TECHNICAL NOTE

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Direct Sequencing of the Human Protamine P1 Gene and Application in Forensic Medicine

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ABSTRACT: Protamines are among the most variable nuclear proteins known in eukaryotes. In order to learn more about their evolution and function in humans and to explore the possibility of potential applications in forensic medicine we have developed a rapid method to amplify and directly sequence the protamine P1 gene simultaneously in many different samples. The method takes only 3.5 h from genomic DNA to the sequencing reactions. Despite the high variability of these genes only one polymorphic site was detected at the coding region level in different individuals. This polymorphic variation does not create a change in the amino-acid sequence of the protamine. Because all the protamine genes sequenced from different species are markedly different among them as well as to the human sequence, amplification and direct sequencing of this gene can be used to unequivocally identify the human or animal origin of biological specimens. Furthermore, the single polymorphic site detected in the human P1 gene could be useful in conjunction with other markers in identification studies in humans.

KEYWORDS: pathology and biology, protamine, genetics, human genome DNA, direct sequencing of genes, allelic variation

Determination of the DNA sequence to identify different individuals or the human or animal origin of biological specimens has been successful using relatively well conserved mitochondrial DNA sequences [*1,2*]. Choosing a relatively well conserved gene has the advantage that it is more likely that the target sequences will be conserved and recognized by a PCR primer, but also has the disadvantage that there is less (or absent) variation in the sequenced DNA region. Thus in many cases discrimination among different human individuals or closely related species is precluded by the lack of differences among sequences. In addition, the use of mitochondrial DNA sequences in these initial strategies makes them unusable to trace the paternal origin of the samples. Thus, we

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decided to explore the possibility of using the most variable nuclear protein encoding autosomal gene (protamine gene) to overcome the problems of lack of differences among the sequences of the different species and the lack of versatility of the existing systems.

Protamines are extremely positively charged proteins which are the major sperm nuclear proteins and condense the DNA in the sperm nucleus [*3–15,44*]. Because of the special interest of protamines to researchers, these proteins and their genes have been characterized in a large number of species. Despite the high variability of protamine genes among different species, no sequence data was so far available in the different human individuals. In order to learn more about the evolution and variability of these proteins and to explore potential applications we have developed a method to amplify the protamine gene using PCR and directly sequence the promoter and coding regions in different samples. Here we show that sequencing of this gene can be used to identify the human or animal origin of biological specimens.

Materials and Methods

Lymphocyte DNA was extracted from 6 mL of blood from different individuals. Isolated lymphocytes were digested for 1 h at 37°C in 10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 0.5% SDS, 20 μg/mL RNAse A after which the solution was supplemented with proteinase K to 100 μg/mL final concentration, digested for an additional 3 h at 50°C, phenol extracted, phenol/chloroform extracted, ethanol precipitated, washed twice with 70% ethanol, once with 100% ethanol, dried under vacuum and dissolved in 10 mM Tris, 1 mM EDTA (TE). Alternatively the DNA can be extracted from biological fluids using other methods such as the Chelex® approach, which we have also used routinely and successfully in our laboratory.

Direct Sequencing of PCR Products

Since we are routinely using in our laboratory the biotin-streptavidin-magnetic particle approach with very good results to prepare template DNA for direct sequencing [*16*], we decided to try and see if this method would also work well to sequence the protamine P1 genes. Amplification and direct sequencing of the human genomic protamine P1 gene has proved to be very efficient using the following conditions: 200 ng of genomic DNA (smaller amounts of DNA can be used if necessary) were amplified in a volume of 50 μL containing 50 mM KCl, 10 mM Tris (pH 8.3), 5 pmoles of each primer (OP13: 5′-ACCTGCTCACAGGTTGGCTG-3′, OP17:5′ BIOTIN-CATTTATTGACAGGTGGCA-3′), $MgCl_2$ at 1.5 mM, dNTPs at 200 μM each and 1 unit of Taq I DNA polymerase. An initial denaturation at 95°C for 10 min was followed by 35 cycles of 2 min at 95°C, 2 min at 55°C, 3 min at 72°C and a final extension at 72°C for 10 min. The resulting amplification product contained only one strand with biotin attached to the 5′ end. The protocol from Dynal® was then used to generate a ssDNA template for sequencing with slight modifications (16) as follows: 20 μL of the PCR reaction was mixed with the prewashed Dynabeads (10 μL of beads prewashed three times with 200 μL of TWS: 0.17% triton-x-100, 500 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA pH 8.0 and resuspended in 80 μL of TWS) at room temperature on a rotatory mixer for 30 min. The Dynabeads are then separated with the aid of a magnetic particle concentrator (MPC) and washed twice with 20 μL of TWS. The PCR product-Dynabeads mix is then resuspended in 32 μL of TE and 8 μL of 1 M NaOH, 4 mM EDTA are added to denature the two strands of the PCR product. At this point the DNA strand containing biotin at the 5′ end remains strongly bound to the streptavidin coated magnetic beads whereas the other strand without biotin remains free in solution. The tube is then placed in the MPC to pull the complex formed by the magnetic particle-streptavidin-5′ biotin-single

stranded DNA to the bottom of the tube and the supernatant removed as above and kept to recover the opposite DNA strand following the protocol described as follows. The beads are finally washed twice with 200 μL of TWS and suspended in 7 μL of H_2O and sequenced using Sequenase (USB), [α-^{35}S]dATP and following manufacturer's instructions.

Precipitation of the Opposite Strand

A total of 40 μL of the supernatant containing the DNA strand without biotin is neutralized with 8 μL of 1 M HCl solution and then precipitated with 0.1 volumes of 3 M sodium acetate pH 8.0 and 3 volumes of 100% ethanol from 2 h to overnight at −20°C. The precipitated DNA is pelleted, washed with 70%, with 100% ethanol, dried, dissolved in 7 μL of distilled water and sequenced as above.

Cloning and Sequencing of PCR Products

Promoter Region—Primer sequences were the following: OP19 (5′-CTGTGACA T/C AG/A GCA G/A CNCCT-3′), and OP11 (5′-CTCACATGCCCATATATGGACATG-3′), and from the coding region facing upstream to the promoter: OP14 (5′-CA-GCATCGGTATCTGGCCAT-3′) and OP112 (3′-CGCCTCCT T/C CGTCTG T/C GAC A/T/G TCT-3′). Touch-down PCR [*17*] was used to amplify the genomic DNA with the following conditions: 200 ng of genomic DNA, 5 pmoles of each primer (OP14 + OP11 to amplify the guinea pig promoter, and OP19 + OP112 to amplify the rest of the species), 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 μM dNTPs and 1 unit of TaqI DNA polymerase. After an initial denaturation at 95°C for 10 min, 15 cycles were performed starting at the annealing temperature of 60°C (extension at 72°C, 1 min, and denaturation at 95°C, 1 min) and decreasing it 1°C every two cycles until the annealing temperature reached 45°C. At this point 25 additional cycles were performed with the same conditions. Cloning and sequencing were done as described [*13,15*]. Three to six independent clones were sequenced for each individual.

Coding Region—200 ng of genomic DNA or 0.5 μg of the cDNA sample were amplified using the following conditions: 50 mM KCl, 10 mM Tris (pH 8.3), 5 pmoles of each primer (OP13: 5′-ACCTGCTCACAGGTTGGCTG-3′, OP17: 5′-CATTTATTGA-CAGGTGGCA-3′), $MgCl_2$ at 1.5 mM, dNTPs at 200 μM each, 1 unit of Taq I DNA polymerase, an initial denaturation at 95°C for 10 min, 35 cycles of 2 min at 95°C, 2 min at 55°C, 3 min at 72°C and a final extension at 72°C for 10 min. The PCR products were cloned into the EcoR V site of Bluescript KS+ (Stratagene®) and sequenced as described [*13,15*]. Three to six independent clones were sequenced for each individual.

Single Strand Conformation Analysis (SSCA)

We performed a pilot assay with the technique SSCA (single strand conformation analysis) [*18,19*] in order to determine if it could be useful in the detection of single nucleotide changes in the human P1 protamine gene. PCR amplification was done with a reaction volume of 25 μL and the following conditions: 100 ng of genomic DNA. 2.5 pmoles of each primer (OP13 + OP17), 50 mM KCl, 10 mM Tris (pH = 8.3), 1.5 mM $MgCl_2$. Final concentrations of 200 μM of all nucleotides were used apart from dATP, which was used at a concentration of 24 μM, 0.38 μL of [α-^{35}S]dATP (specific activity 600 Ci/mmol). One unit of Taq polymerase was added. Incubation times were the same as described in "Coding region amplification." A total of 10 μL of the amplified product was mixed with gel loading buffer [*18–20*] and denatured at 80°C for 10 min and placed

on ice. The samples were loaded on 5% polyacrylamide gel and 10% glycerol [*20*]. Electrophoresis was carried out at 30 W for 8 hours at 25°C. The gel was dried and exposed to X-ray film at −80°C for 1 week.

Results

PCR amplification of genomic DNA with specific protamine P1 gene PCR primers generated a single product at the expected size (Fig. 1). Subsequently single-strand template DNA was prepared using the streptavidin coated magnetic beads purification approach (Fig. 2). The big advantage of this method is that it yields extremely pure template DNA in a very short time. In addition both strands can be successfully sequenced (Fig. 2). Only one polymorphic site became apparent at the coding region level after sequencing 12 unrelated individuals. This is seen as the presence of two independent bands in different lanes at the same level in the sequencing autoradiogram (see arrow in Fig. 2 *left*). Confirmation of the polymorphic nature of this site is provided by the sequence of

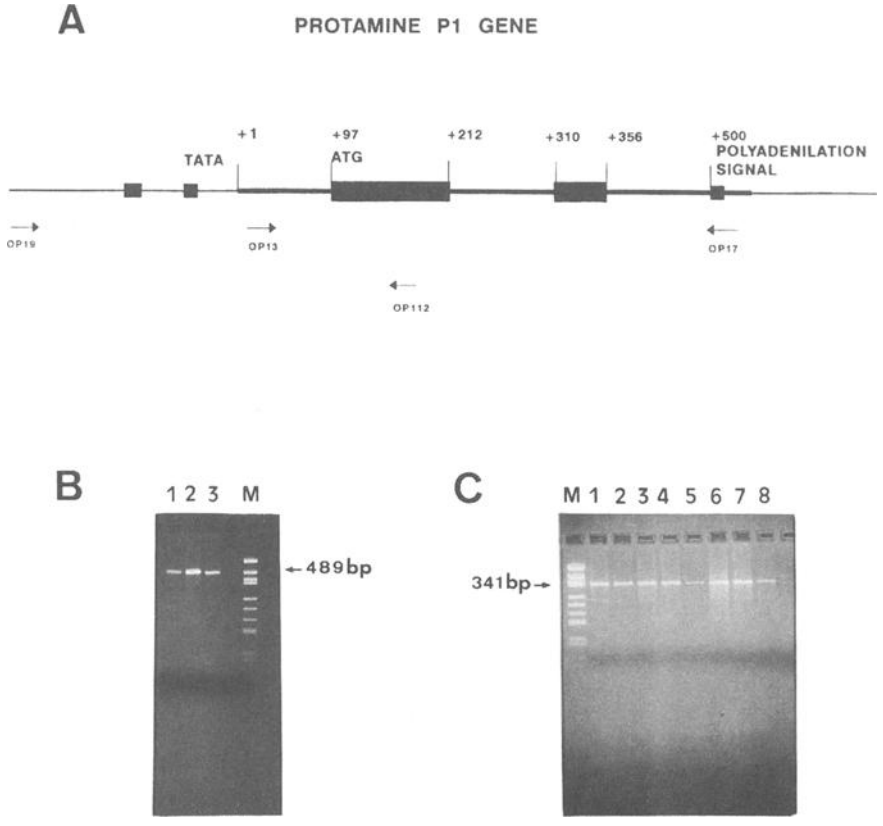

FIG. 1—*PCR amplification of the promoter and coding region of the human protamine P1 gene.* A) *Position of the PCR primers relative to the gene;* B) *Amplification products of the coding region of the protamine P1 gene using the OP13 and OP17 oligonucleotides. 1: Human, 2: Calf and 3: Mouse. M: size marker (Bluescript KS + vector digested with MspI);* C) *Amplification products of the promoter region using the OP19 and OP112 oligonucleotides. 1: Orangutan, 2: Anubis baboon, 3–5: Different Mediterranean individuals, 6: Korean, 7: Sudanese, 8: American Indian, M: size marker (Bluescript KS + vector digested with MspI).*

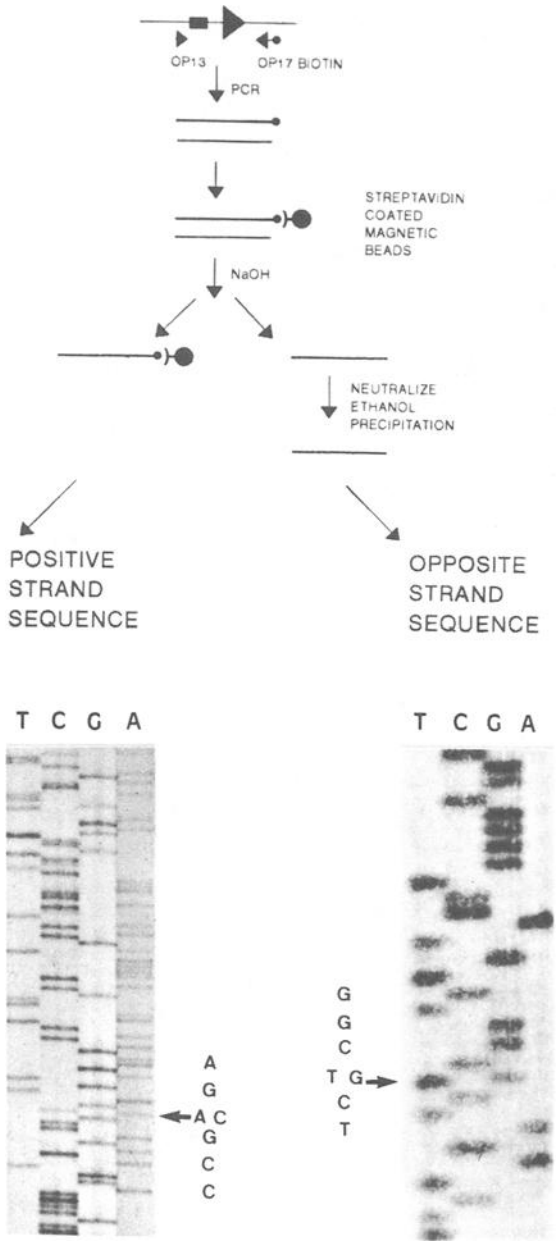

FIG. 2—*Protamine P1 PCR amplification and direct sequencing strategy. The autoradiograms showing the human protamine P1 DNA sequences are shown at the bottom.* Left: *Positive strand sequence.* Right: *opposite strand sequence.*

A

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                                                          *          -78
ACACTCGGGGGCCTGCCCGCCTCTCAAATGCCCATATATGGACATGATGCAGGCCACCTGGCCATGGTTTGTGAG

   *                                          Tata-box                -3
GTCCCAGCCCCTTTGCCCTCACAATGACCAACGGCCCCCTGGCATCTATAACAGGCCGCAGAGCTGGCCCCTGAC

 +1                                                                    +73
TCACAGCCCACAGAGTTCCACCTGCTCACAGGTTGGCTGGCTCAGCCAAGGTGGTGCCCTGCTCTGAGCATTCAG

*                    M   A   R   Y   R   C   C   R   S   Q   S   R   S   R
GCCAAGCCCATCCTGCACC ATG GCC AGG TAC AGA TGC TGT CGC AGC CAG AGC CGG AGC AGA

 Y   Y   R   Q   R   Q   R   S   R   R   R   R   R   R   S   C   Q   T   R
TAT TAC CGC CAG AGA CAA AGA AGT CGC AGA CGA AGG AGG CGG AGC TGC CAG ACA CGG

 R   R   A   M  R                                                     +260
AGG AGA GCC ATG A  GTAAGTGGGCCCAGCTGAGGGTGGGCTGGGGCTGAGGCTGGGAGCTCTCAGGGCCC

                                          C   C   R   P   A   Y   R   P  *R
AGCCTTCCTCTCACCACTTTTCTTGGTCTCACCAG  GG TGC TGC CGC CCC AGG TAC AGA CCG AGA
                                                                      C
 C   R   R   H                                                       +394
TGT AGA AGA CAC TAA TTGCACAAAATAGCACATCCACCAAACTCCTGCCTGAGAATGTTACCAGACTTCA

                                                          +460
AGATCCTCTTGCCACATCTTGAAAATGCCACCATCCAATAAAAATCAGGAGCCTGCTAAGGAACAA
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B

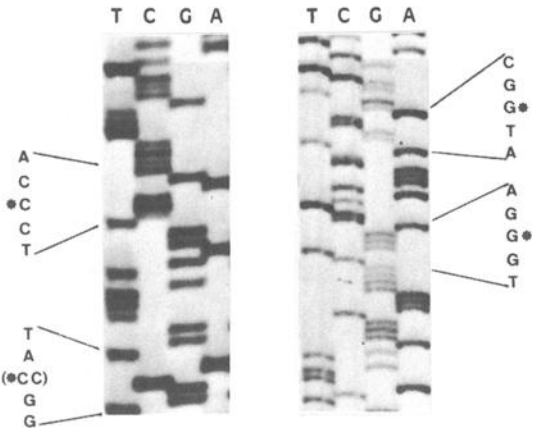

FIG. 3—A) *Nucleotide sequence of the human protamine P1 gene indicating the different gene regions and the position of the polymorphisms found (see "*" at position +322). Three sites of discrepancy were detected when comparing the sequences determined several unrelated individuals to the previously reported human sequence (see "*" at positions −90, −74 and +74).* B) *Sequencing autoradiograms showing a clear-cut presence of one extra C at position −74 in both, in the positive strand sequence* (left) *and in the opposite strand sequence* (right), *and one extra C at position −90 in the opposite strand sequence, as compared to the previously reported sequence.*

the opposite strand which also shows this mutation (Fig. 2 *right*). This polymorphism was found in two independent samples out of 12. No other polymorphisms were found at the coding region or promoter regions (Fig. 3). However three inconsistencies were detected when contrasting our sequences of the human P1 promoter with the sequences reported for this gene [*21*]: our sequence indicates the presence of two extra C's at positions −90 and −74 (Fig. 3) and one extra G at position +74 (Fig. 3). The extra −74 C was present in the sequenced strand in a context without any signs of compression or sequencing artifacts (Fig. 3*B left*). In addition it was also present in all sequenced

samples including those of Europeans, Sudanese, American Indian, and Chinese. This suggested the possibility of a sequencing error in the previously reported human sequences. Thus we sequenced the opposite strand which confirmed the presence of these extra C at position −74 (Fig. 3*B right*). Interestingly the opposite strand sequence at the +74 site showed only one G but of a much stronger intensity suggestive of a compression at this level and in this strand sequence (not shown). Similarly the extra C at position −90 seen in the opposite strand appears as a single band in the positive strand sequence. Thus it is possible that this phenomena had been the origin of the confusion in the previously reported sequence for these specific bases.

Pilot single strand conformation polymorphism of the amplified PCR product indicates a different pattern for the DNA containing the polymorphism (not shown), which means that the polymorphism can be routinely detected without the need for sequencing. This result is encouraging, promising an even faster analysis of many different samples.

Discussion

DNA sequence data should provide the highest quality, most detailed and precise data to unequivocally identify an individual since there are no two individuals on earth with the same genome except for monozygotic twins. Thus the DNA sequence should become is in this sense much superior to existing DNA typing methods that rely mainly in the detection and identification of different sized DNA fragments [*22–24*]. A clear example that the approach of directly sequencing the DNA can be extremely useful in forensic medicine has already been provided by the identification of children from grandmothers in Argentina using mitochondrial DNA sequences [*1*]. Also a system has been proposed to identify the animal origin of biological specimens based on the mitochondrial cytochrome b gene [*2*]. While all these approaches are clearly useful, they also present some major limitations: First of all, the mitochondrial DNA origin of the sequenced gene precludes the use of this method in paternity testing since mitochondrial DNA is passed only from the mother to the next generation. Also in phylogenetic studies only the mother's lineage can be traced for the same reason. Second, the use of a conserved gene results in few (if any) differences present in the DNA sequences of the specimens being compared, especially if they are closely related, which precludes discrimination in many cases. With a view to limit the amount of sequence necessary to make direct DNA sequencing practical in this field, we decided to standardize a method to amplify and rapidly sequence the most variable autosomal gene encoding a nuclear protein known today in eukaryotes, the protamine gene. We have amplified and sequenced the protamine P1 gene promoter and coding region of 12 different individuals (Figs. 1 to 3). Comparison of the sequences in this short region uncovered one polymorphic site at the coding region level. This polymorphism can be valuable as a complement to other available typing systems in the identification of different individuals. For instance, oligonucleotides could be readily designed to detect by hybridization the two alleles (ASO probes). With the constant improvement of DNA sequencing it should be feasible in the future to sequence routinely larger fragments of DNA. Since it can be expected that the regions flanking the protamine genes will even be more variable than the coding and promoter region (which are subject to constrains in evolution due to a functional need), these genes should provide a source of additional polymorphic sites. This should enable by itself efficient discrimination of individuals in most cases. However the most robust potential application of our strategy comes from the finding that the limited variation among humans is clearly distinguishable to the almost bewildering variety of protamine sequences in animal species [*12*] (Fig. 4). Even among the most closely related species to human (chimpanzee, gorilla, orangutan) there is a large number of differences to the human sequence (14, 12, and 19 respectively; [*25*]). Thus amplification and direct se-

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HUMAN      ARYRCCRSQS--RSRYY-RQRQR-SRRRRRRSCQTRRRA--------MRCCRPR--YR-PRCRRH--
GIBBON     ARYRCCRSQS--RSRCY-RRGQR-SRRRRRRSCQTRRRA--------MRCCRPR--YR-LRRRRH--
GORILLA    ARYRCCRSQS--RSRCY-RQRQT-SRRRRRRSCQTQRRA--------MRCCRRR--NR-LRRRKH--
ORANGUTAN  ARYRCCRSQS--QSRCC-RRRQR-CHRRRRRCCQTRRRA--------MRCCRRR--YR-LRCRRH--
REDMONKEY  ARYRCCRSQS--RSRCC-RQRRR-CRRRRRRRCRARRRA--------MKCCRRR--YR-LRCRRY--
CHIMPANZ1  ARYRCCRSQS--RSRCY-RQR-R-SRRRKRQSCQTQRRA--------MRCCRRR--SR-LRRRRH--
CHIMPANZ2  ARYRCCRSQS--RSRCY-RQR-R-SRRRKRQSCQTQRRA--------MRCCRRR--SR-TRRRRH--
REDHOWLER  ARYRCCRSRSLSRSRCY-RQRPR-CRRRRRRSCR-RPRA--------SRCCRRR--YR-LRRRRY--
SAGUINUS   ARYRCCRSQS--RSRCY-RQRRR-GRRRRRRTCR-RRRA--------SRCCRRR--YK-LTCRRY--
MOUSE      ARYRCCRSKS--RSRCR-RRRRR-CRRRRRRCCRRRRRR----------CCRRRRSYT-IRCKKY--
RAT        ARYRCCRSKS--RSRCR-RRRRR-CRRRRRRCCRRRRRR----------CCRRRRSYT-FRCKRY--
RABBIT     VRYRCCRSQS--RSRCR-RRRRR-CRRRRRRCCQRRRVR--------K-CCRR--TYT-LRCRRY--
BULL       ARYRCCLTHS--GSRCR-RRRRRRCRRRRRRFGRRRRRR---------VCCRR---YTVIRCTRQ--
GOAT       ARYRCCLTHS--RSRCR-RRRRRRCRRRRRRFGRRRRRR---------VCCRR---YTVVRCTRQ--
RAM        ARYRCCLTHS--RSRCR-RRRRRRCRRRRRRFGRRRRRR---------VCCRR---YTVVRCTRQ--
STALLION   ARYRCCRSQS--QSRCR-RRRRRRCRRRRRRSVRQRR----------VCCRR---YTVLRCRRRR-
ORCA       ARNRC-RSPS--QSRCR-RPRRR-CRRRIR-CCR-RQRR---------VCCRR---YTTTRCARQ--
OPOSSUM    ARYRR-RSRSRSRSRYGRRRRRSRSRRRR--SRRRRR------RRGRRGRGYHRRSPHRRRRRRRR-
CHICKEN    ARYR--RSRTRSRSP-RSRRRRRRSGRRR--SPRRRRRYGSARRSRRSVGGRRRR-YGSRRRRRRRY
QUAIL      ARYR--RTRTRSRSR-----RRRRSRRRR--SSRR-RRYGRSRRSYRSVGRRRRR-YGRRRRRRRY
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FIG. 4—*Comparison of the human protamine P1 amino-acid sequence with the homologous sequences from some of the described mammalian species. References to the amino-acid sequences are:* S. imperator *(13), rabbit (27), bull (28, 29), goat and rat (30, 31), ram (32), mouse (33, 34), boar (35, 36), human (37–41), other primates (25), stallion (42, 43), and the whale* O. orca *(15). Differences at the DNA level are even more marked than those at the amino acid level shown in here.*

quencing of the protamine genes can be used to identify the human or animal origin of biological specimens. Protamine genes provide the additional advantage that information of the amino-acid or nucleotide sequences in many different species is already available [*12,13,15,25*]. Thus it is possible to readily identify the animal origin of a biological specimen based only on the nucleotide sequence. The determined sequenced does not need to be identical to a previously determined sequence in order to be identified since a large number of DNA sequence comparison programs are now available which can assign the determined sequence to the most closely related species in the databank. This is important because it means that it is not necessary to sample enough individuals of each different species in order to cover all the genetic variation. For the same reason this identification strategy could also tolerate a few sequencing mistakes in the determined nucleotide sequence without changing the final assignment of the species.

Another issue dealt with in this work has been to develop a system to allow rapid and efficient sequencing of protamine genes. Alternative existing methods are:

1. Cloning and sequencing PCR products. This approach requires at least one week and several independent clones must be sequenced to rule out the detection of mutations introduced during the PCR.

2. Direct sequencing PCR products after two rounds of PCR (the first one to amplify and the second one to generate ssDNA) and after laborious and multiple centricon purifications. This method takes at least 11.5 h.

3. Direct sequencing of double stranded PCR products which often gives raise to multiple compressions which preclude reading of the sequences.

4. Direct sequencing during PCR (cycle sequencing at high temperatures) through the use of ^{32}P-labelled primers. This method is quite fast except that the rapid decay of ^{32}P and the fact that is a high energy β-emitter is not as attractive as the use of ^{35}S. The method that we have standardized for the rapid sequencing of the protamine P1 genes requires only one PCR run followed by a fast and simple single strand generation strategy (Fig. 2). Thus, with this method the DNA samples can be processed and sequenced in the same day that they are received in the laboratory. With the increasing introduction of automatic sequencers, the method that we have described can be easily adapted for

the use of fluorescent primers or terminators, and thus still much further facilitate the rapid sequencing of protamine 1 genes. In addition it could be applied to other known hypervariable genomic regions such as the HLADQα with the additional advantage that the alleles of the subtype 4 could be differentiated in this way [*26*].

Acknowledgments

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