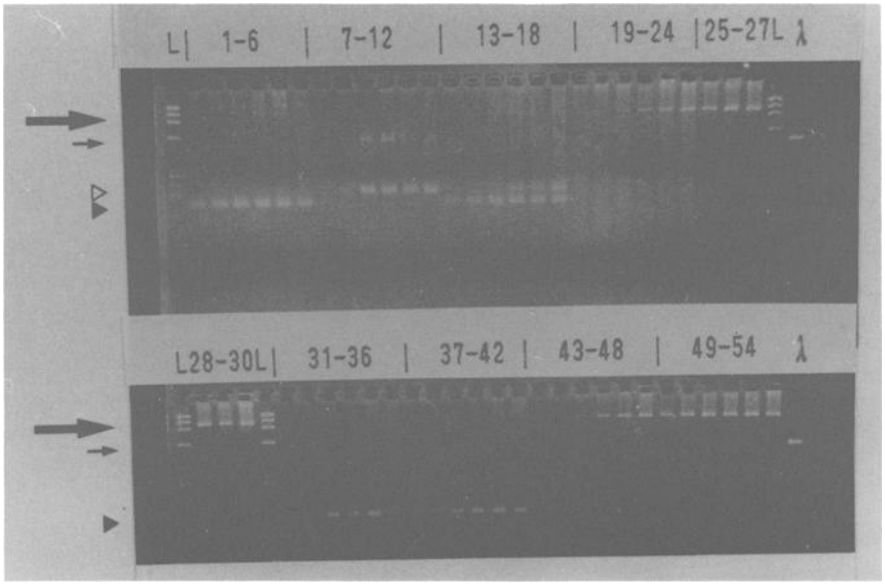

FIG. 2—*Titration of male and female target DNA in X, Y, XY, H, and D17Z1 amplifications using Protocol B: Lanes 1, 7, 13, 19, and 25 contain 0.25 ng of male DNA; Lanes 2, 8, 14, 20, and 26, 1 ng of male DNA; Lanes 3, 9, 15, 21, and 27, 5 ng of male DNA; Lanes 4, 10, 16, 22, and 28, 25 ng of male DNA; Lanes 5, 11, 17, 23, and 29, 50 ng of male DNA; and Lanes 6, 12, 18, 24, and 30, 200 ng of male DNA. Lanes 31, 37, 43, and 49 contain 0.25 ng of female DNA; Lanes 32, 38, 44, and 50, 1 ng of female DNA; Lanes 33, 39, 45, and 51, 5 ng of female DNA; Lanes 34, 40, 46, and 52, 25 ng of female DNA; Lanes 35, 41, 47, and 53, 50 ng of female DNA; and Lanes 36, 42, 48, and 54, 200 ng of female DNA. Lanes 1–6 and 31–36, amplification with X primers; Lanes 7–12, with Y primers; Lanes 13–18 and 37–42, with X and Y primers together; Lanes 19–24 and 43–48, with H primers; and Lanes 25–30 and 49–54, with D17Z1 primers. The L and λ labels and arrows and arrowheads have the same meaning as in Fig. 1.*

Y3,Y4 primers) are shown in Fig. 9. The H, D17Z1, and BOX primers were the same regardless of protocol.

Figure 1 shows PCR products from the amplification of X, Y, H, and D17Z1 sequences in a series of specimens of potential forensic science interest. These data show that X, Y, X+Y, H, and D17Z1 sequences were amplifiable in DNA from a variety of different specimens that might be encountered in casework. Figure 5 shows that similar results were obtained with X, Y, H, and D17Z1 sequences using Protocol A. It may be noted that Y product is visible in both the "male" and the "female" fractions of postcoital vaginal swab extracts (Lanes 21 through 23 and 26 through 28 in Fig. 1 and Lanes 25 through 26 and 29 through 30 in Fig. 5), which indicates that the differential DNA extraction procedure designed to separate these fractions did not completely do so. There were instances (for example, Lanes 11 through 15 and 46 through 50 of Fig. 1 and Lanes 17 through 20 and 25 through 28 of Fig. 5), where D17Z1 product was obtained, along with X and Y products, but H product was barely visible or undetected. These observations can be understood in terms of the copy number of the target sequence in the genome and the quantity of target DNA employed in the PCR reaction.

Target male and female DNA titration results with the various primers are shown in Fig. 2 for Protocol B and in Fig. 6 for Protocol A. X product was amplifiable at all target DNA quantities tested including the lowest, 0.25 ng. Among the loci amplified in these experiments, X sequences have the highest copy number in the genome [*20*]. The D17Z1 sequence copy number is about 10 to 20% as frequent, and the Y sequence about 2%

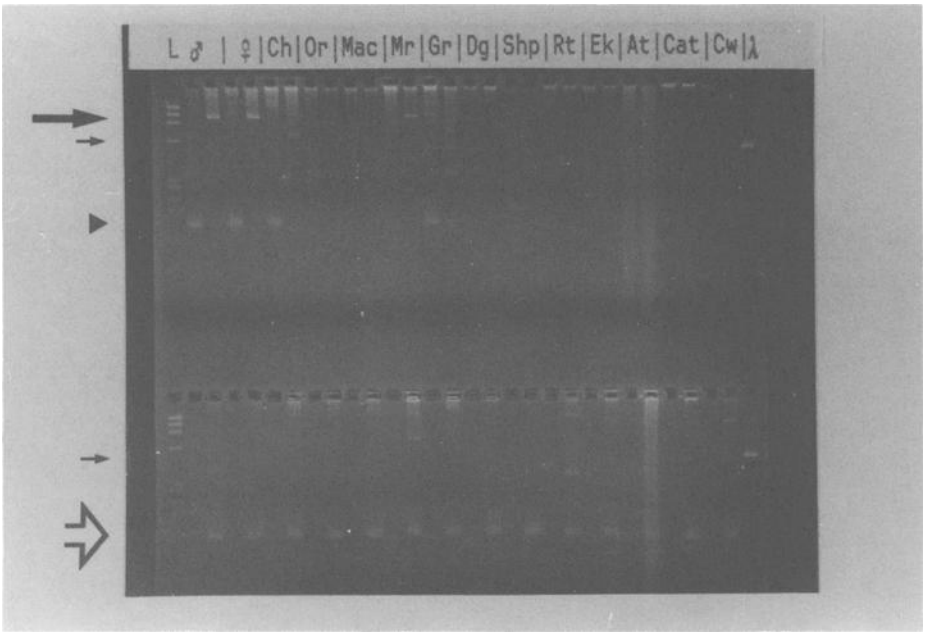

FIG. 3—*Species specificity of X and D17Z1 amplifications using Protocol B: 100 ng of target DNA was amplified throughout. Lane labeling: ♂, human male DNA; ♀, human female; Ch, chimpanzee; Or, orangutan; Mac, macaque; Mr, marmoset monkey; Gr, gorilla; Dg, dog; Shp, sheep; Rt, rat; Ek, elk (wapiti); At, antelope (pronghorn); Cat, cat; and Cw, cow. The odd lanes on the top show amplification with X primers; even lanes on the top, with D17Z1 primers; and bottom lanes directly under D17Z1 lanes, with BOX primers. The end lane labels L and λ, the large and small arrows, and the closed arrowhead have the same meaning as in Fig. 1. The open arrow indicates the position of the 123-bp homeobox amplification product.*

as frequent as that of X [*21,32*]. The H sequence is a single-copy sequence. More target DNA was required for the amplification of Y in male DNA and of H in both male and female DNA than of X sequences for a given number of cycles. D17Z1 product was obtained in both male and female DNA at all target quantities tested with Protocol B. With Protocol A, the D17Z1 titration resembled that of Y. These results probably reflect copy number differences in the sequences in the target DNA, as well as slight differences in the X and Y primers and in the efficiency of amplification with the two enzymes. More target male DNA was required for the coamplification of X and Y sequences than for X or Y alone using Protocol B (compare Lanes 13 through 18 with Lanes 1 through 6 and 7 through 12 in Fig. 2). The presence of Y primers in Protocol B PCR did not materially affect amplification of the X sequence in female DNA (compare Lanes 37 through 42 with Lanes 31 through 36 in Fig. 2).

PCR testing methods involving the amplification of specific sequences, and their detection on gels after electrophoretic separation on the basis of size, seem likely to become more commonly employed in forensic science laboratories. These methods currently include amplification of X and Y sequences for diagnosis of the gender of origin, as well as the amplification and subsequent electrophoretic typing of several variable number of tandem repeats (VNTR) loci [*10,11,23,36–38*]. It would be useful to have primers for different known monomorphic sequences that could serve as amplification controls, whose results might assist in interpreting the results of the overall test.

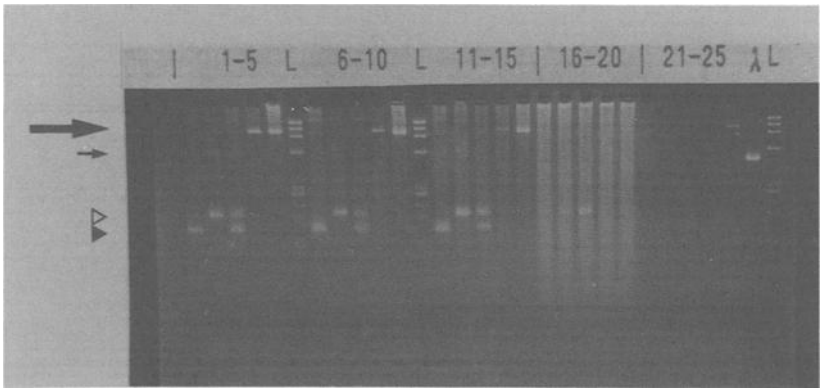

FIG. 4—*Amplification of male DNA from postmortem muscle tissue before and after exposure to selected environmental conditions using Protocol B: DNA was extracted from tissue exposed to the stated condition for 7 days and then analyzed. Lanes 1–5, control human male blood DNA; Lanes 6–10, control male muscle tissue DNA; Lanes 11–15, tissue aged in dry air at room temperature; Lanes 16–20, tissue aged in humid air at room temperature; and Lanes 21–25, tissue aged immersed in salt water at room temperature. Lanes 1, 6, 11, 16, and 21 show amplification with X primers; Lanes 2, 7, 12, 17, and 22, with Y primers; Lanes 3, 8, 13, 18, and 23, with X and Y primers; Lanes 4, 9, 14, 19, and 24, with H primers; and Lanes 5, 10, 15, 20, and 25, with D17Z1 primers. The lane labels L and λ and the large and small arrows and arrowheads have the same meaning as in Fig. 1.*

In this context, we have considered the possibility that X sequence amplification could serve as an X chromosome marker as well as a "permissive" control in PCR testing. Successful amplification of X sequences shows that the target DNA contained sufficient numbers of this sequence to be amplified under the PCR conditions employed with either protocol and demonstrates the absence of polymerase inhibitors in the reaction. As a PCR reaction control, X amplification would be permissive in two ways: first, because of the relatively short sequence amplified and, second, because of the high copy number of the sequence per haploid genome in comparison with Y, D17Z1, and single-copy sequences (such as H and VNTRs).

The H and D17Z1 primers were designed to serve as more "stringent" PCR amplification controls. Amplification of these sequences provides a stringent control for the same reasons that X amplification provides a permissive control: the size of the PCR product and the copy number. As a single-copy sequence, and because of its nearly 1-kb length, H may be too stringent a control for PCR reactions involving X and Y amplifications. Indeed, our data show that X and Y amplifications can be successful in genomic target DNA from specimens in which H is not detected under the same conditions.

The D17Z1 sequence was chosen as an alternative possible stringent PCR control for X and Y amplifications for two reasons. First, its copy number is more comparable to that of the Y sequence. Second, there are data indicating that this sequence is primate-specific, and the use of a probe specific for the D17Z1 sequences has been proposed in

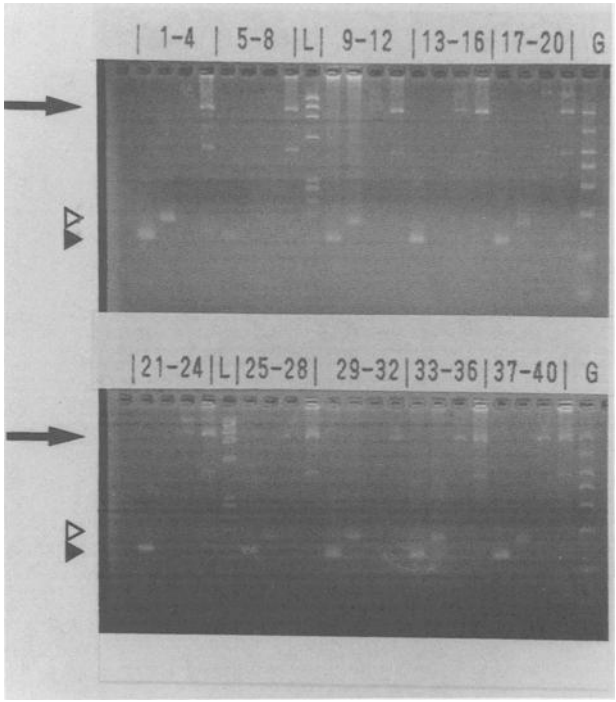

FIG. 5—*Amplification of DNA from a series of specimens of forensic science interest using Protocol A: Lanes 1, 5, 9, 13, 17, 21, 25, 29, 33, and 37, amplification products with X primers; Lanes 2, 6, 10, 14, 18, 22, 26, 30, 34, and 38, with Y primers; Lanes 3, 7, 11, 15, 19, 23, 27, 31, 35, and 39, with H primers; and Lanes 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40, with D17Z1 primers. Lanes 1–4 contain DNA from a known male bloodstain; Lanes 5–8, from a known female bloodstain; Lanes 9–12, from hair root cells (male); Lanes 13–16, from hair root cells (female); Lanes 17–20, from buccal cells (male); Lanes 21–24, from buccal cells (female); Lanes 25–28, from the "female" fraction of a postcoital vaginal swab extract; Lanes 29–32, from the "male" fraction of a postcoital swab extract; Lanes 33–36, from bone (male); and Lanes 37–40, from brain tissue (male). The lanes labeled L contain* Hae*III-digested ϕX-174 DNA; the lanes labeled G contain GelMarker ladder DNA. The arrows and arrowheads have the same meaning as in Fig. 1, except that the X product is 130 bp and the Y product is 172 bp.*

a slot blot format as a means of evaluating the quantity of human DNA in preparations from forensic science specimens [*39*]. DNA isolated from bloodstains, body fluid stains, postmortem tissues, and other specimens of forensic science importance typically contains not only a significant quantity of DNA from microbial and possibly other sources but also degraded DNA. These factors make it very difficult to estimate the quantity of human DNA by minigel or spectrofluorometric methods, and it is the quantity of human

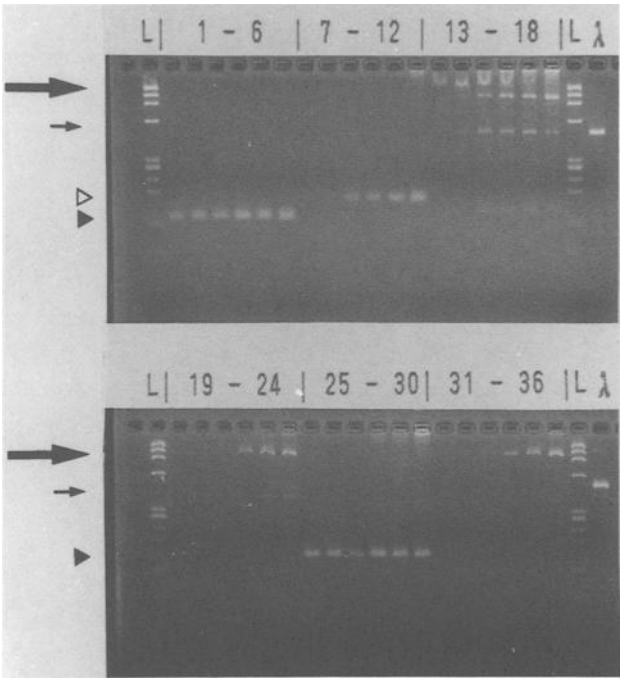

FIG. 6—*Titration of target DNA in X, Y, H, and D17Z1 amplifications using Protocol A: Lanes 1, 7, 13, and 19 contain 0.25 ng of male DNA; Lanes 2, 8, 14, and 20, 1 ng of male DNA; Lanes 3, 9, 15, and 21, 5 ng of male DNA; Lanes 4, 10, 16, and 22, 25 ng of male DNA; Lanes 5, 11, 17, and 23, 50 ng of male DNA; and Lanes 6, 12, 18, and 24, 200 ng of male DNA. Lanes 25 and 31 contain 0.25 ng of female DNA; Lanes 26 and 32, 1 ng of female DNA; Lanes 27 and 33, 5 ng of female DNA; Lanes 28 and 34, 25 ng of female DNA; Lanes 29 and 35, 50 ng of female DNA; and Lanes 30 and 36, 200 ng of female DNA. Lanes 1–6 and 25–30 show amplification with X primers; Lanes 7–12, with Y primers; Lanes 19–24 and 31–36, with H primers; and Lanes 13–18, with D17Z1 primers. The L and λ labels and arrows and arrowheads have the same meaning as in Fig. 1, except that the X product is 130 bp.*

DNA in the preparation that is needed to decide how much specimen will be needed in subsequent restriction endonuclease digestion or PCR reaction steps.

In the context of PCR reaction controls for forensic science testing, we have thus considered X amplification to be a permissive or "relaxed" control, and H and D17Z1 to be stringent controls. We would expect that a given quantity of target DNA from certain specimens might yield satisfactory X-sequence amplification but fail to yield other PCR products from sequences that are either longer, present in a lower copy number, or both. Similarly, the D17Z1 sequence amplification appears to be a suitable stringent control for the X,Y amplification reactions because the PCR product is longer, but its

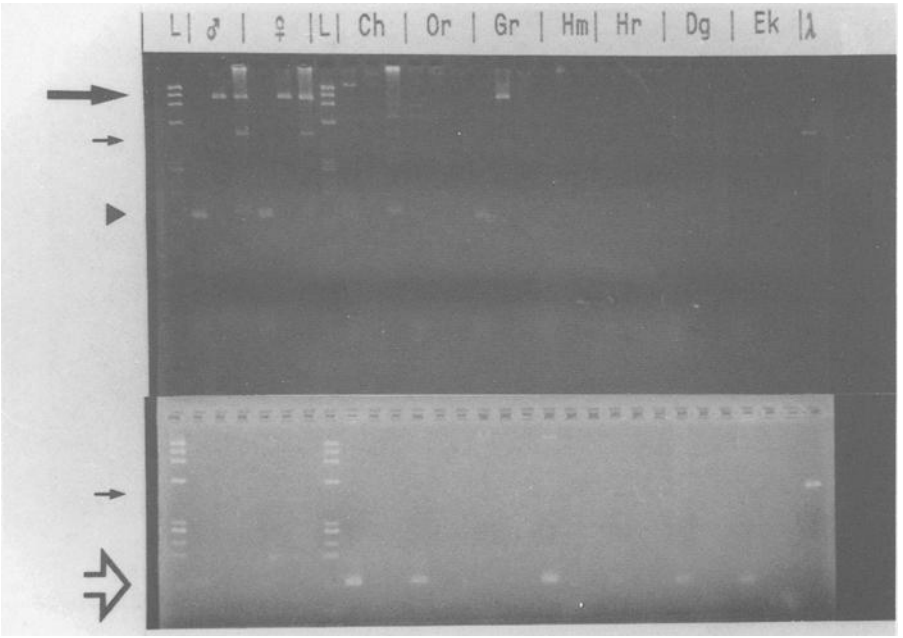

FIG. 7—*Species specificity of X, H, and D17Z1 amplifications using Protocol A: 100 ng of target DNA was amplified throughout. The lane labeling is as follows: ♂, known human male; ♀, known human female; Ch, chimpanzee; Or, orangutan; Gr, gorilla; Hm, chinese hamster; Hr, horse; Dg, dog; Ek, elk (wapiti). The upper lanes show PCR product from amplification of X, H, and D17Z1, in order, for each specimen; the lower lane, below each X lane, shows PCR product from the amplification of the BOX sequence. The lanes labeled L and λ and the arrows and arrowheads have the same meaning as in Fig. 3.*

copy number is more comparable to that of the Y sequence. H sequence amplification might provide a more suitable stringent control for PCR reactions involving VNTR loci, since it is itself a single-copy sequence.

The apparent primate specificity of both the X and D17Z1 sequences, shown in Fig. 3 for Protocol B and in Fig. 7 for Protocol A, adds an additional dimension to their use as PCR reaction controls. Among nonhuman DNAs tested, the X, H, and D17Z1 amplification products were obtained only with human or primate specimens. In both protocols, Y product was obtained only with human male DNA among all the male DNAs tested (data not shown). Thus, the X, H, and D17Z1 sequences were primate-specific in these limited experiments. The species specificity results with X and D17Z1 sequences are generally in accord with what is known about the sequence homology of these higher order α-satellite repeats in human and other primate DNA [*20,32*]. The Y sequence has been reported to be more divergent in humans [*21*], and our limited amplification results with primates are concordant with those data as well. The phylogenetically conserved homeobox sequence yielded amplification product with every DNA tested and thus provided a positive control for the PCR reaction with each specimen.

Thus, amplification of X, Y, and monomorphic control sequences can provide information about gender as well as the species of origin. Amplification of X and H sequences as controls in PCR amplifications of VNTR loci could help in assessing the degree of degradation of the target DNA and perhaps be useful in subsequent interpretation of

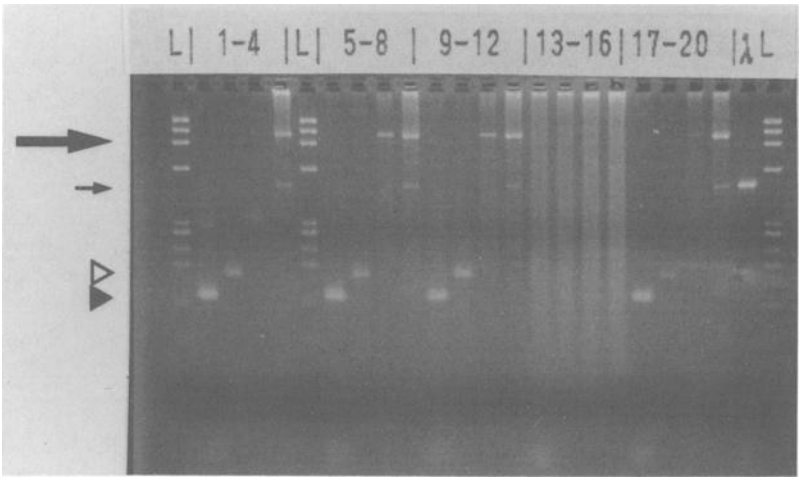

FIG. 8—*Amplification of male DNA from postmortem kidney tissue before and after exposure to selected environmental conditions using Protocol A: DNA was extracted from tissue exposed to the stated condition for 7 days and then analyzed. Lanes 1–4 contain control human male blood DNA; Lanes 5–8, control male kidney tissue DNA; Lanes 9–12, tissue aged in dry air at room temperature; Lanes 13–16, tissue aged in humid air at room temperature; and Lanes 17–20, tissue aged immersed in salt water at room temperature. Lanes 1, 5, 9, 13, and 17 show amplification with X primers; Lanes 2, 6, 10, 14, and 18, with Y primers; Lanes 3, 7, 11, 15, and 19, with H primers; and Lanes 4, 8, 12, 16, and 20, with D17Z1 primers. The lane labels L and λ, and the large and small arrows and arrowheads have the same meaning as in Fig. 6.*

VNTR typing results. Additional studies currently in progress in our laboratories on aged and degraded DNA from forensic science specimens, as well as studies in other laboratories, will shed more light on the value of X, H, and D17Z1 (or other comparable) sequences as controls for PCR amplification tests.

Figure 4 shows the effects of several environmental conditions on postmortem muscle tissue DNA in the subsequent amplification of the sequences studied here using Protocol B. Comparable results with postmortem kidney tissue DNA using Protocol A are shown in Fig. 8. Using Protocol B, humid conditions were more detrimental to X than to Y amplification, and H product was not detectable. Little or no X, Y, or H product was seen in the salt-water-immersed specimen. Curiously, the D17Z1 sequence was detected in both humid air and salt-water specimens. Using Protocol A, none of the sequences amplified in the humid condition specimens, while the salt-water-immersed specimen yielded results with all of them. These observations may reflect minor differences between X and Y primers and protocols. They might further suggest that DNA degradation may not be uniform in different tissues exposed to the same conditions. For example, D17Z1 amplified in the muscle specimen but not in the kidney specimen after exposure to humid conditions. Similarly, the kidney DNA yielded results with all the sequences after salt-water immersion, while X, Y, and H did not amplify in the muscle. These observations are in accord with other data we have collected on the amplification of the 3′APOB VNTR polymorphism in a series of different tissues from the same individual exposed to the same set of environmental conditions for the same period of time. In addition, these observations could suggest that DNA degradation in a given tissue may not be a

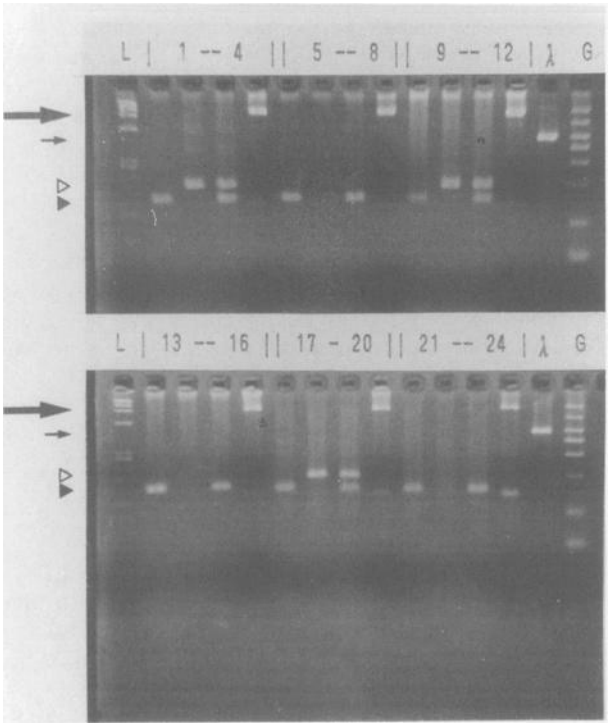

FIG. 9—*Amplification of male and female blood, bone, and tissue DNA using Protocol C: Lanes 1, 5, 9, 13, 17, and 21, amplification products with X primers; Lanes 2, 6, 10, 14, 18, and 22, with Y primers; Lanes 3, 7, 11, 15, 19, and 23, with X and Y primers together; Lanes 4, 8, 12, 16, 20, and 24, with D17Z1 primers. Lanes 1–4 contain target DNA from male blood; Lanes 5–8, from female blood; Lanes 9–12, from bone (male); Lanes 13–16, from bone (female); Lanes 17–20, from thymus (male); and Lanes 21–24, from kidney (female). The lane labels L and λ and the large and small arrows and arrowheads have the same meaning as in Fig. 4. The lane label G has the same meaning as in Fig. 5.*

completely uniform process. In the salt-water-immersed muscle tissue specimen, for example, the 991-bp D17Z1 product was successfully amplified, while the significantly smaller and higher copy number X sequence was not.

Figure 9 shows that X and Y sequences can be separately and simultaneously amplified using Taq polymerase with the X3,X4 and Y3,Y4 primer sets (Protocol C) in DNAs from several bone and tissue specimens of both male and female origin. The results are comparable to those obtained with Replinase (Protocol B). With Protocol C, we found that specificity was enhanced by the addition of formamide to the reaction mixture [*33*].

The X3,X4 and Y3,Y4 primers used in Protocols B and C were designed from the same sequences [*20,21*] used to devise those (X1,X2 and Y1,Y2) employed in Protocol A [*23*]. As these sequences are tandem repeats of 170-bp "monomeric" units, designing primers that specifically amplify a single desired product can be difficult because the

overall sequence contains a number of potential complementary target binding sites. In the X case, we attempted to exploit small differences in sequence within the monomeric repeats to make the primers specific to one monomer species out of twelve by placing the 3′ end of the primer at a position of sequence divergence among repeats. This was not possible in the Y case, as the sequences on which the primers were designed [*21*] have no apparent correlate in later, more refined sequence data [*40*]. Thus, the Y3,Y4 primers were designed primarily to be compatible with the X3,X4 primers in terms of annealing temperature and the size of the PCR product. Both sets of X and Y primers have performed well in our laboratories, as the present data show. Difficulty in coamplifying X and Y sequences using X1,X2 and Y1,Y2 primers while retaining specificity has been reported [*23*]. The successful coamplification of these sequences in the present study may be due in part to the use of the more closely matched primer sets.

Overall, these studies show that X and Y sequence amplification can provide a useful method for determining the sex of origin by PCR in DNA from a variety of different types of specimens. Additional information about the species of origin is obtained simultaneously. Amplification of D17Z1 and H sequences are shown to be potentially valuable controls in PCR amplification tests on DNA from specimens of forensic science interest.

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Address requests for reprints or additional information to
R. E. Gaensslen, Ph.D.
University of New Haven
300 Orange Ave.
West Haven, CT 06516