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The identification of newborns using messenger RNA profiling analysis

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Abstract

The ability to determine the physical characteristics of an individual depositing a bloodstain at a crime scene would be an invaluable tool to investigators, akin to eyewitness information. One useful biometric that may be amenable to molecular genetic analysis is the biological age of an individual. In theory, it may be possible to determine patterns of gene expression that are age specific, thereby permitting the distinction among tissue samples originating from individuals of different ages (e.g., newborn, adolescent, middle-age, elderly). We have discovered two novel isoforms of gamma hemoglobin messenger RNA, designated HBG1n and HBG2n, which exhibit an extremely restricted pattern of gene expression, being confined to newborn individuals. Multiplex quantitative reverse transcription PCR (qRT-PCR) assays incorporating these novel mRNAs have been designed, tested, and evaluated for their potential forensic use. The results indicate that the assays provide the ability to determine whether a bloodstain originated from a newborn.

Keywords: Forensic science; Forensic biochemistry; Biological age; Age determination; Newborn; Hemoglobin; Isoforms; Multiplex quantitative RT-PCR

It is now a matter of routine for the forensic scientist to obtain the genetic profile of an individual from DNA recovered from a biological stain deposited at the crime scene. Either potential contributors of the stain must be known to investigators (i.e., a developed suspect) or the questioned profile must be searched against a database of DNA profiles such as those maintained in the CODIS (Combined DNA Index System)[1] national DNA database [1]. However, in those instances where there is no developed suspect and no match is obtained after interrogation of appropriate DNA databases, the DNA profile per se currently provides no meaningful information to investigators, with the notable exception of gender determination [2]. In these situations, it would be advantageous to the investigation if additional probative information could be obtained from the biological stain. Additional investigative parameters could include determining the physical characteristics of the individual depositing the biological stain. A number of physically recognizable characteristics of an individual are at least partly inherited, including skin color, hair color, eye color, stature (i.e., height and weight), and facial morphology [3–7]. In theory, and given sufficient knowledge of the genetics of complex polygenic traits, DNA analysis on a crime scene sample could provide investigators with information akin to eyewitness identification. Because our understanding of the genetics of these complex traits is somewhat rudimentary (with few exceptions), development of significant forensic applications awaits further advances in our knowledge in this area. One exception may be skin and hair pigmentation given that the genetics of pigmentation has, to a large degree, proved to be amenable to molecular genetic analysis [3,4,8]. An additional useful biometric that could provide important probative information, and that may be amenable to molecular genetic analysis, is the biological age of an individual. The ability to provide

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[1] *Abbreviations used:* CODIS, combined DNA index system; mRNA, messenger RNA; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; RT-PCR, reverse transcription PCR; qPCR, quantitative PCR; dCt, delta cycle threshold.

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investigators with information as to whether a DNA donor is a newborn, an infant, a toddler, a child, an adolescent, an adult, a middle-aged individual, or an elderly individual [9] could be useful in investigating certain cases, particularly those involving young children such as kidnappings, or in providing additional intelligence during terrorist investigations. Currently, no validated molecular assays exist for age determination.

Postulated molecular mechanisms of aging include, inter alia, progressive damage to DNA [10–13], telomere shortening [14,15], long-lived protein glycation [16], and reactive oxygen species (ROS)-mediated oxidative damage to macromolecules [17–19]. However, all such studies concentrate on the degenerative processes of aging and attempt to correlate specific molecular damage with increases in age, particularly in postreproductive individuals [20]. From the forensic standpoint, it would be useful to be able to distinguish among individuals of all age groups, but this may require the detection of more subtle molecular changes. Thus, we have considered an alternative approach to age determination that is based on the epigenetic and developmental control of gene expression that occurs during all stages of human development [9].

The life cycle of humans is composed of a number of developmentally recognized stages [9]. As humans proceed through these developmental stages, subsets of the estimated 20,000–25,000 human genes [21] will be differentially expressed. In theory, a comparison of the gene expression profile from individuals of different ages could reveal constellations of candidate genes whose expression is correlated with a specific age. A limited number of recent reports have described age-associated differential gene expression profiles in skeletal muscle [22], liver [23], brain [24], and skin [25]. A clear example of age-related differential gene expression involves hemoglobin gene switching [26]. The human β-hemoglobin locus is located on the short arm of chromosome 11 (11p15.5) and encodes five functional β-like globin genes (ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β) and a nonfunctional β-pseudogene ($^{\beta}\psi$) [27]. The expression of embryonic hemoglobin (ε-globin) commences in the yolk sac during the early stages of gestational development. During the 5th week of gestation, the fetal liver begins to express the fetal-specific gamma globin chains ($^{A}\gamma$ and $^{G}\gamma$). This up-regulation of γ-globin (HBG) is accompanied by a shutdown of ε-globin synthesis. Shortly before birth, adult hemoglobin (β-globin) gene expression commences in the bone marrow and γ-globin expression is down-regulated.

In this article, we describe an investigation into whether the detection of HBG messenger RNA (mRNA) in a bloodstain would be an accurate predictor of blood from a newborn. Despite our observation that HBG mRNA per se appeared to be expressed throughout the various stages of human development, serendipitously we identified novel newborn-specific HBG mRNA isoforms that form the basis of assays suitable for the detection of blood from newborns.

Materials and methods

Sample preparation

Human blood samples were obtained from donors from Florida Hospital (Orlando, FL, USA) after receiving an exemption from the hospital's institutional review board and in accordance with procedures approved by the University of Central Florida's institutional review board. Bloodstains were made by dispensing 50-μl aliquots onto sterile cotton gauze and were air-dried overnight at room temperature. Other body fluid samples were collected from volunteers in accordance with guidelines approved by the university's institutional review board. Saliva and semen samples were obtained from healthy individuals, and 50-μl stains were prepared. Buccal swabs, vaginal secretion swabs, and menstrual blood swabs were obtained from healthy individuals and allowed to air-dry overnight at room temperature. Venous blood, saliva, and vaginal secretion swabs obtained from an expectant mother at various time points throughout the pregnancy and breast milk swabs (1 month postdelivery) were air-dried overnight. For stability studies, venous blood (50 μl) was prepared on sterile cotton gauze and allowed to sit at room temperature (~25 °C) for 1, 3, 6, 9, 12, and 15 months. Animal blood (with biological age if known) for species specificity testing was collected from two pigtailed macaques (22 days and 5 years) and two rhesus macaques (24 days and 12 years) (Yerkes National Primate Research Center, Atlanta, GA, USA); a calf (10 months), a sheep (3 years), and a lamb (4 months) (Innovative Research, Southfield, MI, USA); a cat and a dog (Tuscawilla Oaks Animal Hospital, Oviedo, FL, USA); a cow and a horse (HemoStat Laboratories, Dixon, CA, USA); a deer (Charles R. Daniels, DeLand, FL, USA); a spider monkey (Coriell Cell Repository, Camden, NJ, USA); two African crown cranes (2 and 3 years), a gopher tortoise (20 years), and a patagonian cavy (1 year) (Wuesthoff Reference Laboratory, Melbourne, FL, USA). One buccal swab from a Chinese muntjac (12 years) (Wuesthoff Reference Laboratory) was also tested for specificity. All stains were stored at −45 °C until needed.

RNA isolation

A guanidine isothiocyanate-phenol/chloroform extraction method was used [28]. Briefly, 500 μl denaturing solution (4 M guanidine isothiocyanate, 0.02 M sodium citrate, 0.5% sarkosyl, 0.1 M β-mercaptoethanol) was preheated in a Spin-Ease extraction tube (Gibco, Life Technologies, Gaithersburg, MD, USA) at 56 °C for 10 min. Prepared stains were then added and incubated at 56 °C for 30 min. The stains were removed into a Spin-Ease extraction tube filter insert, placed back inside the extraction tube, and centrifuged for 5 min at 16,000*g*, after which the filter and the fabric remnants were discarded. Then 50 μl of 2 M sodium acetate and 600 μl of acid phenol/chloroform (5:1, pH 4.5, Ambion, Austin, TX, USA) were added to the extract and

incubated at 4 °C until two phases were resolved (~20 min) and then centrifuged at 16,000*g* for 20 min. The RNA-containing aqueous phase was transferred to a sterile microcentrifuge tube along with 30 μg GlycoBlue glycogen carrier (Ambion) and was precipitated with 500 μl isopropanol overnight at −20 °C. Samples were then centrifuged at 16,000*g* for 20 min to pellet the RNA. The supernatant was carefully removed, and the pellet was washed once with 1 ml of 75% ethanol/25% diethyl pyrocarbonate (DEPC)-treated water and recentrifuged at 16,000*g* for 10 min. The supernatant was discarded, and the pellet was dried in a vacuum centrifuge for 3–5 min and resolubilized in 12–17 μl of RNAsecure Resuspension Solution (Ambion) at 60 °C for 10 min. RNA samples were treated with DNase I immediately or subsequent to storage at −20 °C.

DNase I digestion

Total RNA was treated with 2 U of TURBO DNase (RNase free) (2 U/μl) (Ambion) at 37 °C for 1–2 h. The TURBO DNase was inactivated at 75 °C for 10 min, and the samples were chilled on ice and then stored at −20 °C until needed [29,30].

Quantitation of nucleic acids

RNA was quantified using a sensitive fluorescence assay based on the binding of the unsymmetrical cyanine dye RiboGreen (Molecular Probes, Eugene, OR, USA) [31]. The manufacturer's instructions were followed for the high-range assay, which detects from 20 ng/ml to 1 μg/ml. Briefly, 200-μl assays consisting of 2 μl TURBO DNase-treated RNA extract, 98 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5, in nuclease-free water), and 100 μl of 750 nM RiboGreen reagent in a 96-well plate format. After the addition of RiboGreen and a 3-min incubation at room temperature protected from light, fluorescence emission at 535 nm (excited at 485 nm) was determined using a Wallac Victor^2 microplate reader (PerkinElmer Life Sciences, Boston, MA, USA). RNA concentration was calculated using an appropriate standard curve as described by the manufacturer [31]. All RNA samples were diluted to 5 ng/μl (saliva and buccal swabs were diluted to 10 ng/μl) with nuclease-free water (Ambion).

Reverse transcription (cDNA synthesis)

For all samples 6 μl of RNA (30 ng), and for saliva/buccal 6 μl of RNA (60 ng), was heated at 75 °C for 3 min. For the mixture study, total RNA from three newborns (<24 h) and three juvenile/adult females (16, 22, and 31 years) was combined in different ratio combinations (1:1, 1:5, 5:1, 1:10, and 10:1) to yield the 6 μl (30 ng) necessary for the amplification. To the RNA, 4 μl of a 10-mM dNTP mix (Applied Biosystems, Foster City, CA, USA), 2 μl of 10× first-strand buffer (500 mM Tris–HCl, pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, 50 mM dithiothreitol [DTT]), 2 μl Random Decamer primers (50 μM), 20 U SUPERase-In RNase inhibitor (20 U/μl, Ambion), 100 U Moloney Murine Leukemia Virus–Reverse Transcriptase (100 U/μl, Ambion), and nuclease-free water (Ambion) were added to yield a final reaction volume of 20 μl. Reaction mixtures were incubated at 42 °C for 1 h and at 95 °C for 10 min to inactivate the reverse transcriptase [32].

PCR amplification

All single gene amplification reactions were conducted in a total volume of 25 μl. cDNA (5 ng) was amplified with a standard reaction mix containing 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.125 mM each dNTP, 0.4 μM primers (Table 1), and 1.25 U AmpliTaq Gold DNA polymerase (5 U/μl) (Applied Biosystems). Nuclease-free water (Ambion) was added to yield the final reaction volume.

For the duplex reverse transcription PCRs (RT-PCR), 3 ng of cDNA was amplified in a total reaction volume of 25 μl with the following changes to the standard reaction mix: 0.6 μM S15 primers and 0.05 μM HBG1n or 0.05 μM HBG2n primers (Table 1). The ribosomal protein gene transcript, S15, was included as an internal positive control for the RT and amplification reactions. Amplimer sizes for all genes tested are included in Table 1.

Standard PCR conditions for HBG, HBG1, and HBG2 consisted of an 11-min denaturing step at 95 °C followed by 32 cycles at (i) 94 °C for 20 s, (ii) 55 °C for 30 s, (iii) 72 °C for 40 s, and a final extension step at 72 °C for 5 min [33,34]. PCR conditions for the genes HBG1n and HBG2n along with the two duplexes consisted of the above PCR conditions with the following changes: 28 cycles with a 60 °C annealing temperature.

Cloning and sequencing

Newly identified hemoglobin products were excised from agarose gels and purified using MERmaid spin columns, which specifically isolate low-molecular weight DNA products (10–200 bp) (Q-BIOgene, Carlsbad, CA, USA). Purified products were cloned into TOP10F′ One Shot chemically competent cells using the TOPO TA Cloning Kit (pCR 2.1-TOPO, Invitrogen). Positive colonies were isolated and plasmids were purified using the RapidPURE Plasmid Mini Kit (Q-BIOgene). Plasmids that contained the inserted product were sent to Lark Technologies (Houston, TX, USA) for sequencing analysis.

Post-RT-PCR electrophoresis

RT-PCR amplified products were visualized on 4% NuSieve GTG agarose gels (Cambrex Bio Science Rockland, Rockland, ME, USA). Electrophoresis was carried out at 100 V for 1.25 h in TAE buffer (0.04 M Tris–acetate, 0.001 M EDTA). Gels were stained with SYBR Gold nucleic acid stain (Molecular Probes), visualized on the

Table 1
Primer and probe sequences, and expected DNA and mRNA product sizes, for the gel-based and quantitative RT-PCR assays used to detect newborn-specific transcripts

Gene	Primer and probe sequences	DNA/mRNA amplimer size (bp)
Gel-based RT-PCR assay		
S15		
Forward	5′ TTC-CGC-AAG-TTC-ACC-TAC-C 3′	361/361
Reverse	5′ CGG-GCC-GGC-CAT-GCT-TTA-CG 3′	
HBG		
Forward	5′ GTG-GAT-CCT-GAG-AAC-TTC-AA 3′	1040/154
Reverse	5′ GAG-CTC-AGT-GGT-ATC-TGG-AG 3′	
HBG1		
Forward	5′ ACT-TCC-TTG-GGA-GAT-GCC-AC 3′	1157/277
Reverse	5′ AAA-GCC-TAT-CCT-TGA-AAG-CTC-TGA 3′	
HBG2		
Forward	5′ ACT-TCC-TTG-GGA-GAT-GCC-AT 3′	1160/274
Reverse	5′ GCC-TAT-CCT-TGA-AAG-CTC-TGC 3′	
HBG1n		
Forward	5′ GAA-AGC-TCT-GAA-TCA-TCC-AGG-TG 3′[a]	0/207
Reverse	5′ GGG-CAA-GGT-GAA-TGT-GGA-AG 3′	
HBG2n		
Forward	5′ GGC-AGT-GAG-CTC-AGT-GCA-GTT-C 3′[a]	0/161
Reverse	5′ CAG-CTT-TGG-CAA-CCT-GTC-CT 3′	
qRT-PCR assay		
S15		
Forward	5′ CCA-AAG-CGA-TCT-CTT-CTG-AGG-AT 3′	
Reverse	5′ ACG-CCG-CGG-TAG-GTG-AA 3′	
MGB probe	VIC CGG-CAA-GAT-GGC-AGA-AGT-AGA-GCA-GAA MGBNFQ[b]	
HBG1n		
Forward	5′ GAA-AGC-TCT-GAA-TCA-TCC-AGG-TG 3′	
Reverse	5′ AGT-CAA-GGC-ACA-TGG-CAA-GAA-G 3′	
MGB probe	6FAM TTT - GTG- GCA- TCT- CCC-AAG- GAA- GTC-A GCM GBNF Q[b]	
HBG2n		
Forward	5′ GCA-GTG-AGC-TCA-GTG-CAG-TTC 3′	
Reverse	5′ TTC-CTT-GGG-AGA-TGC-CAT-AAA 3′	
MGB probe	6FAM CAA-AGG-TGC-CCT-TGA-GAT-CAT-CCA-GG MGBNFQ[b]	

[a] Underlined sequence identifies the location of the newborn hemoglobin isoform breakpoint.
[b] MGBNFQ, minor groove binding nonfluorescent quencher.

Omega 10 Chemiluminescence Imaging System (Ultra-Lum, Claremont, CA, USA), and analyzed with ONE-Dscan 2.05 (one-dimensional gel analysis software for Windows, Scanalytics, Fairfax, VA, USA).

Duplex real-time PCR amplification

All quantitative PCR (qPCR) assays were performed in a 25-μl total reaction volume consisting of a standard reaction mix containing 3 ng of cDNA (blood, semen, vaginal secretions, menstrual blood, and breast milk) or 6 ng of cDNA (saliva/buccal), 12.5 μl Taqman Universal PCR Master Mix (Applied Biosystems), 0.25 μM of each probe, and nuclease-free water (Ambion).

For the newborn assays (⩽4 months), 0.6-μM (S15) primer and 0.1-μM (HBG1n) or 0.05-μM (HBG2n) primer were added to the standard reaction mix. For the newborn assays (<24 hour), 0.9 μM S15 primer and 0.05 μM HBG1n or 0.05 μM HBG2n primer were added to the standard reaction mix.

All primer and probe sequences are listed in Table 1. Real-time PCRs were carried out on a 7000 Sequence Detection System (Applied Biosystems). Amplification conditions consisted of (i) 1 cycle at 50 °C for 2 min, (ii) 1 cycle at 95 °C for 10 min, and (iii) 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Data were collected at the second step of the third stage (60 °C for 1 min). Delta cycle threshold (dCt) values were calculated by subtracting the Ct value generated from the newborn-specific gene from the Ct value of the housekeeping gene, that is, $dCt = Ct\ (S15) - Ct\ [HBG(1/2)n]$ [35]. Samples that fail to amplify the newborn genes are given a default Ct [HBG(1/2)n] value of 40.00 (the amount of qPCR cycles used).

Results and discussion

Hemoglobin gamma mRNA analysis

The tetrameric fetal and adult hemoglobin protein complexes are composed of either two alpha and two gamma ($\alpha_2\gamma_2$) or two alpha and two beta ($\alpha_2\beta_2$) hemoglobin chains, respectively. This well-characterized variation in fetal versus adult hemoglobin was the basis for the initial design of a newborn-specific assay. The gamma hemoglobin locus was analyzed by RT-PCR using three different sets of primers. The universal set amplifies both gamma hemoglobin

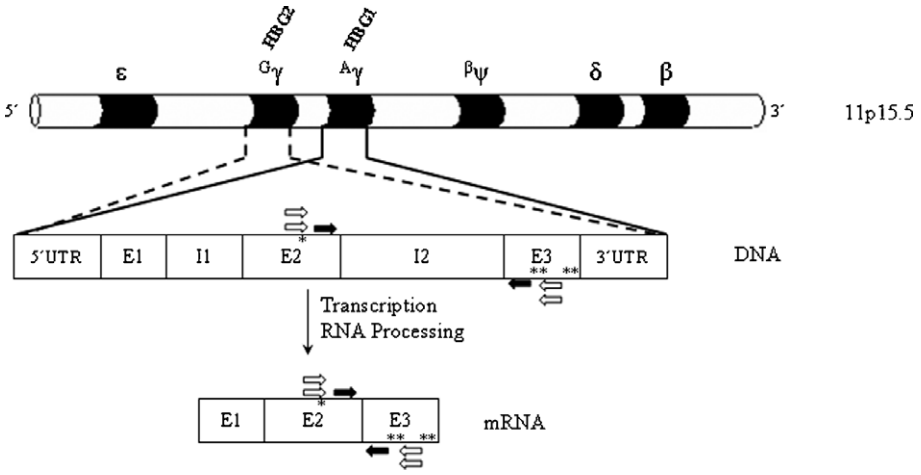

Fig. 1. Structure of the human β-hemoglobin locus. DNA and mRNA structures of the fetal gamma hemoglobin genes, HBG1 ($^{A}\gamma$) and HBG2 ($^{G}\gamma$), are shown. The human β-hemoglobin locus on chromosome 11 encodes five functional β-like globin genes (ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β) and a nonfunctional β-pseudogene ($^{\beta}\psi$). Both gamma hemoglobin genes are composed of three exons (E1, E2, and E3) and two intronic sequences (I1 and I2). All primer sets were designed to flank the second intron (I2) to distinguish mRNA- and DNA-amplified products. Three different sets of primers were designed for gamma hemoglobin amplification using the five polymorphic sites that are indicated by asterisks (∗). Universal primers (HBG) will bind to the nonpolymorphic regions in both gamma hemoglobin genes and amplify a total gamma hemoglobin product (filled arrows). Gene-specific primers (HBG1 and HBG2) were designed to land on polymorphic sites for individual A-gamma and G-gamma amplifications, respectively (open arrows).

genes simultaneously (HBG), whereas two sets of gene-specific primers amplify either the HBG1 (A-gamma) or HBG2 (G-gamma) genes individually (Fig. 1). RT-PCR was performed using total RNA extracted from bloodstains from individuals 1 hour to 91 years of age. Contrary to the initial hypothesis, an mRNA-amplified product corresponding to total HBG (154 bp, Fig. 2A) or individual HBG1 (277 bp, Fig. 2B) or HBG2 (274 bp, Fig. 2C) genes was amplified in all ages tested. These results demonstrate that, in contrast to the expression pattern for the fetal hemoglobin protein, HBG mRNA production is not restricted solely to the fetal and newborn stages of development. Detailed inspection of the results from the individual HBG1 and HBG2 RT-PCR assays revealed the presence of an additional lower molecular weight product in younger individuals 1 hour, 13 days, and 3 months of age (Figs. 2B and C, respectively). The RT-PCR experiments were repeated with multiple individuals 1 hour to 89 years of age. All newborns tested (1 hour to 4 months of age, $n = 16$) successfully produced a lower molecular weight product with both HBG1 and HBG2 primer sets, whereas all other individuals (5 months to 89 years of age, $n = 20$) did not produce the low-molecular weight amplimers (Figs. 2B and C and data not shown).

Cloning and sequencing of newborn-specific HBG isoforms (HBG1n, HBG2n)

The newborn-specific low-molecular weight amplimers were cloned into chemically competent cells and sequenced as described in Materials and methods. Sequence alignment of the low-molecular weight amplimers to the known gamma hemoglobin sequences (HBG1 or HBG2) revealed that these novel products were actually truncated forms of the standard gamma hemoglobin mRNA sequences. We term the novel newborn isoforms HBG1n and HBG2n based on their alignment with the HBG1 and HBG2 genes, respectively. A comparison of HBG1n and HBG2n sequences with those of HBG1 and HBG2 revealed that these newborn transcripts contained deletions that spanned the 3′ end of exon 2 and the 5′ end of exon 3. The genic location of the HBG1n and HBG2n deletion breakpoints differed, although both occurred between different duplicated pentanucleotide sequences present in exon 2 and exon 3. Specifically, the pentanucleotide sequences and their locations within the gene are ATGAT (292–296, 509–513 [GenBank: NM_000559]) and CACTG (330–334, 492–496 [GenBank: NM_000184]) for HBG1 and HBG2, respectively. Although the sequence at the breakpoint is known, the actual position within the direct repeat where the break occurs is unknown. The deletion spanned 217 bp in HBG1 and 162 bp in HBG2. The biological function of the HBG1n and HBG2n, if any, is unknown at this juncture, as is the mechanism of their formation, although a novel cellular DNA or RNA recombination event is postulated (Fig. 3).

Gel-based RT-PCR assays for newborn gamma hemoglobin isoforms

Based on the HBG1n and HBG2n sequences, gel-based RT-PCR assays were designed. Forward primers for the HBG1n and HBG2n assays were designed to span the breakpoints in each of the two isoforms, thereby precluding the amplification of the HBG genes (Table 1, underlined sequence). Total RNA from bloodstains from three individuals 8 days, 15 years, and 84 years of age were tested along with a genomic DNA control. As expected, a single

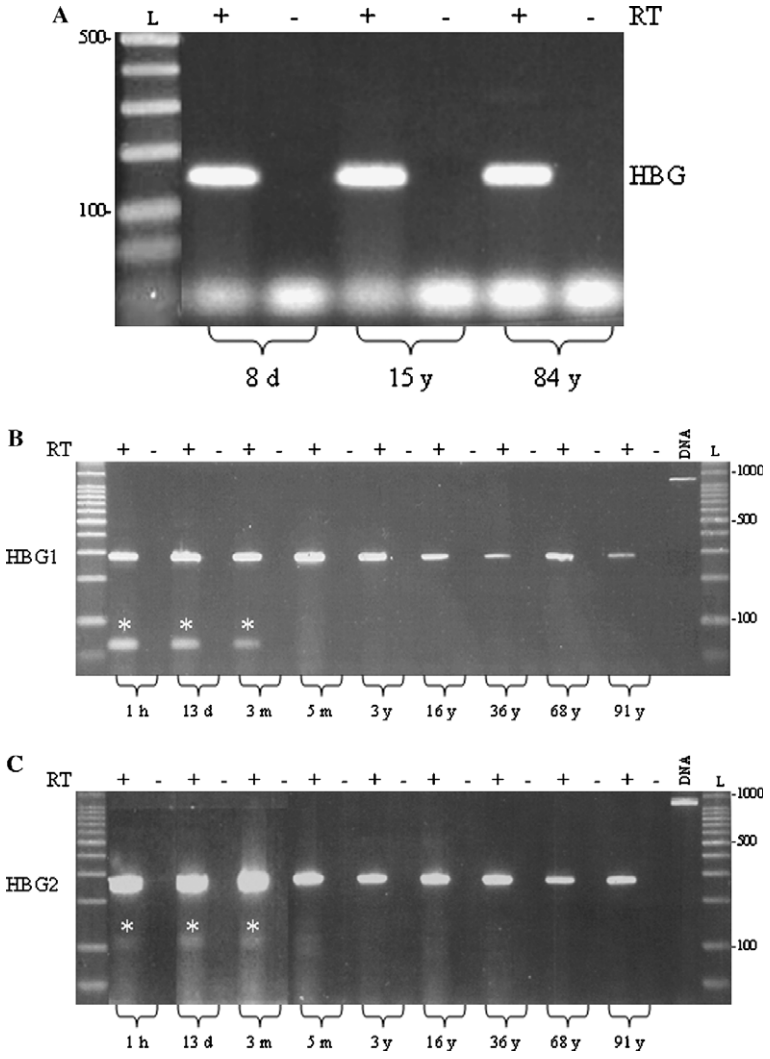

Fig. 2. Identification of gamma hemoglobin transcripts in blood from different age groups. Total RNA was isolated from 50-μl bloodstains of individuals 1 hour to 91 years of age and was tested for the presence of total gamma hemoglobin (HBG, 154 bp) (A), HBG1 (277 bp) (B), and HBG2 (274 bp) (C). For HBG1 and HBG2, genomic DNA was amplified as a control (far right lane). As a control for DNA contamination, the RNA isolates were also amplified without prior reverse transcriptase (–RT) treatment. A low-molecular weight transcript (denoted by ∗) was detected in newborn samples up to 3 months old with the HBG1 (B) and HBG2 (C) primers. Gels were stained with SYBR Gold nucleic acid stain. RT, reverse transcriptase; L, 100-bp DNA ladder; h, hour; d, day; m, month; y, year.

amplified product consistent with the detection of HBG1n and HBG2n was detected only in the 8-day-old (Figs. 4A and B, respectively).

An internal positive control (IPC), the ribosomal protein S15, was incorporated into the assays, resulting in two duplex RT-PCR reactions. S15 was chosen as the IPC instead of either of the commonly used housekeeping genes, GAPDH and β-actin, because S15 exhibited significantly fewer processed pseudogene-derived artifacts in RNA isolates containing trace quantities of genomic DNA (data not shown). Each duplex reaction contained primers for the housekeeping gene, S15 [34], and one of the two newborn gamma isoforms: HBG1n or HBG2n. The S15–HBG1n (Fig. 5A) and S15–HBG2n (Fig. 5B) duplexes demonstrated the presence of S15 mRNA in all ages tested, whereas the newborn gamma hemoglobin gene transcripts were found only in individuals 1 hour to 3 and 4 months of age, respectively.

qRT-PCR for newborn-specific genes

The two gel-based duplex RT-PCR assays for the identification of HBG1n and HBG2n were reconfigured for analysis using a real-time qPCR platform. The resulting prototype qRT-PCR assays, as formulated, should detect the HGB(1/2)n-derived amplicons at a significantly higher level in newborns ($\leqslant$4 months) compared with those of older age groups (>4 months). To accomplish this, the amount of HBG(1/2)n expression in different age groups was characterized by a dCt metric, Ct (S15) − Ct [HBG(1/2)n], which measures the expression of HGB(1/2)n isoforms in relation to the S15 internal positive control. Samples from newborns ($\leqslant$4 months) typically generated Ct [HBG(1/2)n] values less than those of S15 (indicating the relatively high level of expression of HBG(1/2)n in newborns compared with the S15 housekeeping gene) (Figs. 6A and B, left pan-

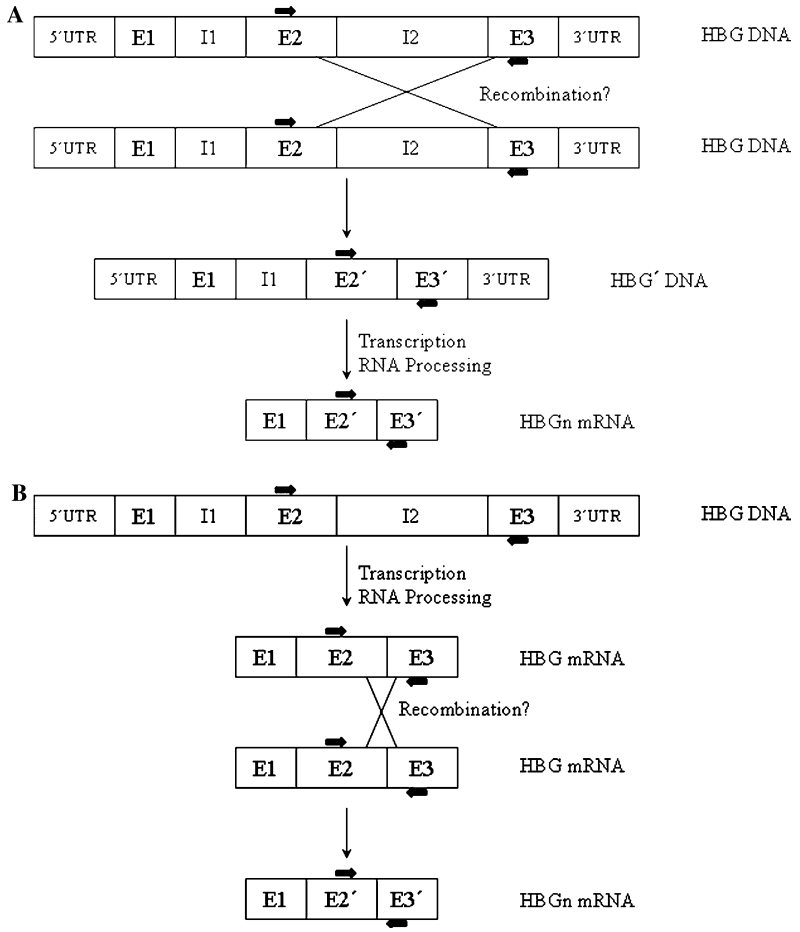

Fig. 3. Postulated rearrangement mechanisms for the formation of the novel newborn-specific gamma hemoglobin transcripts. Repeated sequences located within exons 2 and 3 of the gamma hemoglobin genes (HBG) mediate a DNA or RNA rearrangement event to yield the newborn-specific forms of gamma hemoglobin. (A) A somatic DNA recombination event could produce an alternate hemoglobin sequence (HBG′), which contains an intact exon 1 and intron 1 (E1 and I1, respectively) and two truncated exons (E2′ and E3′), no longer separated by an intronic sequence. Transcription of this gene would then generate the newborn-specific form of gamma hemoglobin. (B) An RNA-based rearrangement between two gamma mRNA species (HBG) could produce newborn-specific gamma hemoglobin mRNA isoforms (HBGn).

els). In contrast, Ct [HBG(1/2)n] values from non-newborns (>4 months) were greater than generated S15 Ct values (Figs. 6A and B, right panels). In some non-newborns (>4 months), the HBG(1/2)n transcripts were present in insufficient quantity to reach the Ct threshold. In these instances, the Ct [HBG(1/2)n] was given a default value of 40.00, which represents the total number of PCR cycles used. Therefore, the qRT-PCR assays, as configured, should produce positive dCt results for newborn blood samples, whereas all other age groups should produce negative dCt results. For example, the dCt values of the newborns illustrated in Figs. 6A and B were +1.60 (HBG1n) and +5.60 (HBG2n), whereas the non-newborns were −2.61 (HBG1n, 72 years old) and −8.03 (HBG2n, 15 years old).

In certain circumstances (e.g., newborns who have been illegally removed from the hospital), it would be useful to determine whether an individual was less than 24 hours old. It was possible (see below), by altering the primer concentrations, to modify the two duplex qRT-PCR assays described above such that they were predictive (i.e., based on a positive dCt metric) of blood from an individual less than 24 hours old (Figs. 6C and D). Examples of the results from the less than 24-hour-old individual assays are provided in Figs. 6C (HBG1n) and 6D (HBG2n). The corresponding dCt values for a 1-hour-old individual were +3.75 (HBG1n) and +5.89 (HBG2n), whereas an 8-day-old individual produced values of −1.40 and −1.66, respectively.

The precise Ct that an amplified gene product attains is dependent on two factors: the amount of target gene present in the sample and the concentration of primer and probe used in the PCR. The two newborn duplex real-time PCR assays (⩽4 months and <24 hours) illustrate the effect that these two factors have in real-time PCR amplification (Supplementary Fig. 1). Ubiquitously expressed genes (i.e., housekeeping genes) are expressed at relatively the same levels in all cell types. Differentially expressed genes have regulated expression patterns and either are turned on/off (i.e., present/not present) or are expressed at different levels (i.e., increased/decreased) in a tissue or developmental stage-specific manner. In both newborn assays, the concentration of the newborn gamma hemoglobin isoform primers is similar (HBG1n = 100 nM [⩽4 months] and 50 nM

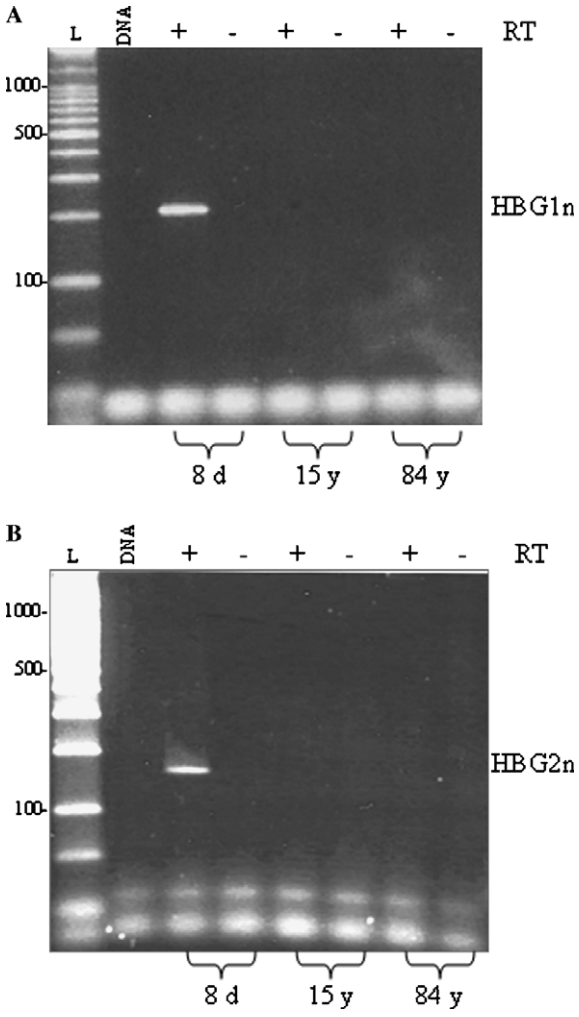

Fig. 4. Amplification of two newborn-specific gene transcripts. Total RNA was isolated from 50-μl bloodstains from a newborn (8 days), a juvenile (15 years) and an elderly individual (84 years). RT-PCR was performed with primers designed to amplify only the newborn-specific isoforms of gamma hemoglobin: HBG1n (207 bp) (A) and HBG2n (161 bp) (B). Amplification of genomic DNA was included as a control. Gels were stained with SYBR Gold nucleic acid stain. L, 100-bp DNA ladder; RT, reverse transcriptase; d, day; y, year.

[<24 h]; HBG2n = 50 nM [⩽4 months] and 50 nM [<24 h]). Therefore, because the primer/probe concentrations are the same in both assays, amplification of these hemoglobin isoforms is dependent on the initial gene copy number. Supplementary Fig. 1 illustrates that a sample amplified with all four duplex reactions from both assays should produce a relatively constant Ct [HBG(1/2)n] value. Because the S15 housekeeping gene exhibits a constant level of expression (constant initial copy number), all samples should reach the threshold at relatively the same cycle number. Thus, increasing or decreasing the S15 primer/probe concentration will shift all amplification response plots to the left or right, respectively. The S15 primer concentrations vary significantly between the two newborn assays. In the ⩽4-month assay, 600 nM is used, compared with 900 nM in the <24-hour assay. Therefore, by increasing the S15 primer concentration, all amplification plots (Ct values) are leftward shifted in the <24-hour assay when compared with the ⩽4-month assay, and with the hemoglobin genes remaining constant, this allows older newborns to produce negative dCt values (more S15 product than HBG[1/2]n) compared with younger newborns (more HBG[1/2]n product than S15) (Supplementary Fig. 1).

Specificity of the HBG(1/2)n qRT-PCR assays

Age specificity

The ability of the qRT-PCR assays to identify newborns (⩽4 months or <24 hours) was tested by analyzing 132 blood samples from multiple donors varying in biological age from 1 hour to 92 years (<24 h [$n = 10$], 1 day–1 month [$n = 19$], 2–4 months [$n = 22$], 5 months–3 years [$n = 37$], 4–18 years [$n = 20$], and 19–92 years [$n = 24$]). The results are summarized in the form of two-dimensional scatter plots in which each sample's dCt (HBG1n) and dCt (HBG2n) are displayed (Fig. 7). Positive results from newborns are expected to be confined to the upper right quadrant (positive dCt [HBG1n] and dCt [HBG2n]), whereas negative results from non-newborns would be found in the lower left quadrant (negative dCt [HBG1n] and dCt [HBG2n]).

In the ⩽4-month assay, 98% (i.e., 50) of the 51 newborn samples (⩽4 months) samples yielded at least one positive dCt value, whereas 96% (i.e., 78) of the 81 non-newborns yielded two negative dCt values (Fig. 7A and Table 2). Indeed, the vast majority of ⩽4-month-old individual samples (90%, i.e., 46/51) gave two positive dCt values (upper right quadrant). The one non-newborn sample that appears in the upper right quadrant in Fig. 7A originates from a 7-month-old. Subsequent repeat analysis (2×) places it in the lower right quadrant (i.e., one positive dCt and one negative dCt). Of the samples that generated one positive dCt value and one negative dCt value, four of the six individuals were 4 months old. This is consistent with the occurrence of a transitional developmental state that occurs approximately 4 months after birth in which transcription of the HBG(1/2)n isoforms is curtailed.

For the <24-hour assay, all newborn samples 1–24 hours of age generated positive dCt values (10/10) for each duplex (Fig. 7B and Table 2). Fully 97% (i.e., 100/103) of individuals more than 1 month of age generated two negative dCt values, as expected.

No sex-specific differences were observed with either the <24-hour or ⩽4-month assays (data not shown).

Body fluid specificity

Saliva ($n = 18$), semen ($n = 2$), vaginal secretions ($n = 2$), and menstrual blood ($n = 7$) from healthy donors, as well as venous blood, saliva, and vaginal secretions from a pregnant female and breast milk (1-month postdelivery), were assayed with the ⩽4-month and <24-hour duplexes. HBG1n and HBG2n Ct values were undetermined for all body fluids tested, illustrating that the two duplexes are specific for venous newborn blood (data not shown).

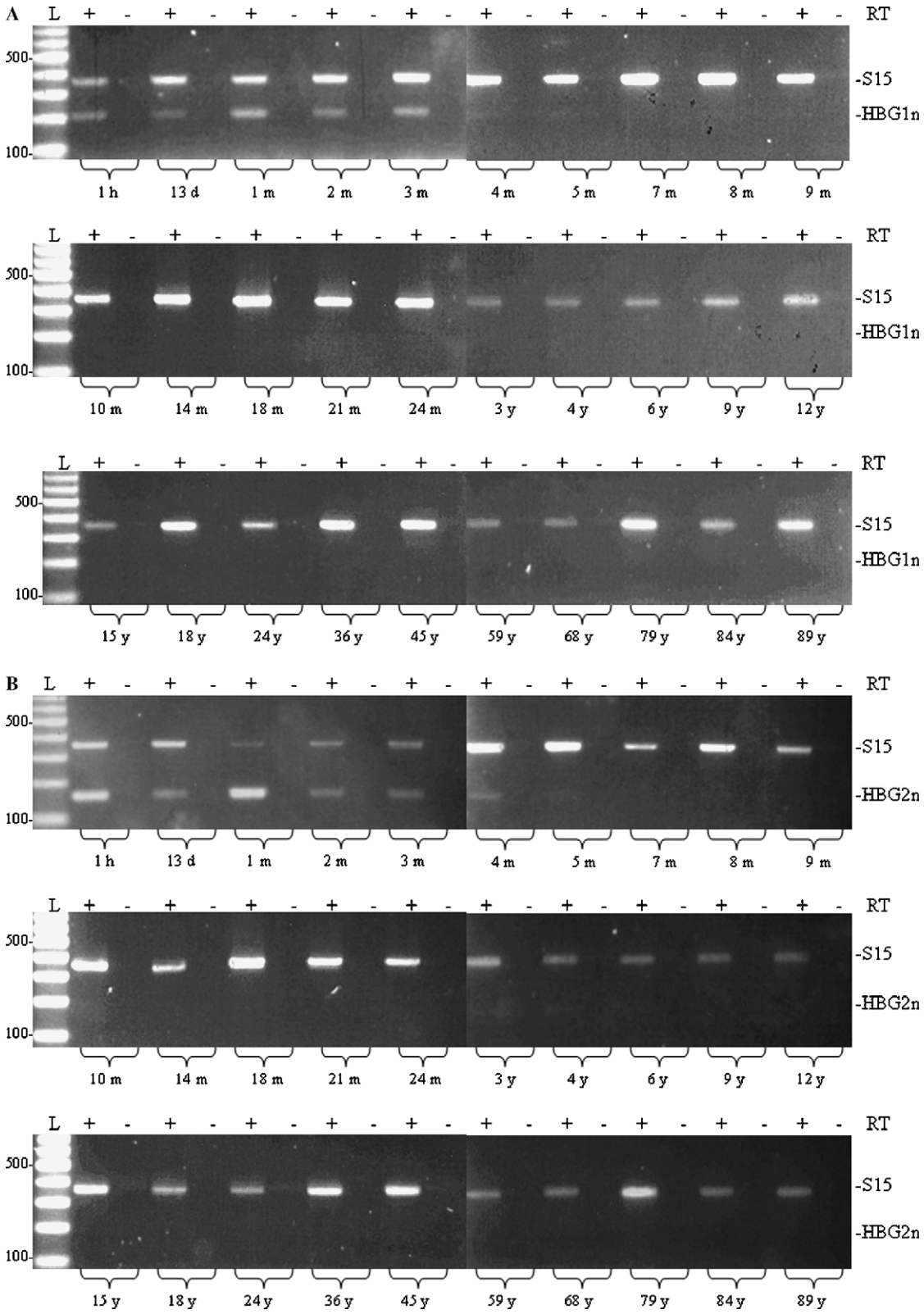

Fig. 5. Age specificity of the HBG1n and HBG2n transcripts. Total RNA isolated from 50-μl bloodstains from 30 different male and female individuals was amplified with two duplex RT-PCR assays that incorporate each newborn gene together with a housekeeping gene as an internal control. The S15–HBG1n (A) and S15–HBG2n (B) duplexes illustrate that the amplification of the newborn gamma hemoglobin product is restricted to the biological ages ranging from 1 hour to 3 or 4 months, respectively, whereas all ages amplify the S15 housekeeping control gene. Gels were stained with SYBR Gold nucleic acid stain. L, 100-bp DNA ladder; RT, reverse transcriptase; h, hour; d, day; m, month; y, year.

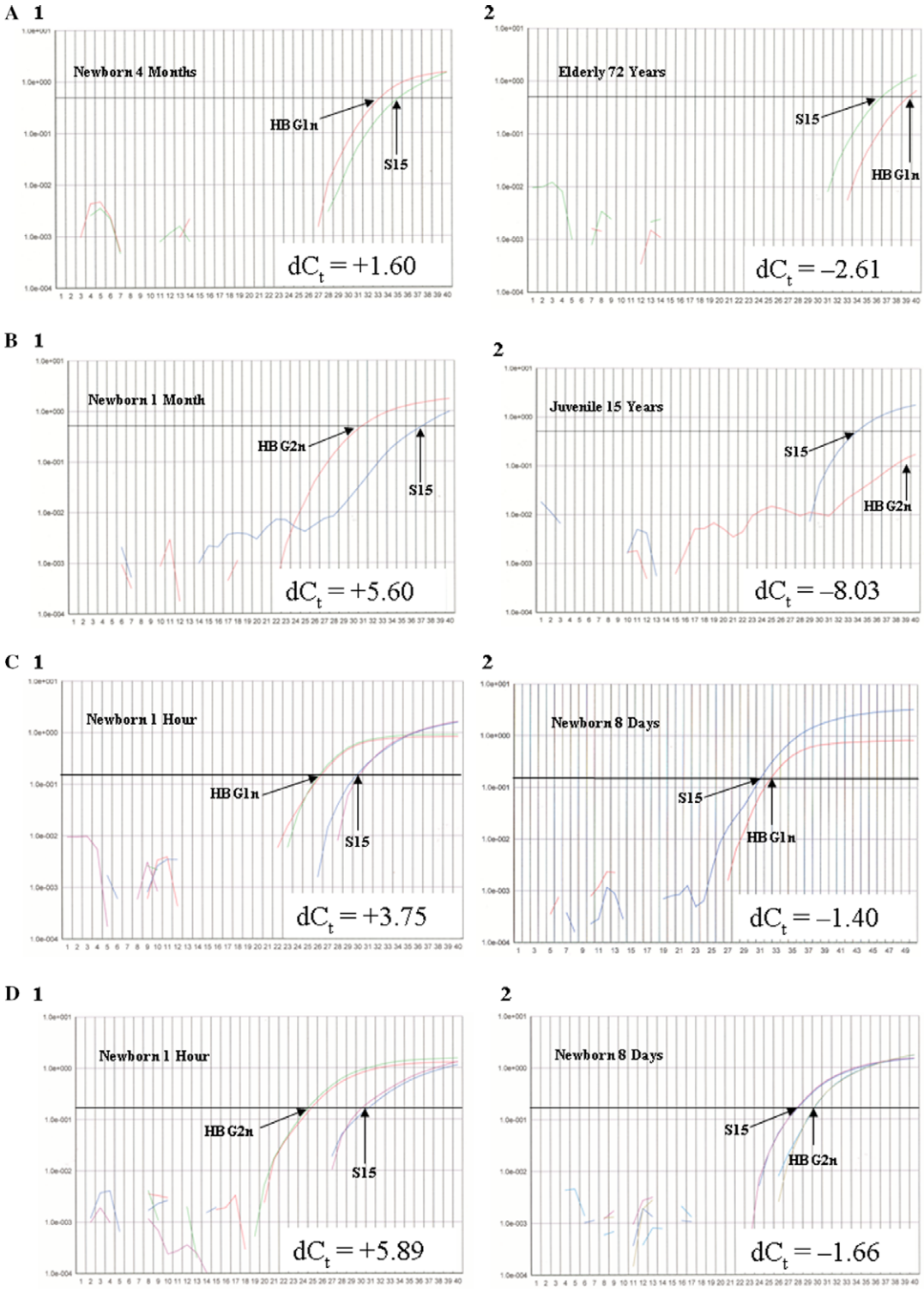

Fig. 6. Quantitative real-time PCR assays for the identification of newborns. Duplex qRT-PCR assays were designed to specifically identify blood originating from individuals ⩽4 months old (A and B) or <24 h old (C and D). The response curves demonstrate that the HBG(1/2)n transcripts are more abundant than the S15 housekeeping gene in newborns but are less abundant than the S15 gene in older age groups. (A) Response curve of the S15–HBG1n duplex (⩽4 months) comparing a newborn and an elderly individual. (B) Response curve of the S15–HBG2n duplex (⩽4 months) comparing a newborn and a juvenile. (C) S15–HBG1n duplex (<24 h) comparing newborns 1 hour and 8 days old. (D) S15–HBG2n duplex (<24 h) comparing newborns 1 hour and 8 days old. The dCt metric, Ct (S15) – Ct [HBG(1/2)n], which measures the expression of HBG(1/2)n isoforms in relation to the S15 internal positive control, is displayed for each sample. Ct values, the cycle numbers at which the response curves cross the threshold line (solid horizontal line), are identified (arrows).

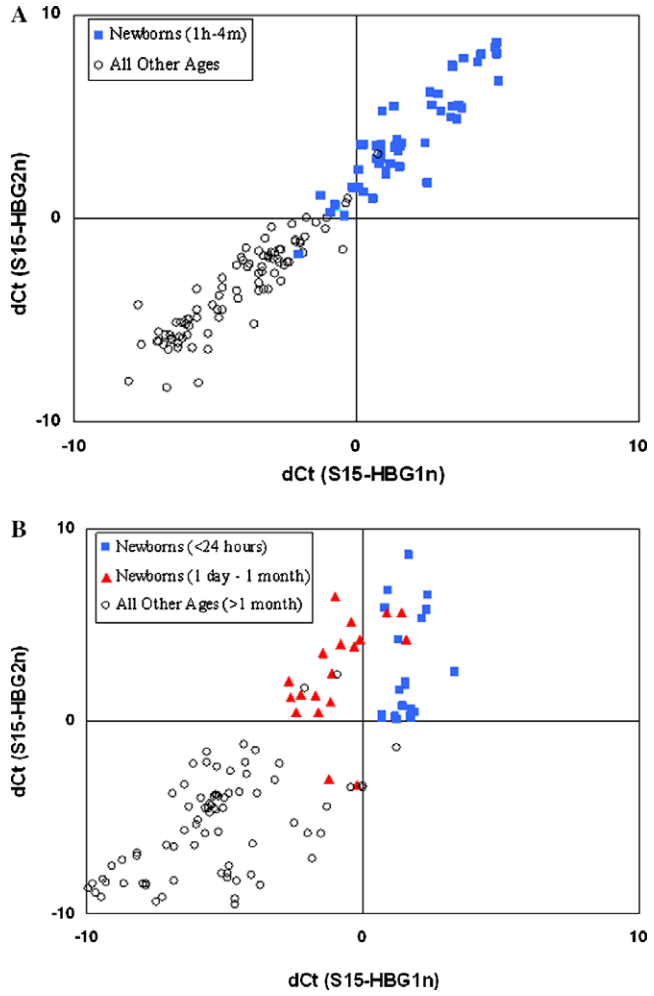

Fig. 7. Specificity of the HBG(1/2)n qRT-PCR assays. To test the specificity of the qRT-PCR assays for the identification of newborns, 132 individuals of different age groups (1 hour to 92 years) were analyzed with the four real-time PCR duplexes: HBG1n (⩽4 months), HBG2n (⩽4 months), HBG1n (<24 h), and HBG2n (<24 h). The results are displayed as scatter plots in which each sample's dCt (S15–HBG1n) and dCt (S15–HBG2n) obtained from using the ⩽4-month (A) or <24-h (B) assay format is plotted. Newborn samples (from different age groups) and non-newborn samples are labeled separately.

Table 2
Biological age specificity results for the two newborn duplex real-time PCR assays

Biological age	*n*	Assay results					
		⩽4 months			<24 h		
		(+/+)[a]	(+/−)[b]	(−/−)[c]	(+/+)[a]	(+/−)[b]	(−/−)[c]
<24 hours	10	10	—	—	10	—	—
1 day to 1 month	19	19	—	—	3	14	2
2 months to 4 months	22	17	4	1	—	1	21
5 months to 3 years	37	1	2	34	—	2	35
4 years to 18 years	20	—	—	20	—	—	20
19 years to 92 years	24	—	—	24	—	—	24

[a] Represents two positive delta Ct values (+/+).
[b] Represents one positive delta Ct value and one negative delta Ct value (+/−).
[c] Represents two negative delta Ct values (−/−).

Human specificity

RNA was extracted from bloodstains from a variety of animal species, including two pigtailed macaques (one newborn and one adult), two rhesus macaques (one newborn and one adult), a calf (newborn), a cow (adult), a lamb (newborn), a sheep (adult), a cat, a dog, a horse, a deer, a spider monkey, two African crown cranes, a gopher tortoise, and a patagonian cavy, and was tested with the newborn qPCR assays. One buccal swab from a Chinese muntjac was also tested. HBG1n and HBG2n Ct values were undetermined for all animal samples tested, illustrating that the two duplexes are specific for human newborn blood (data not shown).

Mixture study

The newborn assays are expected to be of use in the investigation of criminal abortion cases. In such instances, putative products of conception sometimes are recovered and expected to comprise mixed samples, typically the newborn (or fetus) and that of an adult. Therefore, to ensure the detectability of newborn blood in the presence of adult blood, controlled mixture studies were carried out. Total RNA from the blood of newborns (<24 hours) and of either juveniles (16 years) or adults (22 or 31 years) was combined to simulate mixtures from criminal abortion cases. Three separate newborn/non-newborn admixed pairs were studied, with each pair composed of a sample set of the same admixture ratios (1:1, 1:5, 5:1, 1:10, and 10:1). The 15 mixed RNAs were reverse-transcribed and amplified with both newborn duplexes using the ⩽4-month and <24-hour assay formats.

In the ⩽4-month assay, all 15 mixtures generated two positive dCt values except for one of the 1:10 mixtures (24-hour-old newborn:31-year-old adult) (data not shown). This latter sample generated one positive dCt value (S15–HBG1n) and one negative dCt value (S15–HBG2n). In the <24-hour assay, all of the 1:1, 5:1, and 10:1 mixtures generated two positive dCt values (data not shown). The three 1:5 mixtures and two of the three 1:10 mixtures generated one positive dCt value and one negative dCt value in the S15–HBG2n and S15–HBG1n assays, respectively. The other 1:10 mixture (24-hour-old newborn:31-year-old adult) generated two negative dCt values (data not shown).

The above results indicate that the assays can detect newborn/non-newborn admixed samples and are likely to be of use to demonstrate the presence of newborn blood in putative products of conception.

Sensitivity

The sensitivities of the qRT-PCR newborn assays were determined by varying the amount of total RNA input into the assays using RNA isolated from bloodstains from two newborns (both 1 hour old) and two non-newborns (a 13-year-old and a 53-year-old). The average dCt values from both newborns and both adults are shown for each duplex reaction (Fig. 8). The ⩽4-month newborn assay generated

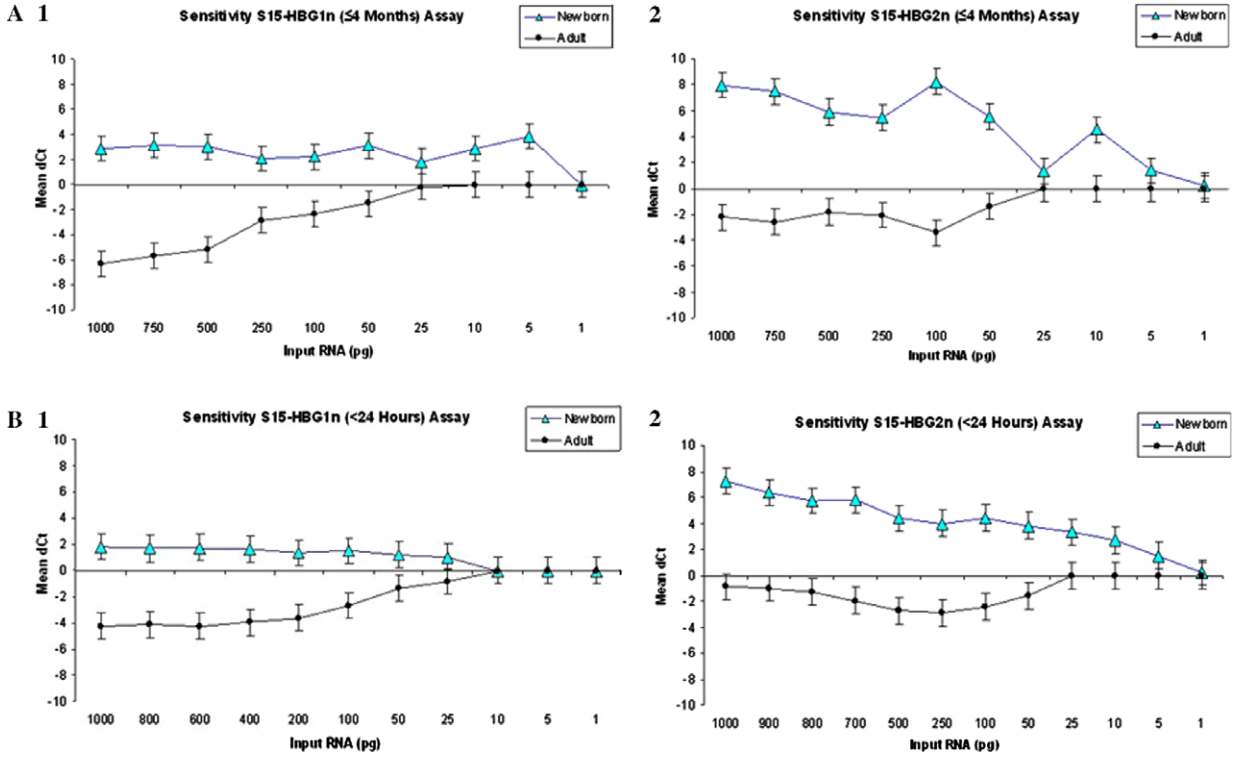

Fig. 8. Sensitivity of the HBG(1/2)n qRT-PCR assays. The sensitivities of the qRT-PCR assays were determined by varying the amount of total RNA input into the assays using RNA isolated from bloodstains from two newborns (both 1 hour old) and two non-newborns (a 13-year-old and a 53-year-old). The average dCt values from both newborns and both adults are shown for each duplex assay for different input RNA quantities. (A1) HBG1n (⩽4 months); (A2) HBG2n (⩽4 months); (B1) HBG1n (<24 h); (B2) HBG2n (<24 h).

positive dCt values with at least 5 pg RNA with the newborn samples, whereas the adult samples generated negative dCt values with at least 50 pg RNA (Fig. 8A). Input RNA less than these concentrations did not produce detectable housekeeping gene or newborn gene products that reached the Ct threshold (Fig. 8A). With the <24-hour newborn assay, the S15–HBG1n duplex generated the expected positive and negative dCt values (newborns and adults, respectively) with at least 25 pg RNA input (Fig. 8B1). With the S15–HBG2n assay, newborns generated positive dCt values with at least 5 pg input RNA, whereas the adult samples generated negative dCt values with at least 50 pg input RNA (Fig. 8B2).

Based on these sensitivity studies, a minimum input of 50 pg RNA is recommended for the qRT-PCR newborn assays.

Stability of HBG(1/2)n transcripts in aged bloodstains

To be useful in forensic casework, the HBG(1/2)n transcripts should be stable over time in dried stains. To assess the stability of the newborn transcripts in the dried state, blood from two newborns (1 hour and 2 months old), two juveniles (14 and 15 years old), and two elderly individuals (84 and 86 years old) was deposited on cloth, allowed to air-dry, and stored at room temperature (~25 °C) for various time points (1, 3, 6, 9, 12, and 15 months). Total RNA was isolated from the bloodstains and then assayed for HBG1n and HBG2n transcripts by qRT-PCR. The results are displayed in a two-dimensional scatter plot as before (Fig. 9). In both newborn assays (⩽4 months and <24 hour), the 1-hour-old newborn generated two positive dCt values in all aged samples, whereas the juvenile and elderly individuals generated two negative dCt values at all time points tested.

Despite the excellent specificity exhibited by the assays with 15-month aged bloodstains (i.e., aged newborn bloodstains cluster separately from aged bloodstains from other developmental age groups), caution must be exercised in aged samples from older newborns. Although the 2-month-old newborn sample produced two positive dCt values when stored at room temperature up to 1 month with the ⩽4-month assay, it produced one positive and one negative dCt value for the S15–HBG2n and S15–HBG1n duplexes, respectively, with stains aged 3–15 months (Fig. 9A). In the <24-hour assay, the same 2-month-old produced one positive dCt value and one negative dCt value after 1, 3, 6, and 9 months of storage but produced two negative dCt values after 12 and 15 months of storage (Fig. 9B).

Conclusions

We have identified two novel gamma hemoglobin transcripts (HBG1n and HBG2n) that exhibit restricted expression in the blood of (human) newborn children. Individual qRT-PCR assays were developed to measure both of these

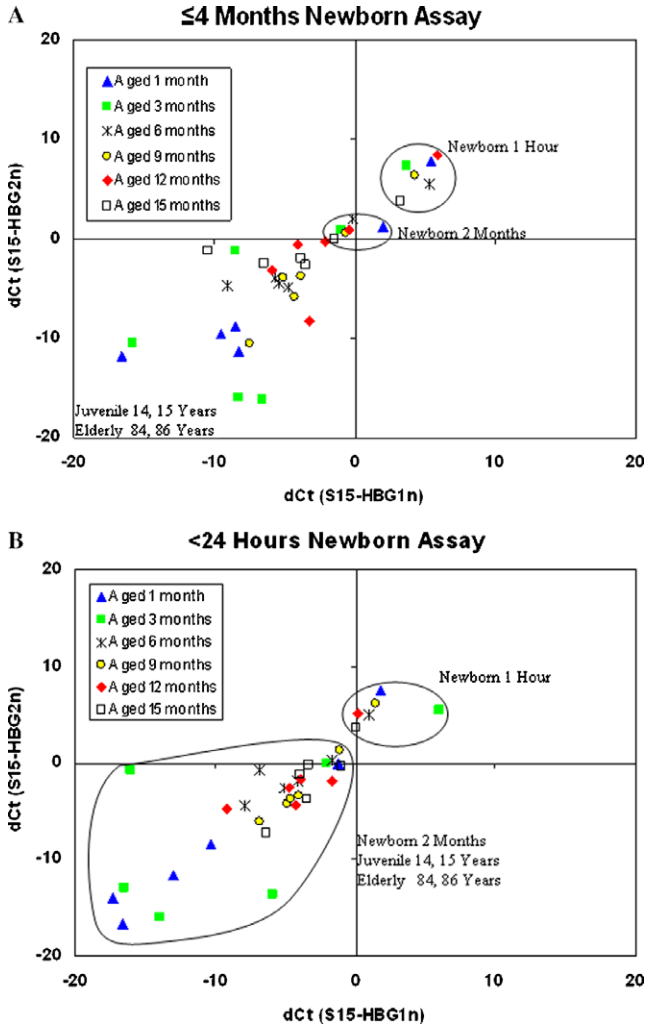

Fig. 9. Temporal stability of the HBG(1/2)n transcripts in bloodstains. Bloodstains (50 µl) from two newborns (1 hour and 2 months old), two juveniles (14 and 15 years old), and two elderly individuals (84 and 86 years old) were stored at room temperature (~25 °C) for 1, 3, 6, 9, 12, and 15 months. Total RNA was isolated from the bloodstains and then assayed for HBG1n and HBG2n transcripts by qRT-PCR. The results are displayed in a two-dimensional scatter plot similar to that shown in Fig. 7. (A) ⩽4-month assay; (B) <24-hour assay. Newborn samples and non-newborn samples are labeled.

transcripts in forensic specimens. Adjustment of the primer concentrations in the qRT-PCR permitted the establishment of two temporally delimited assays, one of which was specific to blood from newborns age 4 months or under (⩽4 months) and the other of which was specific to blood from newborns who were just hours old (<24 hours). Both assays may be useful in a variety of child kidnapping, assault, and criminal abortion investigations, with the latter assay (<24 hours) being of particular use for those cases involving hospital abductions.

A series of specificity performance checks carried out on the qRT-PCR assays revealed that the HBG(1/2)n transcripts appear to be restricted to blood from newborns in the human (or at least primate) lineage. The assays appear to be sensitive and robust enough for forensic use in that only a few cell equivalents of total RNA are required (i.e., 50 pg) and more than 100 ng of total RNA is recoverable from typical-sized (50-µl) bloodstains [36]. Thus, the sensitivity of the assay is 50 to 500 cells assuming 0.1–1.0 pg total RNA per cell [37–39]. The newborn blood-specific transcripts are detectable at least up to 15 months in the dried state.

A comprehensive developmental validation study carried out in accord with the guidelines established by the Scientific Working Group on DNA Analysis Methods, including an evaluation of environmentally compromised and degraded bloodstains, will be the subject of a separate report. The biological function of the novel newborn-specific transcripts, if any, is unknown. Future studies will include an evaluation of the rearrangement events that result in the formation of HBG(1/2)n transcripts (Fig. 3) as well as a determination of whether the gene transcripts produce protein products.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.06.024.

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