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Size Separation of Circulatory DNA in Maternal Plasma Permits Ready Detection of Fetal DNA Polymorphisms

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Background: **Analysis of fetal DNA in maternal plasma has recently been introduced as a new method for noninvasive prenatal diagnosis, particularly for the analysis of fetal genetic traits, which are absent from the maternal genome, e.g., RHD or Y-chromosome-specific sequences. To date, the analysis of other fetal genetic traits has been more problematic because of the overwhelming presence of maternal DNA sequences in the circulation. We examined whether different biochemical properties can be discerned between fetal and maternal circulatory DNA.**

Methods: **Plasma DNA was examined by agarose gel electrophoresis. The fractions of fetal and maternal DNA in size-fractionated fragments were assayed by real-time PCR. The determination of paternally and maternally inherited fetal genetic traits was examined by use of highly polymorphic chromosome-21-specific microsatellite markers.**

Results: **Size fractionation of circulatory DNA indicated that the major portion of cell-free fetal DNA had an approximate molecular size of <0.3 kb, whereas maternally derived sequences were, on average, considerably larger than 1 kb. Analysis of size-fractionated DNA (≤0.3 kb) from maternal plasma samples facilitated the ready detection of paternally and maternally inherited microsatellite markers.**

Conclusions: **Circulatory fetal DNA can be enriched by size selection of fragment sizes less than ~0.3kb. Such selection permits easier analysis of both paternally and maternally inherited DNA polymorphisms.**

Since its discovery in 1997 by Lo et al. *(1)*, circulatory fetal DNA in maternal plasma or serum has rapidly emerged as the prime focus for the development of risk-free methods for prenatal diagnosis of fetal genetic traits *(2, 3)*. Indeed, because of the relative abundance of this fetal genetic material, which is present in concentrations several orders of magnitude higher than those of trafficking fetal cells *(4)*, the determination of fetal genetic loci that are totally absent from the maternal genome is relatively easy. Consequently, the analysis of circulatory fetal DNA in maternal plasma is already being offered clinically by several centers to determine fetal RHD status in pregnancies with a Rhesus constellation or fetal sex by the detection of Y-chromosome-specific sequences in pregnancies at risk for an X-linked disorder (e.g., hemophilia, fragile X syndrome) *(3)*.

Quantitative analysis of this new-found fetal analyte by real-time PCR strategies has also indicated that the concentrations of circulatory fetal DNA are increased in a variety of pregnancy-related pathologies, including preeclampsia *(5, 6)*, preterm labor *(7, 8)*, and hyperemesis gravidarum *(8)*, and in pregnancies with fetal aneuploidies, most notably trisomy 21 *(9, 10)*. These studies have suggested that fetal DNA concentrations may serve as a new screening marker for such pregnancy-related anomalies *(11)*.

A caveat of current investigations is that the overwhelming amount of circulatory DNA in the maternal circulation is of maternal origin (>90%) *(4)*, which has rendered the differentiation of more subtle genetic differences between mother and child considerably more difficult *(2, 3)*. This is generally true for Mendelian genetic disorders involving point mutations *(12)* or those instances where both parents are carriers for the same

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disease allele, as well as for the examination of DNA polymorphisms *(13, 14)* that could be used for the determination of fetal ploidy *(15)*. Consequently, few reports exist regarding the successful use of circulatory fetal DNA for such applications *(12, 16–18)*.

To date, few studies have addressed the biochemical properties of circulatory fetal DNA *(19, 20)*. In the most recent of these, Chan et al. *(20)* used differently sized PCR amplicons to discern the respective size distributions of circulatory fetal and maternal DNA species; their results indicated that fetal DNA molecules are generally smaller than comparable maternal ones. Because this approach permitted a precise delineation of only rather small DNA species (<800 bp), we have examined this aspect, using a combination of agarose gel electrophoresis, Southern blotting, and real-time PCR, because this would permit an analysis of large DNA molecules (>10 to 20 kb). Our studies have shown that circulatory DNA has apoptotic characteristics, displaying a typical ladder obtained by nucleosomal cleavage. We also observed that circulatory fetal DNA is generally of a smaller size than maternally derived cell-free DNA fragments, in good agreement with recent observations *(20)*. By exploiting this observation, we have shown that even a simple strategy, such as size separation using conventional agarose gel electrophoresis and subsequent PCR analysis *(21)*, can lead to the selective enrichment of circulatory fetal DNA sequences. These in turn can be used for the determination of DNA polymorphisms that are masked by maternal sequences in the native plasma sample.

Materials and Methods

SOUTHERN BLOT ANALYSIS

Plasma sample collection. After receiving approval by the Cantonal Institutional Review Board of Basel, Switzerland, we obtained 18 mL of peripheral blood from pregnant women who had given written informed consent. EDTA (Movovette tubes; Sarstedt) was used as anticoagulant. The blood samples were first cleared by centrifugation at 1600*g* for 10 min, after which the plasma was subjected to a second centrifugation step at 16 000*g* for 10 min. Plasma was immediately used for DNA extraction in each analysis.

Southern hybridization. Circulatory DNA was extracted from ~7–10 mL of maternal plasma (gestational age, 11–17 weeks) and 18 mL of cord blood plasma by a conventional phenol–chloroform procedure, with a slight modification in that the plasma sample was first treated with a chaotropic guanidinium isothiocyanate solution to denature any nucleases (Qiagen) *(22)*. As a control, we used plasma from nonpregnant women.

The extracted DNA was separated on a 1.0% agarose gel. A 100-bp ladder and *Hin*dIII-digested Lambda phage DNA were used to estimate molecular size (New England Biolabs). The DNA was transferred to nylon membranes (Roche) with 20× standard saline citrate by a standard capillary transfer method *(22)*.

Transferred DNA was detected with the Roche® DIG labeling and detection system, according to the manufacturer's instructions (Roche) *(23)*. The highly repetitive Alu sequence was used as a hybridization probe and was directly digoxigenin-labeled by a PCR process using the PCR DIG Probe Synthesis Kit (Roche). The primer for the Alu sequence was as follows: 5′-ATC TCG GCT CAC TGC AA-3′. Prehybridization was carried out at 42 °C in DIG Easy Hyb solution (Roche). Hybridization was performed at 42 °C overnight, after which the membrane was washed at high stringency and incubated with the chemiluminescent alkaline phosphatase substrate (CSPD) according to the manufacturer's instructions. The resulting blot was exposed on x-ray film.

DETERMINATION OF SIZE DISTRIBUTION OF CIRCULATORY FETAL-DERIVED DNA IN MATERNAL PLASMA

Preparation of circulatory DNA. Plasma samples were prepared as described above. Peripheral blood was collected from pregnant women carrying a singleton male fetus. Six samples were obtained early in pregnancy (median gestational age, 13 + 2 weeks), and eight samples were collected in the third trimester (median gestational age, 34 + 4 weeks). Blood from three nonpregnant women and three healthy males was used as controls. Routinely, 5–7 mL of plasma was used for DNA extraction, which was performed with a combination of the Roche High Pure Template DNA Purification Kit (Roche) and a custom-made vacuum pump for the isolation of circulatory plasma DNA. In brief, as described in the manufacturer's instructions, the plasma sample was incubated with binding buffer and proteinase K at 70 °C for 10 min, after which the required volume of isopropanol was added and the sample was passed through the Roche column under reduced pressure. The column was then washed with inhibitor removal buffer and twice with wash buffer, respectively, as recommended by the manufacturer. The column-bound circulatory DNA was eluted in 40 μL of elution buffer.

Gel electrophoresis and isolation of circulatory DNA fragments. The total circulatory DNA was subjected to agarose gel electrophoresis on a 1.0% agarose gel (Invitrogen) containing 0.5 mg/L ethidium bromide (Sigma). Size markers were a 100-bp ladder and *Hin*dIII-digested Lambda phage DNA (New England Biolabs). Electrophoresis was carried out at 80 V for 1 h. Each lane of the gel containing circulatory DNA was then cut with a sterile scalpel blade into six discrete sections, with the molecular weight markers used as a guide. The sizes of the fragments in the sections, based on the molecular weight markers, were 0.09–0.3, 0.3–0.5, 0.5–1.0, 1.0–1.5, 1.5–23, and >23 kb. Because the location of the 23-kb marker is imprecise in 1% gels, we used it only as a rough guide to estimate the

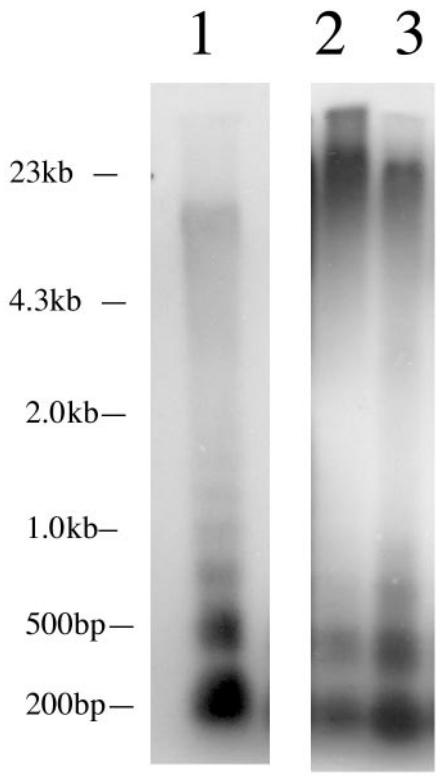

Fig. 1. Southern blot analysis of plasma circulatory DNA, using a highly repetitive Alu probe.

Lane 1, plasma from cord blood; *lane 2*, maternal plasma (13 weeks of gestation); *lane 3*, plasma from nonpregnant woman.

size of DNA fragments larger than 10 kb. The circulatory DNA was extracted from the agarose sections with a QIAEX®II Gel Extraction Kit (Qiagen) and eluted in 40 μL of sterile 10 mmol/L Tris-HCl (pH 8.0). Procedures to prevent contamination were used during these experiments, including ultraviolet irradiation of the gel tray and tank, use of fresh buffers with each electrophoretic run, use of plasma samples from women carrying female fetuses (n = 2), and examination of blank gel slices (n = 14) in parallel with each analysis. In no instance were any false-positive results recorded.

Determination of the proportion of circulatory fetal and total DNA. The relative proportions of fetal and total circulatory DNA eluted from the individual agarose gel sections were determined by use of a well-established TaqMan® real-time PCR assay for the *SRY* gene on the Y chromosome and the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene *(6)*. The only modifications were that a new-generation Perkin-Elmer Applied Biosystems 7000 Sequence Detector was used and that minor groove binding probes were used instead of the previous 6-carboxytetramethylrhodamine-conjugated probes (Applied Biosystems). The PCR reactions were carried out in a final reaction volume of 25 μL, which consisted of 6 μL of eluted DNA, 300 nM each primer, 150 nM each probe, and 2× TaqMan Universal PCR Master Mix (Applied Biosystems). PCR was carried out with an initial incubation at 50 °C for 2 min to activate uracil-N-glycosylase, followed by incubation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. This analysis permitted determination of the total amount of DNA present in each fraction, by use of the *GAPDH*-specific assay, and the fraction that was fetal, by use of the *SRY*-specific assay. The relative proportions of each were expressed as percentages.

DETECTION OF HIGHLY POLYMORPHIC MICROSATELLITE MARKERS

Sample preparation. We collected 18 mL of maternal blood from three third-trimester pregnancies and collected cord blood subsequent to delivery. After plasma separation by high-speed centrifugation, the buffy coat was collected, washed with phosphate-buffered saline, and used for the preparation of maternal genomic DNA. Fetal genomic DNA was prepared similarly from the cord blood sample. To verify that this same approach can be used to examine clinically relevant samples, we examined four samples taken early in pregnancy (median gestational age, 13 + 5 weeks). In this instance the fetal genotype was determined from archived amniocyte or chorionic villus cultures. One of these samples was obtained from a trisomy 21 fetus.

Fluorescent PCR analysis of highly polymorphic microsatellite markers. We used the same standardized fluorescent PCR assay for highly polymorphic short tandem repeat (STR)

Table 1. Size distribution of total and fetal circulatory DNA in third-trimester maternal plasma samples.[a]

	Median (range)		
Size of DNA fraction, kb	Size distribution of total DNA,[b] %	Size distribution of fetal DNA,[c] %	Proportion of fetal DNA per fraction,[d] %
<0.3	22.4 (15.7–26.7)	70.0 (51.0–82.3)	68.7 (22.2–87.1)
0.3–0.5	28.4 (15.7–35.2)	24.3 (13.8–31.6)	15.4 (6.4–31.4)
0.5–1.0	23.0 (15.0–26.8)	3.8 (0.0–17.4)	2.6 (0.0–7.8)
1.0–1.5	7.5 (2.2–11.4)	0.0 (0.0–8.7)	0.0
1.5–23	21.1 (10.3–35.7)	0.0	0.0

[a] Six samples were analyzed in this study. Median gestational age was 34 + 4 weeks.

[b] Size distribution of total circulatory DNA was determined by a real-time PCR assay for the *GAPDH* gene. The values are indicative of the percentage of total DNA in each fraction examined.

[c] Size distribution of circulatory fetal DNA as determined by a real-time PCR assay for the *SRY* gene. These values are indicative of the percentage of fetal DNA with regard to the total amount of fetal DNA in each fraction examined.

[d] The proportion of fetal DNA indicates the percentage of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction.

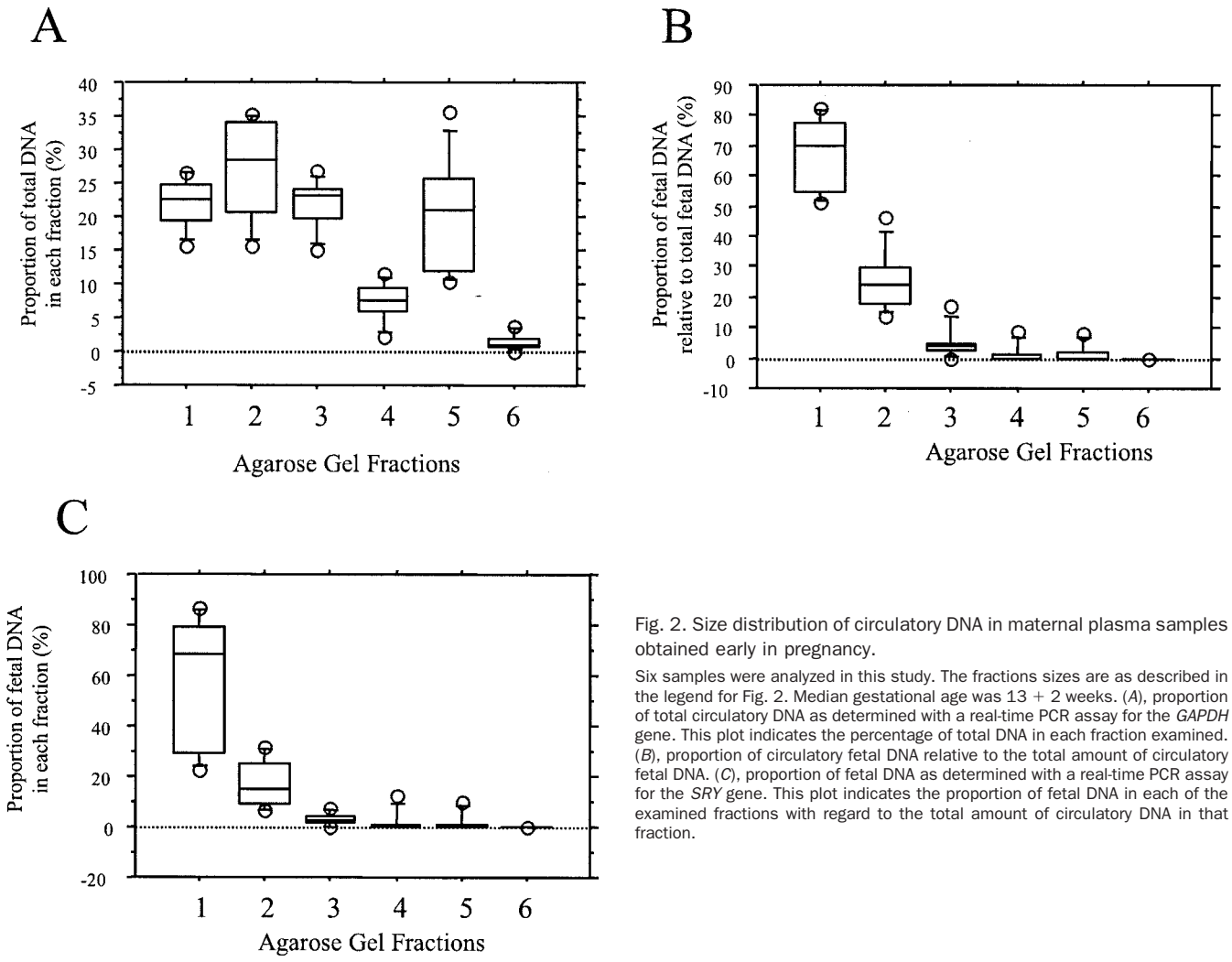

Fig. 2. Size distribution of circulatory DNA in maternal plasma samples obtained early in pregnancy.

Six samples were analyzed in this study. The fractions sizes are as described in the legend for Fig. 2. Median gestational age was 13 + 2 weeks. (*A*), proportion of total circulatory DNA as determined with a real-time PCR assay for the *GAPDH* gene. This plot indicates the percentage of total DNA in each fraction examined. (*B*), proportion of circulatory fetal DNA relative to the total amount of circulatory fetal DNA. (*C*), proportion of fetal DNA as determined with a real-time PCR assay for the *SRY* gene. This plot indicates the proportion of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction.

markers on chromosome 21 that we used previously for our analysis of urinary DNA *(24)*. The fetal and maternal genomic DNA sources were used to determine which STR markers on chromosome 21 were informative. These informative STR markers were then used for the detection of paternally and maternally inherited fetal alleles in total maternal plasma circulatory DNA as well as in size-separated circulatory DNA fractions. The low-molecular-weight circulatory DNA fraction was prepared as described above.

Because the concentration of circulatory DNA after size fractionation was very low, we used a seminested PCR assay as described previously *(24)*. The PCR products were analyzed by capillary electrophoresis on an ABI 310 gene analyzer (Applied Biosystems). For samples taken early in pregnancy, in which the concentration of circulatory fetal DNA has been shown to be lower than at term, our investigation showed that the PCR procedure had to be modified to obtain optimum results. For this reason,

Table 2. Size distribution of total and fetal circulatory DNA in maternal plasma samples obtained early in pregnancy.[a]

	Median (range)		
Size of DNA fraction, kb	**Size distribution of total DNA, %**	**Size distribution of fetal DNA, %**	**Proportion of fetal DNA per fraction, %**
<0.3	26.9 (12.7–41.3)	85.5 (67.8–100.0)	28.4 (11.6–56.6)
0.3–0.5	29.1 (26.1–54.4)	11.7 (0.0–15.5)	4.0 (0.0–13.5)
0.5–1.0	28.5 (14.5–25.9)	1.2 (0.0–16.8)	0.4 (0.0–5.2)
1.0–1.5	8.2 (4.5–12.1)	0.0	0.0
1.5–23	8.6 (7.6–23.7)	0.0	0.0

[a] Eight samples were analyzed in this study. Median gestational age was 13 + 2 weeks. Size distributions of total circulatory DNA, circulatory fetal DNA, and the proportion of fetal DNA were determined as described in the footnotes for Table 1.

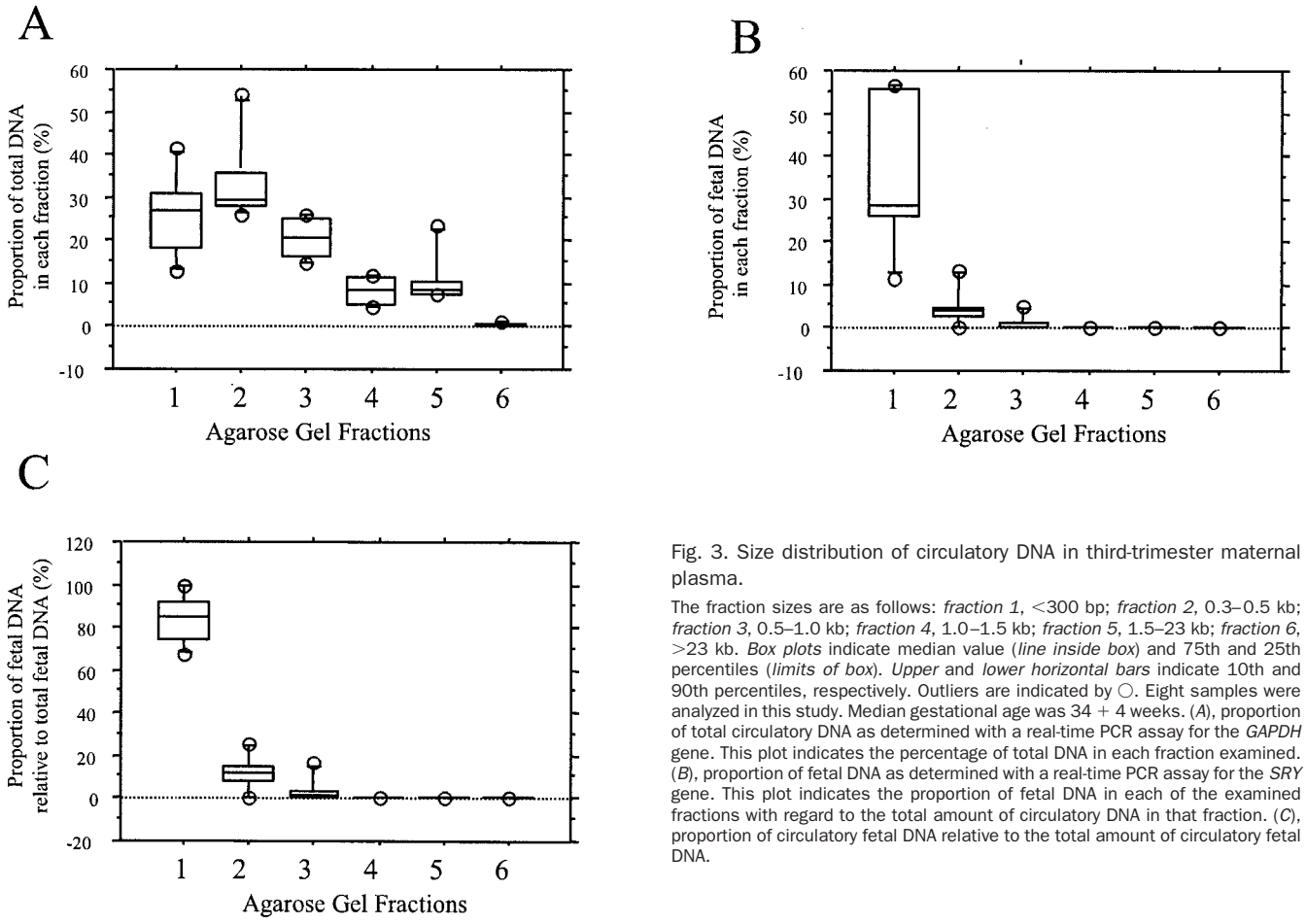

Fig. 3. Size distribution of circulatory DNA in third-trimester maternal plasma.

The fraction sizes are as follows: *fraction 1*, <300 bp; *fraction 2*, 0.3–0.5 kb; *fraction 3*, 0.5–1.0 kb; *fraction 4*, 1.0–1.5 kb; *fraction 5*, 1.5–23 kb; *fraction 6*, >23 kb. *Box plots* indicate median value (*line inside box*) and 75th and 25th percentiles (*limits of box*). *Upper* and *lower horizontal bars* indicate 10th and 90th percentiles, respectively. Outliers are indicated by ○. Eight samples were analyzed in this study. Median gestational age was 34 + 4 weeks. (*A*), proportion of total circulatory DNA as determined with a real-time PCR assay for the *GAPDH* gene. This plot indicates the percentage of total DNA in each fraction examined. (*B*), proportion of fetal DNA as determined with a real-time PCR assay for the *SRY* gene. This plot indicates the proportion of fetal DNA in each of the examined fractions with regard to the total amount of circulatory DNA in that fraction. (*C*), proportion of circulatory fetal DNA relative to the total amount of circulatory fetal DNA.

we used a "touch-down" PCR method instead of our conventional seminested PCR method described above. In this procedure, a total of 50 cycles were run, using PCR reactions containing 3 μL of 10× buffer, 3.5 mM $MgCl_2$, 160 μM deoxynucleotide triphosphates, 0.1 μM each of forward and reverse primers *(24)* (one primer of each set was fluorescent labeled), 1U of AmpliTaq Gold polymerase, and 3 μL of DNA in a final volume of 30 μL. After incubation at 95 °C for 10 min, 10 cycles (95 °C for 15 s, 65 °C for 15 s, 72 °C for 30 s) of thermal cycling were carried out in which the annealing temperature was decreased by 1 °C/cycle. This was then followed by 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 30 s. The PCR was terminated after an extension phase at 72 °C for 7 min. As described above, the PCR products were analyzed by capillary electrophoresis on an ABI 310 gene analyzer *(24)*.

Results

Circulatory DNA has been proposed to exhibit apoptotic hallmarks such as oligonucleosomal laddering and nucleosome association *(25, 26)*. In our initial investigation, we attempted to determine whether circulatory DNA in pregnant women also displayed such characteristics. For this examination we used Southern blot analysis of total circulatory plasma DNA that had been subjected to agarose gel electrophoresis. The analysis of such blots with the ubiquitous highly repetitive Alu sequence indicated that oligonucleosomal fragments could indeed be detected and were present in all three of the plasma sources examined: maternal blood, nonpregnant female control blood, and cord blood (Fig. 1). This examination also indicated that a substantial proportion of the circulatory DNA had a molecular size >10 or even >23 kb (Fig. 1). The presence of such high-molecular-weight DNA species cannot be attributed to the plasma sample being contaminated by maternal cells because we took extreme care to obtain cell-free plasma samples. It is of interest that these high-molecular-weight DNA molecules are quite similar to the very large ones we observed in terminally differentiated erythroblasts before enucleation (S. Hristoskova et al., manuscript in preparation).

Unfortunately, we were not able to determine the characteristics of circulatory fetal DNA in the samples obtained from pregnant women because the concentrations of fetal DNA were too low to be detectable even when we used a very high copy probe (DYS14) specific for the Y chromosome.

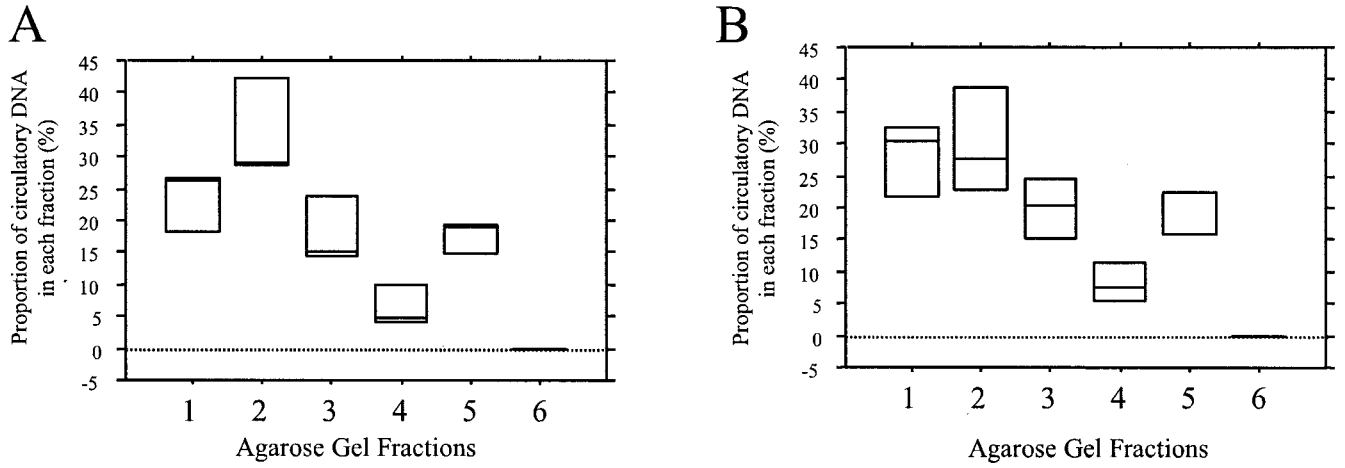

Fig. 4. Size distribution of circulatory DNA in plasma samples from healthy nonpregnant women or healthy males.

Three samples per group were analyzed in this study. The fractions sizes are as described in the legend for Fig. 2. (*A*), proportion of total circulatory DNA in each fraction examined in samples obtained from nonpregnant women. (*B*), proportion of total circulatory DNA in each fraction examined in samples obtained from healthy male volunteers.

Because we were, however, able to detect circulatory apoptotic DNA fragments in our Southern blot analysis, we used an alternative strategy to determine whether circulatory fetal DNA displayed a similar pattern. For this analysis, we used an approach that had previously been used successfully for the characterization of rare linear extrachromosomal DNA species *(21)*. In this procedure, the circulatory DNA was first subjected to agarose gel electrophoresis, after which individual gel fragments containing the size-fractionated DNA were examined by PCR. For our examination, after electrophoresis the agarose gel was cut into six discrete sections containing fragments with approximate sizes of <0.3, 0.3–0.5, 0.5–1.0, 1.0–1.5, 1.5–23.0, and >23 kb. Once the circulatory DNA was extracted from these gel fragments, the proportions of fetal and maternal DNA in these fractions were then determined by well-established real-time PCR assays for the *SRY* locus on the Y chromosome and the ubiquitous *GAPDH* gene *(6)*. To ensure that we were not being misled by any PCR artifacts, we included several procedures to prevent contamination in our study, including use of plasma samples from women pregnant with female fetuses (n = 2) and the parallel examination of blank gel slices in each analysis (n = 14). No false-positive results were recorded in any of these instances.

Our examination of plasma samples from third-trimester pregnancies with a male fetus indicated that the vast proportion of circulatory fetal DNA, as detected by the *SRY*-specific PCR assay, had an approximate molecular size <300 bp (Table 1 and Fig. 2), with very little or no

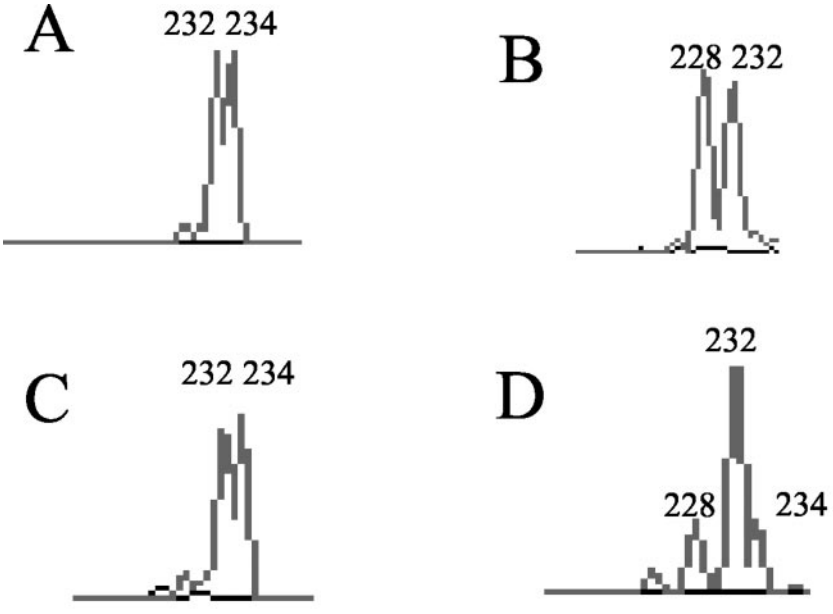

Fig. 5. Detection of both paternally and maternally inherited highly polymorphic microsatellite markers in size-fractionated circulatory DNA.

In this analysis a maternal plasma taken near term was used. Shown are capillary electropherograms of D21S11 alleles. (*A*), maternal genomic DNA. Two maternal alleles with sizes of 232 and 234 bp were detected. (*B*), fetal genomic DNA. The paternally inherited allele with a size of 228 bp and the maternally inherited allele with a size of 232 bp were detected. (*C*), total circulatory DNA. The predominant alleles detected were those of the mother (D21S11 alleles with sizes of 232 and 234 bp). (*D*), size-fractionated circulatory DNA with a size <300 bp. Three D21S11 alleles with sizes of 228, 232, and 234 bp were detected.

fetal DNA having a molecular size >1 kb. On the other hand, maternally derived sequences, as determined by the *GAPDH*-specific PCR assay, were estimated to be larger than 0.5–1.0 kb and included molecular-weight species larger than 10–20 kb. A similar pattern for both fetal and maternal circulatory DNA was also observed in plasma samples obtained early in the second trimester of pregnancy (Table 2 and Fig. 3).

With regard to the size distribution of total circulatory DNA, we determined that the pattern we had observed in pregnant women was very similar to that observed in samples taken from nonpregnant women as well as healthy male volunteers (Fig. 4). In none of these analyses were we able to detect large amounts of DNA with a molecular size greater than that indicated by the 23-kb molecular weight marker, in contrast to what we observed in our Southern blot analysis (Fig. 1). The reason for this anomaly may be that these large fragments are not easily eluted from the agarose gel under the conditions we are using, unlike in the Southern blotting, where the DNA is first treated with alkali to generate the small fragments required for efficient capillary transfer.

Our data also indicated that selective enrichment of circulatory fetal DNA sequences may be possible by examination of DNA fragments with a size less than ~300 bp. Our next step, therefore, was to determine whether such size-dependent separation would facilitate the determination of more subtle fetal genetic traits.

For this purpose we examined whether both paternally and maternally inherited DNA polymorphisms could be discerned from such size-fractionated circulatory DNA. For our analysis we used highly polymorphic STR sequences on chromosome 21, which have previously been demonstrated to be suitable for the reliable distinction of mother and child *(24)*. To test the feasibility of this approach, we first examined samples taken close to term because they would contain the maximum concentrations of circulatory fetal DNA and because it was possible to obtain the fetal genotype, a prerequisite for such studies, from a cord blood sample after birth. In this manner, we could select easily discernible polymorphic markers between mother (Fig. 5A) and fetus (Fig. 5B).

In this analysis, the benefits of selectively enriching for circulatory fetal DNA species became readily apparent in that the paternally inherited STR allele (with a size of 228 bp) was barely detectable in the total plasma extracted DNA (Fig. 5C) but was clearly present in the DNA fraction with a size <300 bp (Fig. 5D). Furthermore, the method could not differentiate the maternally inherited STR allele (with a size of 232 bp) from the predominantly maternal pattern obtained from the analysis of total plasma extracted DNA (Fig. 5C). This fetal allele, however, was detected in the analysis of the DNA fraction with a size <300 bp (Fig. 5D) because the peak for that STR allele had a much larger area than either the paternally inherited fetal allele (228 bp) or the solitary maternal allele (234 bp). This indicates that both fetal and maternal loci are contributing to the presence of this particular PCR product. Similar results were obtained in the analysis of the DNA fraction with a size of 300–500 bp, although the results in this instance were less evident than those obtained with the smaller DNA fraction (<300 bp; data not shown). The reproducibility of this approach was verified in the analysis of two additional samples, which were analyzed at a several different polymorphic loci; we obtained similar results for these samples (Table 3).

Table 3. Detection of paternally and maternally inherited fetal highly polymorphic microsatellite markers in size-fractionated circulatory DNA obtained from maternal plasma samples taken close to term.[a]

Case no. (D21 locus)	Method of sample preparation[b]	Maternal alleles detected	Fetal alleles detected
1 (D21S11)	Maternal genomic DNA	232/234	
	Fetal genomic DNA		228/232
	Total plasma DNA	232/234	Not detectable
	Plasma DNA <300 bp	232/234	228/232
1 (D21S1435)	Maternal genomic DNA	172/180	
	Fetal genomic DNA		172/176
	Total plasma DNA	172/180	176[c]
	Plasma DNA <300 bp	172/180	172/176
2 (D21S1270)	Maternal genomic DNA	184/188	
	Fetal genomic DNA		180/184
	Total plasma DNA	184/188	Not detectable
	Plasma DNA <300 bp	184/188	180/184
2 (D21S1432)	Maternal genomic DNA	138/152	
	Fetal genomic DNA		134/138
	Total plasma DNA	138/152	134[c]
	Plasma DNA <300 bp	138/152	134/138
3 (D21S1435)	Maternal genomic DNA	168/176	
	Fetal genomic DNA		168/172
	Total plasma DNA	168/176	172[c]
	Plasma DNA <300 bp	168/176	168/172

[a] Three samples were analyzed in this study.

[b] Genomic DNA was prepared directly from maternal or fetal lymphocytes. "Total plasma DNA" indicates analysis of circulatory DNA extracted from an nonfractionated DNA samples, whereas "Plasma DNA <300 bp" indicates analysis of a discrete fraction of circulatory DNA that had been size-fractionated by gel electrophoresis.

[c] Minimal detection of paternally inherited allele.

To determine whether this approach could also be applied to clinically relevant samples, we examined four samples taken early in the second trimester of pregnancy. In these analyses the fetal genotype was determined from archived amniocyte or chorionic villus cultures. One of these samples was from a fetus affected by Down syndrome (trisomy 21). This was evident from our microsatellite analysis for the D21S1432 marker, in which three equivalent peaks with sizes of 133, 137, and 141 bp, respectively, were detected (Fig. 6B), implying that the fetus had inherited a copy of each of the two maternal chromosomes 21 in addition to the paternally inherited chromosome 21. In our analysis of the total circulatory cell-free DNA in the maternal plasma sample, only the

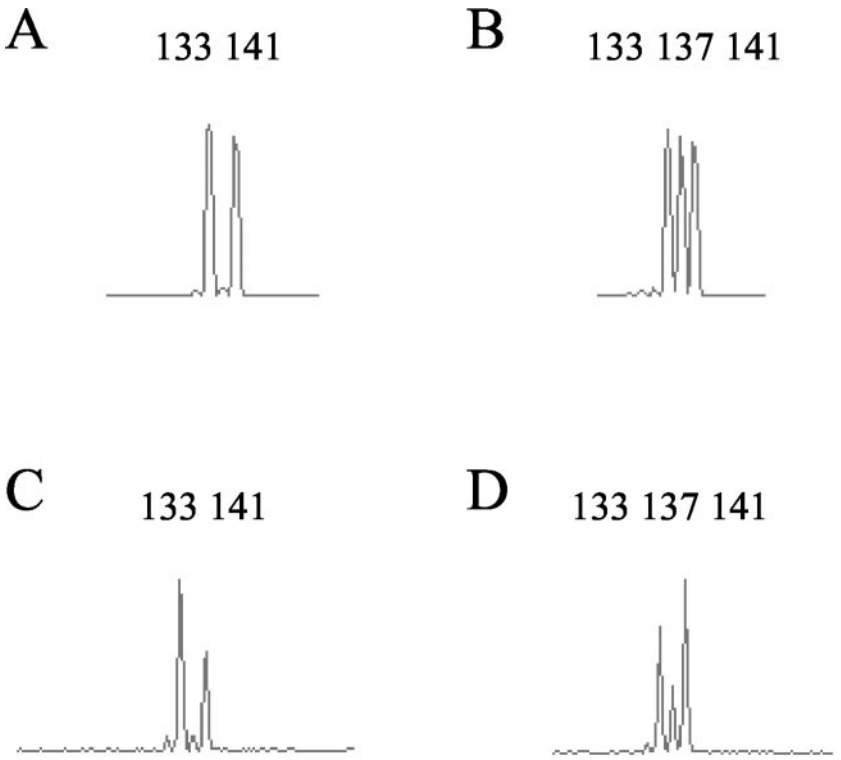

Fig. 6. Analysis of highly polymorphic microsatellite markers in the plasma of a mother carrying a fetus with trisomy 21.

In this analysis we used an early second-trimester maternal plasma sample. Shown are capillary electropherograms of D21S1432 alleles. (*A*), maternal genomic DNA. Two maternal alleles with sizes of 133 and 141 bp were detected. (*B*), fetal genomic DNA. The paternally inherited allele (137 bp) and both of the inherited maternal alleles (133 and 141 bp) were detected. (*C*), total circulatory DNA. The predominant alleles detected were those of the mother (D21S1432 alleles; 133 and 141 bp). (*D*), size-fractionated circulatory DNA with a size <300 bp. The paternal D21S1432 allele, with a size of 137 bp, was readily detected.

two maternal markers were readily detected (133 and 141 bp; Fig. 6C), whereas our analysis of size-fractionated DNA with an approximate size of <300 bp facilitated the ready detection of the paternally inherited 137-bp marker (Fig. 6D). We observed a similar feature when we examined this sample for a different microsatellite marker, D21S1270 (Table 4). In this instance, it was not possible to determine whether the maternally inherited polymorphisms could be detected because the fetus had the same pattern as the mother for both loci. In the three other cases, which had a normal karyotype, we were in all three cases readily able to detect the paternally inherited polymorphic locus in the size-fractionated DNA sample (Table 4). However, in only one instance could we discern the presence of the maternally inherited locus (Table 4, case 3). In case 2, this was not possible because the mother was homozygous for the locus interrogated, whereas in the other cases [case 2 (D21S1435) and case 4 (D21S1440)], the maternal locus that had not been inherited by the fetus appeared to have been preferentially amplified (Table 4).

Discussion

Our investigation supports the current hypothesis that circulatory DNA has apoptotic attributes *(25, 26)* in that we could readily discern oligonucleosomally cleaved fragments by Southern blot analysis. Our analysis also indicated that a substantial proportion of the circulatory DNA has a very large molecular size (>20 kb). Independent investigations in our laboratory indicated that these large circulatory DNA species may be derived from the erythropoietic system, in that DNA isolated from terminally differentiating erythroblasts exhibited similar characteristics (S. Hristoskova et al., manuscript in preparation). It is currently unclear whether these large DNA molecules are subsequently cleaved into smaller oligonucleosomal fragments in the maternal plasma or whether the smaller fragments we detected are derived from another source.

Using an approach that had previously been used to examine rare linear extrachromosomal DNA species *(21)*, in which DNA size-fractionated by gel electrophoresis was subsequently extracted and analyzed by PCR, we made the surprising finding that a large discrepancy existed in the size of circulatory fetal and maternal DNA species. In this regard our study indicated that fetal DNA molecules predominantly have an approximate size ≤300 bp, whereas most maternally derived DNA molecules are considerably larger than this. The fact that no large circulatory fetal DNA species were detected (i.e., >20 kb) implies that the mechanism contributing to the formation of the large maternally derived DNA species is not involved in the liberation of circulatory fetal DNA. The explanation for this difference could be that circulatory fetal DNA appears to be exclusively derived from the placenta *(27)*, whereas the vast proportion of normal maternal circulatory DNA is of hemopoietic origin *(28)*.

With regard to the size distribution of circulatory maternal and fetal DNA species, our results are remarkably similar to those reported recently by Chan et al. *(20)*, who also observed that fetal cell DNA molecules were

Table 4. Detection of paternally and maternally inherited fetal highly polymorphic microsatellite markers in size-fractionated circulatory DNA obtained from maternal plasma samples obtained early in the second trimester.[a]

Case no. (D21 locus)	Method of sample preparation[b]	Maternal alleles detected	Fetal alleles detected
1 (D21S1270)	Maternal genomic DNA	182/191	
	Fetal genomic DNA		182/187/191
	Total plasma DNA	182/191	Not detectable
	Plasma DNA <300 bp	182/191	187
1 (D21S1432)	Maternal genomic DNA	133/141	
	Fetal genomic DNA		133/137/141
	Total plasma DNA	133/141	Not detectable
	Plasma DNA <300 bp	133/141	137
2 (D21S1440)	Maternal genomic DNA	154	
	Fetal genomic DNA		154/157
	Total plasma DNA	154	Not detectable
	Plasma DNA <300 bp	154	157
2 (D21S1435)	Maternal genomic DNA	141/172	
	Fetal genomic DNA		172/176
	Total plasma DNA	141/172	Not detectable
	Plasma DNA <300 bp	141/172	176
3 (D21S1440)	Maternal genomic DNA	157/160	
	Fetal genomic DNA		154/157
	Total plasma DNA	157/160	Not detectable
	Plasma DNA <300 bp	157/160	154/157
4 (D21S1440)	Maternal genomic DNA	154/157	
	Fetal genomic DNA		157/160
	Total plasma DNA	154/157	Not detectable
	Plasma DNA <300 bp	154/157	160

[a] Four samples were analyzed in this study.

[b] Genomic DNA was prepared directly from maternal or fetal lymphocytes. "Total plasma DNA" indicates analysis of circulatory DNA extracted from an nonfractionated DNA samples, whereas "Plasma DNA <300 bp" indicates analysis of a discrete fraction of circulatory DNA that had been size-fractionated by gel electrophoresis.

generally smaller than those of maternal origin. For their study, they made use of differently sized PCR amplicons ranging in size from 105 to 798 bp to determine the size of predominantly maternally derived DNA molecules, and amplicons of 107–524 bp to examine circulatory fetal DNA molecules. Although their approach permitted a much more precise delineation of the size distribution of circulatory DNA molecules within this given range, they were not able to demonstrate the existence of very large (>10 to 20 kb) maternal circulatory DNA species, as our analysis could.

Another important point that the study of Chan et al. *(20)* did not address, and which has been the major focus of our study, is that this observation permits the development of a strategy facilitating the selective enrichment of circulatory fetal DNA sequences. The exploitation of this approach in turn permits the determination of highly polymorphic fetal genetic traits not discernible from the analysis of total extracted plasma circulatory DNA. In this manner we were able to detect the presence of both paternally and maternally inherited STR markers in size-separated circulatory DNA fractions, which was not possible when the same analysis was attempted on unfractionated samples *(13, 14)*. Our study does, however, indicate that the method we have chosen for this proof-of-concept study is too imprecise and inefficient to be used for potential clinical applications. This became clear in our analysis of paternally and maternally inherited polymorphic markers; we could not determine the precise proportion that the fetal markers contribute to the analyzed pattern. This feature is very important because numerous clinical studies have clearly shown that the analysis of such highly polymorphic STR markers can be very useful for the determination of fetal ploidy *(15)*. Hence, if our approach using size fractionation were optimal, it should be feasible to determine fetal chromosomal anomalies directly from maternal plasma. Because in our study we were not able to determine fetal ploidy from the size-fractionated DNA sample for the one case with a trisomy 21 fetus (Fig. 6 and Table 4), we believe that this application will have to await developments that permit better separation of maternal and fetal DNA species. It is, however, possible that even in its current form, our approach could be used for the noninvasive determination of paternity.

Another important aspect of our observation is that it may in future aid in the examination of Mendelian disorders, particularly those involving point mutations, because these analyses should no longer be hindered by the large excess of maternal DNA sequences in the circulation *(2, 3)*. Indeed, the use of quantitative assays may make it possible to determine the fetal genotype in those instances where both partners are carriers for the same disease allele.

In summary, our findings showed that circulatory fetal DNA molecules are generally of a smaller size than comparable maternally derived sequences and that selective enrichment of fetal DNA sequences can be achieved by size-dependent separation. This latter feature permits the detection of fetal genetic traits not detectable in total plasma circulatory DNA. For clinical applications, more efficacious separation modes will need to be developed that facilitate better differentiation and recovery of fetal and maternal circulatory DNA species.

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