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Low Levels of β Hexosaminidase A in Healthy Individuals with Apparent Deficiency of This Enzyme

R. NAVON,[1] B. GEIGER,[2] Y. BEN YOSEPH,[2] AND M. C. RATTAZZI[3]

INTRODUCTION

The association between Tay-Sachs disease (TSD; G_{M2} gangliosidosis type I) and generalized deficiency of β hexosaminidase A (hex A) activity with artificial substrates, first reported by Okada and O'Brien [1], is now well established. Using natural substrates, it has also been shown that tissues from patients with TSD cannot degrade G_{M2} ganglioside in vitro [2, 3] and that purified hex A, but apparently not purified β hexosaminidase B (hex B), can hydrolyze G_{M2} ganglioside to a measurable extent [3, 4]. Thus, hex A deficiency is commonly believed to be the cause of the accumulation of G_{M2} ganglioside in patients with TSD. However, it has recently been reported that purified hex B can cleave G_{M2} ganglioside in vitro [5], raising the question of its participation in the catabolism of this glycolipid in vivo.

In a recent publication [6], Navon et al. described a Jewish family in which four healthy adult individuals showed a severe deficiency of hex A activity. Using a heat inactivation method based on the differential thermal lability of hex A and hex B [7, 8], it was shown that, in these subjects, hex A activity with artificial substrates was comparable to that of patients with TSD [6]. Further studies employing radioactive natural substrates, however, showed that leukocytes from these "variant" individuals were capable of hydrolysis of G_{M2} ganglioside in vitro [9]. This activity was about 50% of normal, comparable to that of leukocytes from heterozygotes for the TSD gene [9]. These data suggested that low levels of hex A activity in leukocytes from variant individuals not detected by the indirect thermal inactivation method were responsible for the observed G_{M2} ganglioside hydrolysis. If this were true for other tissues, this could account for the absence of clinical signs

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[1] Department of Human Genetics, Tel Aviv University Medical School, Sheba Medical Center, Tel Hashomer, Israel.

[2] Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel.

[3] Division of Human Genetics, Department of Pediatrics, Children's Hospital, State University of New York at Buffalo, Buffalo, New York.

of G_{M2} gangliosidosis in these subjects [6]. Using more sensitive and direct electrophoretic and immunological assay systems, we have been able to demonstrate the presence of low but significant levels of hex A activity with artificial substrates in fibroblasts and melanoma cells from variant individuals, as opposed to total hex A deficiency in patients with TSD. The observed hex A activity with artificial substrates, however, in these preparations is much lower than that in fibroblasts from heterozygotes for the TSD gene; this does not correlate well with G_{M2} ganglioside-cleaving activity reported in leukocytes from these same variant individuals [9].

MATERIALS AND METHODS

Enzymatic Assay

The activity of hexosaminidases was determined with the substrate 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (Pierce, Rockland, Ill.), as previously described [8]. Heat inactivation assay was carried out at 50°C for 3 hr [8].

Tissue Samples

Liver slices from normal individuals and patients with TSD were obtained at autopsy. Melanoma tumor tissue was obtained from one of the variant individuals (variant H) and an unrelated normal individual following surgical removal of the tumor. Enzyme was extracted from the tissues by homogenization in 0.01 M Na phosphate buffer pH 6.0 at 0°C. Skin fibroblasts were cultured in F-10 +30% fetal calf serum (Gibco, Grand Island, N.Y.) [12]. Extracts of cultured fibroblasts were prepared by freezing and thawing cells in 0.05 M citrate buffer pH 5.0 containing 0.1% bovine serum albumin.

Electrophoresis

Extracts of fibroblasts in 0.05 M Na phosphate-citric acid buffer pH 5.5 were electrophoresed on cellulose acetate gel (Cellogel, Kalex Chemical Co., Manhasset, N.Y.) at pH 5.5 and at pH 6.0 [13, 14]. After incubation of the gels at 37°C with 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide solution (0.264 mM in 0.1 M Na phosphate-citric acid buffer, pH 4.5), the UV fluorescence was enhanced by short incubation in 0.25 M Na carbonate-glycine buffer, pH 10.0.

The relative activity of hex A and hex B was quantitated by excision of the fluorescent bands and appropriate blank zones, elution of the enzymatically liberated 4-methylumbelliferone in 3 ml of Na carbonate-glycine buffer (18 hr at 4°C), and fluorometry [14].

Ion-Exchange Chromatography

Microcolumns of DEAE cellulose (DE-52, Whatman) in Pasteur pipettes were pre-equilibrated with 0.01 M Na phosphate buffer pH 6.0, containing 0.05 M NaCl. Dialyzed tissue extracts were applied to the columns with the same buffer. Hex B was not bound by the ion exchanger, and the bound hex A was subsequently eluted with phosphate buffer containing 0.3 M NaCl. The active fractions were pooled, and the relative activities of hex B and hex A determined.

Immunological Procedures

Hex A and hex B were purified to homogeneity by affinity chromatography [10, 11] and were used for immunizations and for the standardization of the immunoassays.

One mg of each purified isozyme in complete Freund's adjuvant was injected intradermally into goats. The injection was repeated 2 weeks later, and blood was collected weekly, starting 2 weeks after the last injection. Specific anti-hex A was prepared by

exhaustive absorption of anti-hex A on Sepharose-bound hex B [15]. The absorbed antiserum reacted with only hex A, as judged by immunodiffusion [15] or by the exclusive binding of radioactive hex A; hex B was not bound by the specific antibody preparation [11].

Radial immunodiffusion and radio immunoassays were carried out as described previously [11]. Radial immunodiffusion took place in agarose gels containing anti-hex B or specific anti-hex A (diluted 1:500 and 1:100, respectively). The precipitin rings were visualized using the substrate Naphthol AS-BI-*N*-acetyl-β-D-glucosaminide and Fast Garnet GBC (Sigma, St. Louis, Mo.). The combined amounts of both hex A and hex B were calculated from the net area of the rings obtained in the plate containing anti-hex B, using standards of pure hex A and hex B; hex A alone was determined similarly in the plates containing the specific anti-hex A.

Radioimmunoassays were performed with both antisera. Standards of pure hex A and hex B as well as tissue extracts were allowed to compete with lactoperoxidase-iodinated [16] hex A in the binding to anti-hex B (diluted 1:160) and to specific anti-hex A preparation (diluted 1:20). The goat antibodies were precipitated with rabbit anti-goat IgG, and the radioactivity in the precipitate was determined. The relative amount of hex A was calculated from the ratio between the amount of hex A, determined by the assay with specific anti-hex A, and the total amount of β hexosaminidase determined with anti-hex B.

RESULTS

Low levels of hex A in different tissues of the variant individuals were demonstrated using electrophoresis on cellulose acetate gel, ion-exchange chromatography, radial immunodiffusion, and radioimmunoassay.

Electrophoresis on cellulose acetate gel at pH 5.5 and pH 6.0 of cultured fibroblasts from variant individual H, examined at four successive culture passages, showed the presence of a weak band of β hexosaminidase activity, migrating with the same mobility as hex A from control fibroblasts and obligate TSD heterozygotes; no such band was visible in fibroblasts from TSD patients (figs. 1 and 2).

Electrophoretic quantitation of the relative activity of hex A from variant fibroblasts gave an average of 3.54% of the total (hex A + hex B) activity in the sample (two independent determinations, in duplicate, on the same sample). The specific activity of β hexosaminidase in the variant fibroblasts was about 1,080 nmol/mg protein per hour (average of two determinations on the same extract, stored at −80°C for 6 months) compared to an average of 365 nmol/mg protein per hour for similarly stored normal fibroblasts, assayed at the same time.

Similar results were obtained by quantitative ion-exchange chromatography on DEAE cellulose. The relative activity of hex A and hex B in cells from normal individuals, patients with TSD, and variant individuals is given in table 1. Hex A activity in variant individuals was significantly higher than in TSD patients (i.e., about 6% of the total β hexosaminidase activity). These findings were confirmed for all four variant individuals.

In the radial immunodiffusion assay, 10 μl aliquots of tissue extracts of TSD and variant individuals were applied to agarose gels containing either of the two antibody preparations. The samples were tested in several dilutions (usually 8 serial 1:2 dilutions) to allow the detection of small amounts of hex A.

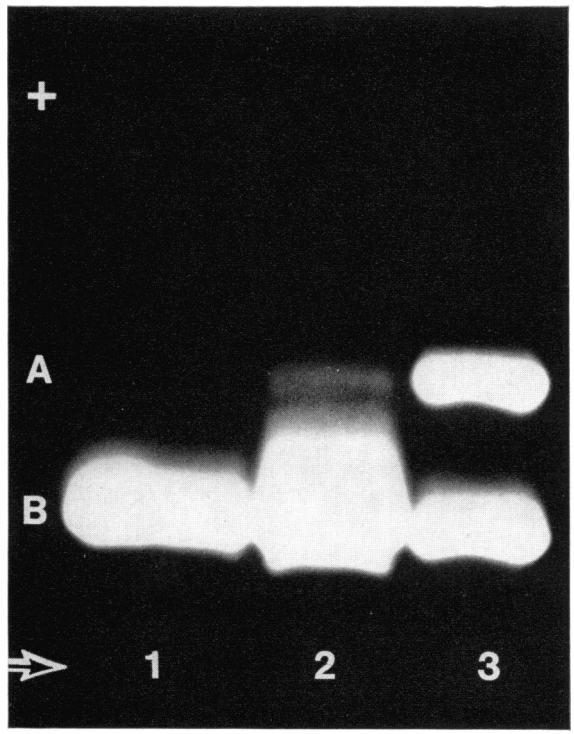

FIG. 1.—Fluorescent bands of β hexosaminidase activity after electrophoresis of cultured fibroblasts on cellulose acetate gel at pH 6.0 and development with fluorogenic substrate (see text for details). *Lane 1*, Patient with Tay Sachs disease; *lane 2*, variant individual H; *lane 3*, normal individual. *A*, Hex A; *B*, hex B; *arrow*, point of application. Samples have similar protein concentration. A minor band of enzyme activity, migrating between hex B and the point of application, was consistently observed in fibroblasts from the variant individual. Its significance is unknown. Exposure time necessary for the photographic documentation of the hex A band in *lane 2* caused this minor slow-moving band, quite distinct by visual inspection, to appear as part of the hex B band.

As shown in figure 3, tissues from normal, TSD, and variant individuals demonstrated immunoprecipitate rings of comparable size in the anti-hex B-containing gels. Quantitation was possible by using the results from the few lower concentrations which were in the linear range (20–80 mm^2 of ring area). In gels containing the specific anti-hex A (fig. 3), normal tissues produced rings similar in size to those obtained with the anti-hex B gels; tissues from TSD patients formed no visible rings. Tissues from variant individuals generated distinct rings in only the two or three most concentrated samples which corresponds to 5%–7% of the total (table 2). In all samples tested, total enzymatic activity agreed with the ring area obtained in the gel containing anti-hex B antiserum.

Tissue extracts from normal, TSD, and variant individuals were tested by radioimmunoassay for their capacity to compete with radioactively labeled hex A in binding to either the cross-reactive anti-hex B antibodies or the specific anti-hex A

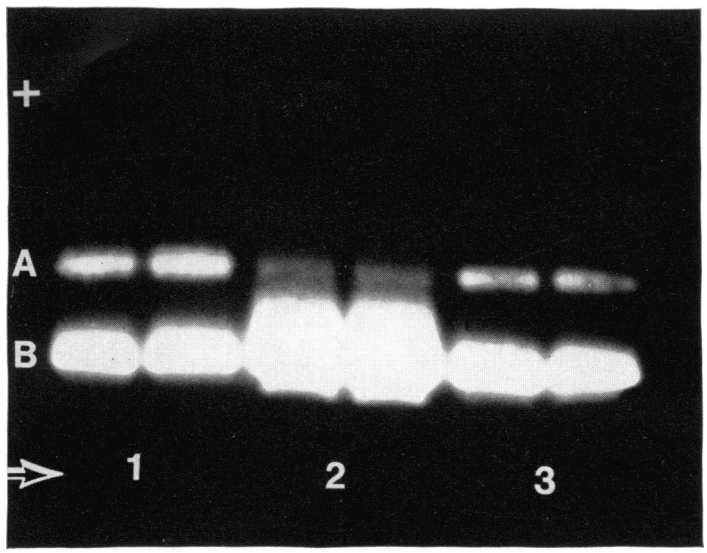

FIG. 2.—Electrophoresis of cultured fibroblasts at pH 6 (duplicate samples in adjacent lanes). *Lane 1*, Obligate heterozygote for the TSD gene (mother of TSD patient in fig. 1); *lane 2*, variant individual H; *lane 3*, obligate TSD heterozygote (father of patient in fig. 1). Notation and remarks as in figure 1.

antibodies. As shown in table 3, the results were practically identical to those obtained by ion-exchange chromatography and radial immunodiffusion; in Tay-Sachs tissues virtually no hex A ($< 1\%$) could be detected, while significant amounts of hex A, representing approximately 6% of total β hexosaminidase, were found in all tissues from the variant individuals.

TABLE 1

SEPARATION OF HEXOSAMINIDASES ON DEAE CELLULOSE

SAMPLE	PERCENT ACTIVITY	
	Hex B	Hex A
Normal liver	46.7	53.3
Normal placenta	42.0	58.0
Melanoma tumor	32.1	67.9
Tay-Sachs liver (K)	98.9	1.1
Tay-Sachs liver (O)	98.0	2.0
Variant melanoma (H)	94.4	5.6
Variant fibroblasts (H)	93.8	6.2
Variant fibroblasts (A)	94.3	5.7
Pure hex A	2.3	97.7
Pure hex B	98.8	1.2

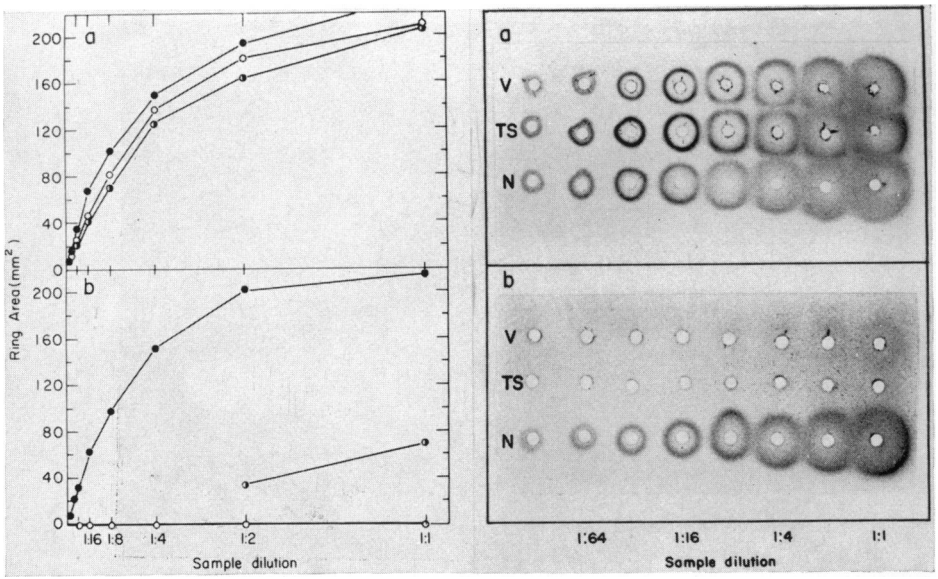

FIG. 3.—Radial immunodiffusion plates after histochemical stain for β hexosaminidase activity (*right*), and corresponding plots of precipitin ring area vs. sample dilution (*left*) in gels containing cross-reactive anti-hex B immune serum (*upper half*) or specific anti-hex A immune serum (*lower half*). *V*, Extract of melanoma from variant individual H (*half-filled circles* in the graph); *TS*, extract of liver from TSD patient (*open circles* in the graph); *N*, extract of normal liver (*closed circles* in the graph).

DISCUSSION

The four variant individuals were previously reported to be indistinguishable from patients with TSD by heat inactivation enzyme assay of leukocytes, serum, skin fibroblasts and urine, and starch gel electrophoresis of fibroblast extracts [6]. Reexamination was prompted by the finding of G_{M2} ganglioside-cleaving activity

TABLE 2

DETERMINATION OF THE CONTENT OF HEXOSAMINIDASE A BY RADIAL IMMUNODIFFUSION

SAMPLE	Percent Hex A
Normal liver	62.0
Melanoma tumor	64.6
Normal placenta	61.2
Normal fibroblasts	63.0
Tay-Sachs liver (K)	...*
Tay-Sachs fibroblasts (N)	...*
Variant melanoma (H)	6.8
Variant fibroblasts (H)	6.9
Variant fibroblasts (A)	5.0
Variant fibroblasts (P)	5.9

* No visible ring.

TABLE 3

DETERMINATION OF HEXOSAMINIDASE A BY RADIOIMMUNOASSAY

SAMPLE	DILUTION	PERCENT INHIBITION		PERCENT HEX A
		With Anti-hex B	With Specific Anti-hex A	
Normal liver (I)	1:3	86.8	84.7	
	1:9	58.1	39.0	63.0
	1:27	25.0	9.9	
Normal liver (II)	1:3	91.3	88.8	
	1:9	62.7	40.6	57.2
	1:27	32.1	10.0	
Tay-Sachs liver (O)	1:3	92.4	2.0	
	1:9	66.3	−2.1	< 1
	1:27	32.2	4.2	
Tay-Sachs liver (K)	1:3	75.0	2.0	
	1:9	43.2	2.9	< 1
	1:27	16.1	1.5	
Variant melanoma	1:3	94.1	19.6	
	1:9	76.7	4.3	6.3
	1:27	42.2	0.9	
Variant fibroblasts (H)	1:3	94.9	18.0	
	1:9	70.8	4.6	6.9
	1:27	40.2	−1.0	
Variant fibroblasts (A)	1:3	93.2	11.3	
	1:9	68.3	2.9	5.6
	1:27	37.1	0.4	

in their leukocyte preparations, an amount comparable to that found in TSD heterozygotes [9], and by the detection of a clear hex A band after cellulose acetate gel electrophoresis of fibroblasts from one of the subjects (figs. 1 and 2). We, therefore, examined cultured skin fibroblasts from these subjects to substantiate the presence of hex A by more direct and sensitive methods than previously [6] employed. Cellulose acetate gel electrophoresis, ion-exchange chromatography, radial immunodiffusion, and radioimmunoassay gave results which agreed with one another and showed significant amounts of hex A activity (3.5%–6.9% of total, or approximately 5%–10% of hex A activity in normal tissues) in skin fibroblasts from the four subjects. Correspondingly low levels of hex A protein were measured by radioimmunoassay (table 3), which measures the amount of enzyme only by its antigenic properties, independent of its enzymatic activity. Similar results were obtained by examining melanoma tissue from one of the subjects. Tissues from patients with TSD contained neither active hex A nor inactive, immunologically reactive enzyme (tables 2 and 3), which agrees with earlier studies [11, 17, 18].

The family of the four variant individuals includes individuals with normal and intermediate levels of hex A activity and three cases of TSD as discussed in our previous publication [6]. Pedigree analysis led us to postulate that the variant

individuals are compound heterozygotes for the common TSD mutant gene and a rare allelic mutant. The small amount of hex A detected in these individuals would thus be coded for by the rare mutant allele since the common TSD mutant apparently is not capable of coding for the expression of hex A. A similar genetic hypothesis has been proposed by Vidgoff et al. [19] to explain the apparent profound deficiency of heat labile hex A activity in plasma and leukocytes from a healthy, non-Jewish woman whose son had died with TSD. However, the subsequent finding of levels of hex A activity comparable to those of TSD heterozygotes in tears and cultured skin fibroblasts from the same woman led these authors to suggest that the mutation may affect the tissue distribution of the enzyme [20]. The results of our present work and of previous studies [6, 9] do not provide evidence of such a heterogeneous distribution of hex A activity in the tissues of the four subjects examined, indicating that a different mutant may be involved in the case described by Vidgoff et al. [19, 20].

The hex A activity with artificial substrate in leukocytes and fibroblasts from the four variant individuals is much lower than that usually found in heterozygotes for the TSD gene [6, 8, 21] (i.e., undetectable by the thermal denaturation assay [6]). If the results of the present studies on skin fibroblasts can be extended to leukocytes, one can assume that hex A in these cells amounts to no more than 5%–10% of normal. However, leukocytes from these subjects have 50% of the normal G_{M2} ganglioside-cleaving activity [9]. Although we have not determined G_{M2} ganglioside-cleaving activity in fibroblasts, its presence in cells other than leukocytes could explain the absence of clinical signs of G_{M2} gangliosidosis in these subjects [6]. This is difficult to reconcile with the very low levels of hex A activity with artificial substrates in different cells and tissues from the variant individuals ([6] and table 2) and the correspondingly low amounts of hex A protein detected in fibroblasts and melanoma tissue (table 3). The immunological data argue against the possibility that in these cells the non-TSD mutant codes for the expected amount of hex A protein, which retains normal activity towards G_{M2} ganglioside but has very little activity with artificial substrates (as a result of the mutation). This has been proposed in the case of an adult individual with marked deficiency of hex A and hex B activity with artificial substrates but without signs of G_{M2} gangliosidosis [22].

It could be argued that hex A may be heterogeneous and that only a minor subfraction of the enzyme is active with G_{M2} ganglioside as proposed by Bach and Suzuki [23]. Accordingly, in the four individuals studied, the enzyme fraction with G_{M2} ganglioside-cleaving activity coded for by the non-TSD gene could be normal, while the rest of hex A, active with artificial substrate, could be affected by the mutation. However, a single mutation in TSD affects hex A in its entirety, indicating that the minor and the major subfractions are controlled by the same gene. It would be difficult to explain how the rare mutation in the variant individuals would result in absence of the major subfraction without impairment of the minor, G_{M2} ganglioside-cleaving subfraction.

Thus, if one assumes that only hex A is responsible for the catabolism of G_{M2}

ganglioside in vivo [4, 23–25], there does not appear to be a simple explanation for the discrepancies discussed above. However, the finding that purified hex B can cleave G_{M2} ganglioside in vitro [5], has raised the possibility that this enzyme may normally participate in the degradation of the ganglioside in vivo [5, 9]. This observation supports the contention that the observed residual activity with G_{M2} ganglioside in crude extracts of leukocytes from the variant subjects [9], in which hex A activity is extremely low [6], may be due primarily to hex B. We have no direct experimental data to validate this hypothesis. However, if hex B participated in G_{M2} ganglioside catabolism in vivo, this enzyme should be altered in TSD [14, 26]; in fact, data have been presented indicating that the kinetic properties [25] and the "corrective activity" [27] of hex B from tissues of TSD patients are abnormal. Thus, identification of the enzyme form responsible for G_{M2} ganglioside degradation in the tissues of the variant individuals may clarify the discrepancies discussed above and indicate whether hex B is affected by the TSD mutation.

Finally, the finding of intermediate hex A activity with artificial substrates in both parents and in all six of the non-TSD offspring of these variant individuals [6] indicates that the heterozygotes for the rare mutant may not be distinguishable from heterozygotes for the common TSD mutant using the thermal inactivation method. Even if the methods of the present study were employed, it is doubtful that this distinction could be made, in view of the small amount of hex A associated with the presence of the postulated rare mutant allele in the variant individuals. Thus, it does not seem possible at present to estimate the frequency of matings between the two types of heterozygotes such as the one that, according to our hypothesis [6], produced the four healthy variant individuals. However, the application of the sensitive direct quantitation methods described above to the monitoring of pregnancies in which both parents are apparently heterozygous for the TSD gene, should help to distinguish between a true TSD fetus and a fetus with detectable hex A, thus preventing unnecessary terminations of pregnancy.

SUMMARY

Appreciable β hexosaminidase A (hex A) activity has been detected in cultured skin fibroblasts and melanoma tissue from healthy individuals previously reported as having deficiency of hex A activity indistinguishable from that of patients with Tay-Sachs disease (TSD). Identification and quantitation of hex A, amounting to 3.5%–6.9% of total β hexosaminidase activity, has been obtained by cellulose acetate gel electrophoresis, DEAE-cellulose ion-exchange chromatography, radial immunodiffusion, and radioimmunoassay. Previous family studies suggested that these individuals may be compound heterozygotes for the common mutant TSD gene and a rare (allelic) mutant gene. Thus, the postulated rare mutant gene appears to code for the expression of low amounts of hex A. Heterozygotes for the rare mutant may be indistinguishable from heterozygotes for the common TSD mutant. However, direct visualization and quantitation of hex A by the methods described may prevent false-positive prenatal diagnosis of TSD in fetuses having the incomplete hex A deficiency of the type described in the four healthy individuals.

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