Deficiency of the Hexosaminidase A Activator Protein in a Case of GM₂ Gangliosidosis; Variant AB

PETER HECHTMAN,⁽⁴¹⁾ B. A. GORDON, AND N. M. K. NG YING KIN

MRC Human Genetics Research Group and Centre for Human Genetics, Biology Department, McGill University, Montreal, Quebec, Canada [P.H.]; Children's Psychiatric Research Institute Biochemistry Laboratory, London, Ontario, Canada [B.A.G.]; and Donner Laboratory for Neurochemistry, Montreal Neurological Hospital, Montreal, Quebec, Canada [N.M.K.N.Y.K.]

Summary

A patient is described whose clinical course and pathologic features, including massive brain storage of GM₂ ganglioside in grey matter, are identical with those of classical Tay-Sachs disease despite normal levels of β -N-acetyl hexosaminidase and normal isozyme distribution. The kinetic properties and thermolability of the patient's hexosaminidase are normal. Crude extracts of a postmortem sample of patient's liver can catalyze the hydrolysis of 5.1 pmoles of labeled GM₂ ganglioside/16 h/mg of protein (control liver = 69.9 pmoles/16 h/mg). Addition of partially purified human liver hexosaminidase A activator protein stimulated the hydrolysis of substrate by the patient's liver extract by 27-fold, compared to 3-fold for control livers.

Measurement of "activator" in enriched fractions of patient's and control liver showed a reduced (25-30% of control) amount of stimulation of hexosaminidase A catalyzed hydrolysis of GM₂ ganglioside as well as of Asialo-GM₂ ganglioside. The addition of sphingomyelin to reaction mixtures, which is known to inhibit surfactant stimulation of hexosaminidase A, reduced activation of hexosaminidase A by patient's liver preparation to undetectable levels.

Polyacrylamide gel electrophoresis of enriched preparations of control and patient's liver showed a rapidly migrating protein band in control liver corresponding to the activator protein and the absence of this protein band in the patient's liver.

Speculation

We believe that this patient is a genocopy of classical Tay-Sachs disease as a result of a mutation at a gene locus controlling the expression of the hexosaminidase A activator protein. The *in vivo* requirement of activator protein for hydrolysis of sphingolipid substrates by lysosomal hydrolases may explain the occurrence of other storage disease entities in which normal levels of enzyme are present in the homozygote when assayed using synthetic hydrophilic substrates.

Classical Tay-Sachs disease (GM₂ gangliosidosis, type B) is marked by severe, progressive and fatal neurodegeneration as a consequence of massive storage of GM₂ ganglioside [N-acetyl-galactosaminyl-(N-acetyl-neuraminyl) galactosyl glucosyl ceramide] in the neurons. Storage of this compound occurs due to a deficiency of the enzyme β -N-acetyl hexosaminidase A (Hex A) (EC 3.2.1.30) (28). In addition to the classical form of GM₂ gangliosidosis, Sandhoff *et al.* (29) described two other variants; type 0, marked by a complete deficiency of both Hex A and Hex B and type AB in which normal levels of Hex A and B activity were measured in tissues. In the last of these variant forms of Tay-Sachs disease, normal levels of hexosaminidases were determined using synthetic hydrophilic substrates (19).

Li and co-workers (20) and Hechtman (12) showed that the *in vitro* hydrolysis of the N-acetyl galactosaminyl linkage of GM₂-ganglioside by purified preparations of human liver Hex A requires the presence of an activator (AP). Hydrolysis of synthetic hexosaminidase substrates is unaffected by AP. This activator has been purified and shown to be a heat stable protein with a molecular weight of 36,000 (13). An *in vivo* metabolic function for the activator protein was demonstrated by Conzelman and Sandhoff (2) who showed that kidney extracts of the original AB variant patient were deficient in the Hex A activator protein.

In this report we describe clinical and biochemical features of a new case of GM₂ gangliosidosis, type AB. The patient shows massive storage of GM₂ ganglioside in grey matter as well as a pattern of liver glycolipid accumulation different from that found in the type 0 variant of the disease. Activity of Hex A in the patient's tissues is both quantitatively and qualitatively normal. The patient's liver, however, is deficient in a specific Hex A activator protein.

Clinical and pathological report. The patient (G.M.) was the first child of a 17-year-old diabetic non-Jewish mother. He was seen initially at 14 months for investigation of suspected psychomotor retardation. His motor milestones had been delayed and he was able to roll over and sit unsupported although he had difficulty with head control. Development assessment placed him within the moderately retarded range. He was seen again approximately 4 months later when he developed seizures; now obviously hypotonic, he had made no further developmental progress. Although his head circumference, at the 75th percentile, appeared disproportionately large, an air encephalogram was normal. At 24 months of age, bilateral macular cherry red spots were observed. By this time he was showing evidence of quadriplegia. Over the subsequent 3 months, his neurologic status continued to deteriorate. He experienced repeated respiratory tract infections and died with bronchopneumonia at 27 months of age.

Pathology reports, previously published in abstract form (10), indicated storage of both lipid and carbohydrate material in the neurons.

MATERIALS AND METHODS

Source of tissues. Liver obtained from the patient at autopsy was stored at -70°C. Control livers stored under these conditions for up to 2½ years did not experience a decrease in specific activity of Hex A activator protein. The control liver samples do lose 40-50% of Hex A activity following a year of storage at -70°C.

Identification of gangliosides, glycolipids and oligosaccharides from brain, liver and urine. Gangliosides were isolated from brain according to the procedure of Folch *et al.* (8), as modified by Suzuki *et al.* (33). The isolated ganglioside extract of both brain and liver was chromatographed with a single ascending development on a precoated silica gel plate in chloroform:methanol:2.5

N-ammonia::60:40:9 and the individual ganglioside bands located by spraying with resorcinol.

The frozen liver sample was thawed and extracted with 10 volumes of chloroform:methanol (2:1) and subjected to Folch partition as modified by Wolfe *et al.* (38). The oligosaccharides and gangliosides from the upper phase were then separated using a Sep-Pak C18 cartridge (Water's Associates, Mississauga, Ontario). Water was used to elute the oligosaccharides (24) and methanol to elute the gangliosides (37). Neutral glycolipids were isolated from the lower phase by silica gel chromatography (36).

The oligosaccharide fraction was analyzed by thin layer chromatography on silica gel using butanol:acetic acid:H₂O::2:1:1 as the developing solvent and orcinol spray reagent for visualization (16). High pressure liquid chromatography was also used for both analysis and preparation of oligosaccharides (24). Composition of the major oligosaccharide thus isolated was determined by gas liquid chromatography after subjecting the oligosaccharide successively to acid hydrolysis, borohydride reduction and acetylation (17).

Neutral glycolipids isolated by silica gel chromatography were freed from contaminating phospholipids by mild base methanolysis. Thin layer chromatography on silica gel plates was performed using chloroform:methanol:H₂O::100:42:6 as the developing solvent and orcinol as the spray reagent.

A 24-hour urine sample (220 ml) was centrifuged at 3000 X g for 20 min at 4°C. The supernatant was then analyzed by thin layer chromatography and high pressure liquid chromatography for oligosaccharides. The urinary sediment was extracted with 2 x 10 ml of chloroform:methanol::2:1 and analysed for gangliosides and glycolipids.

Measurement of hexosaminidase. Hexosaminidase activity and β -galactosidase activities reported in Table 1 were determined spectrophotometrically using the appropriate p-nitrophenyl- β -glycoside substrate. Other hexosaminidase assays were performed fluorometrically (19). Substrates and 4-methylumbelliferone standard were purchased from Koch-Light (Colnbrook, Buckinghamshire, England).

Purification of hexosaminidase A from human liver. Hex A was purified according to a previously published procedure (12) except that before the resolution of Hex A from Hex B by DEAE-Sephadex chromatography, enzyme activity in the liver extract was enriched by adsorption onto concanavalin A-sepharose (Pharmacia, Uppsala, Sweden) and elution by α -methylmannoside containing buffer using the elution system described by Mahuran and Lowden (22).

The final preparation had a specific activity of 11,200 nmoles 4-methylumbelliferyl N-acetylglucosaminide cleaved/min/mg of protein (1350-fold purified over crude liver supernatant) and was completely labile to heating at 50°C for 30 min. Crude Hex A was prepared from small samples of patient's and control liver by

homogenization of 10 g of liver with 20 ml of 20 mM NaH₂PO₄-NaOH pH 5.5. The homogenate was centrifuged at 16,000 X g for 30 min at 4°C and the supernatant was dialysed overnight against 6 l. of 2.5 mM citric acid Na₂HPO₄ pH 4.4. Denatured protein was removed by centrifugation as previously described. The dialysed supernatants were dialysed against 20 mM NaH₂PO₄-NaOH pH 6.0 and applied to a column of DEAE sepharose (50 ml) equilibrated in the same buffer. Hexosaminidase B was eluted by washing the column with application buffer and Hex A was then recovered by elution with 20 mM NaH₂PO₄-NaOH pH 6.0-0.5 M NaCl. The Hex A-containing eluate was concentrated using aquacide II (Calbiochem, La Jolla, CA) and dialysed against phosphate buffer.

Preparation of ganglioside and glycolipid substrates. Isolation of GM₂ ganglioside from brain of patients with Sandhoff disease, radiochemical synthesis of [³H]-(N-acetylglucosamine)-GM₂ ganglioside and radiochemical purity of the labeled product have previously been described (12). The [³H]-GM₂ ganglioside had a specific radioactivity of 18.2 mCi/mmol, and was >98% radiochemically pure by thin layer chromatography in chloroform:methanol:2.5 N NH₃::60:40:9.

[³H]-Asialo GM₂ ganglioside (N-acetylglucosaminyl galactosyl glucosyl ceramide) was prepared from [³H]-GM₂ ganglioside by heating an aqueous solution of the radiochemically labeled ganglioside for 40 min at 80°C in 0.03 N HCl. The hydrolysate was then shaken with 5 volumes of chloroform:methanol::2:1 and the phases separated by centrifugation. The lower phase material was used as a source of [³H]-asialo GM₂ ganglioside.

Enzymic hydrolysis of ganglioside and glycolipid substrates. The composition of reaction mixtures, incubation conditions and the quantitation of the [³H]-N-acetyl galactosamine released have been reported in a previous communication (34). Additions to reaction mixtures included Triton X-100 (Fisher, Montreal, Quebec, Canada), sodium taurocholate, (Calbiochem, La Jolla, CA) and sphingomyelin (Sigma, St. Louis, MO). Sphingomyelin and asialo GM₂ ganglioside were added to reaction tubes as chloroform:methanol (2:1) solutions and the solvent evaporated under a stream of N₂.

Purification of the hexosaminidase A activator protein. Activator protein was purified as described previously (13) except that the Sephadex G-100 gel filtration step was omitted. The specific activity of the partially purified activator protein prepared used was 10,500 Units/mg protein. This value is 40% of the purest preparations obtained. Enriched liver fractions refer to liver homogenates prepared from 10 g of patient and control livers and processed through the first two steps of the AP purification procedure, resulting in a 10-fold enrichment of AP from the control liver homogenate. Comparable amounts of protein were obtained from control liver (144 mg) and from patient liver (138 mg).

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Davis (5). Protein bands were visualized using Coomassie Brilliant Blue.

Protein determination. Protein was determined by the method of Lowry (21).

RESULTS

Nature of the storage disease. The progressive nature of the neurodegeneration in patient GM strongly suggested the presence of a sphingolipid storage disease. Massive storage of GM₂ ganglioside in the patient's grey matter, which shows a chromatographic pattern of resorcinol staining bands nearly identical to that of the lipid extract of grey matter of a Tay-Sachs brain, is seen following thin layer chromatography (Fig. 1). GM₂ ganglioside accounts for 70% of the sialic acid content of the patient's total brain ganglioside fraction compared to less than 5% for normal brain tissue. The presence on the chromatogram of higher gangliosides, GM₁, GD_{1a}, GD_{1b} and GT (according to the nomenclature of Svennerholm) rules out the possibility that storage of GM₂ ganglioside is due to a defect in a biosynthetic enzyme such as has been recently proposed for GM₃ gangliosidosis (23).

Table 1. Activity of lysosomal enzymes in postmortem tissues

Liver sample	Liver	Brain
<i>p</i> -Nitrophenyl- β -D-N-acetyl glucosaminide (μ moles hydrolyzed/g/min)		
Total Activity		
G.M.	19.5	38.3
Control 1	22.7	28.2
Control 2	32.2	26.3
Heat Labile Activity ¹		
G.M.	9.2	18.0
Control 1	7.9	9.9
Control 2	11.3	9.2
<i>p</i> -Nitrophenyl- β -D-galactoside (μ moles hydrolyzed/g/min)		
G.M.	1.86	0.58
Control 1	0.22	0.27
Control 2	0.99	0.29

¹ Determined after incubation for 30 min at 50°C.

Fig. 1. Thin layer chromatography of brain gangliosides. Brain gangliosides were spotted on a precoated silica gel plate and after a single ascending development in chloroform:methanol:2.5 N ammonia::60:40:9, visualized by spraying with resorcinol: (1) normal control brain grey matter; (2) Tay-Sachs Disease (B variant) brain grey matter; (3) patient GM brain grey matter; (4) GM white matter; (5) Tay-Sachs Disease (B variant) white matter; (6) normal control brain white matter; and (7) ganglioside GM₁.

Traces of GM₂ ganglioside were found in the patient's liver although none were present in the urine.

The major glycolipid identified in liver was a trihexosylceramide which migrated similarly to asialo GM₂ ganglioside. Globoside was absent from liver samples. Neutral glycolipids were not detectable in the patient's urine.

Despite the storage of GM₂ ganglioside, enzyme assays performed on extracts of postmortem brain and liver provide no evidence for a defect of either total hexosaminidase activity or for an abnormal distribution of hexosaminidase isozymes. The data are presented in Table 1.

GM₂ ganglioside hydrolysis in crude liver extracts. Crude liver extracts were prepared as indicated in Methods up to the dialysis against acidic buffer. The supernatants were then concentrated by dialysis against 90% saturated (NH₄)₂SO₄ and the precipitated protein redissolved in 20 mM H₂PO₄-NaOH pH 6.5. After dialysis to remove traces of (NH₄)₂SO₄, the crude concentrated liver supernatants were used to measure hydrolysis of [³H]-GM₂ ganglioside. The results of these assays are shown in Table 2.

Hydrolysis of [³H]-GM₂ ganglioside by mutant liver extracts unsupplemented by AP was negligible (50% above blank values). Addition of partially purified AP (which is free of Hex A activity) to the reaction mixture resulted in a 3-fold stimulation of the reaction catalyzed by the control liver extract, which indicates that in this crude liver preparation the Hex A is not saturated with respect to AP. By contrast, the reaction catalyzed by the mutant liver extract was stimulated 27-fold by the addition of AP. The results are stated as pmoles GM₂ ganglioside hydrolysed/mg of protein. If, however, enzyme activity in the two extracts in the presence of AP is normalized to Hex A activity the rate of cleavage of substrate per unit of Hex A is 3.4 for the control and 7.2 pmoles/16 h/Unit Hex A for the mutant liver extracts.

Properties of mutant liver hexosaminidase A. Although the results reported in Table 1 suggest that the gene defect in patient Gm is not at the locus controlling the activity of Hex A, some attempts were made to determine the properties of Hex A from the patient's liver in order to rule out a "qualitative" defect in the enzyme. Figures 2 and 3 respectively, present the thermolability characteristics and the kinetic properties of Hex A enriched from the two liver preparations. Both the K_m and thermolability are substantially identical for the two enzyme preparations.

Table 3 shows the activation by detergents of crude Hex A from control and GM liver as well as purified human liver Hex A. Hex A in both crude preparations is stimulated to the same extent by sodium taurocholate and Triton X-100, although neither enzyme preparation is activated to the same extent by detergents as the purified Hex A. The limited detergent stimulation of crude liver

Table 2. Hydrolysis of [³H] GM₂ ganglioside by crude liver extracts

	Control liver	GM liver
Total hexosaminidase ¹	86.6	24.4
Hexosaminidase A	62.9	19.5
GM ₂ ganglioside hydrolysis ²		
– Activator	69.9	5.1
+ Activator	214.8	140.8

¹ nmoles/min/mg protein.

² pmoles/16h/mg protein.

Fig. 2. Thermolability of crude human liver Hex A: (circles) enzyme heated at 52°C, (triangles) enzyme heated at 41°C, (open symbols) control liver Hex A, (closed symbols) Gm liver Hex A.

Hex A is probably due to the presence of lipid in the crude enzyme preparations.

Activation of purified Hex A by patient and control liver preparations. In order to undertake direct measurements of AP in both liver samples, the liver supernatants were processed through the first two steps of a purification procedure (13). The second step of this procedure consists of heating the supernatant at 58°C for 30 min followed by removal of denatured protein by centrifugation. In this step all Hex A in the liver supernatant is inactivated, and AP activity is determined in reaction mixtures containing purified Hex A.

The effect of addition of these enriched liver fractions on the

Fig. 3. K_m of human liver Hex A for methylumbelliferyl- β -D-N-acetylglucosaminide: (open circles, dashed line) control liver Hex A, (closed circles, solid line) GM liver Hex A.

Table 3. Stimulation of GM_2 ganglioside hydrolysis by sodium taurocholate and Triton X-100

	Crude Hex A control liver ¹	Crude Hex A GM liver ¹	Purified Hex A ¹
Enzyme	0.34	0.25	0.12
+ Triton X-100 0.25 mg/ml	0.46	0.34	1.0
+ Sodium taurocholate 10 mg/ml	0.72	0.76	1.2

¹ pmoles GM_2 hydrolyzed/Unit hex A/16 h.

catalytic activity of purified Hex A toward [3H]- GM_2 ganglioside and [3H]-asialo GM_2 ganglioside is shown in Figure 4. The enriched fraction prepared from the patient's liver is not completely devoid of ability to stimulate hydrolysis of either substrate. These results were surprising in view of the virtual absence of [3H]- GM_2 ganglioside hydrolysis in the unsupplemented crude liver extract reported in Table 2. The low level of stimulation observed by addition of the enriched patient's liver fraction to reaction mixtures containing purified Hex A is of a nonspecific nature and is most likely an artefact caused by heating of the liver supernatant. Activation due to AP and detergent are distinguishable by the occurrence in the latter case of decreased stimulation at postoptimal concentrations of detergent. A more precise distinction between the two modes of Hex A activation is seen if the reactions occur in the presence of sphingomyelin as shown in Figure 5. The presence of a 35-fold molar excess of sphingomyelin (relative to substrate) completely abolished Triton X-100 stimulation of Hex A whereas the sphingomyelin has no effect on AP enhanced Hex A activity (14). We speculate that Triton X-100 activation occurs by formation of a mixed micelle of Triton and substrate in which the substrate is exchangeable with other lipids, whereas the binding between substrate and AP is more specific.

When the enriched liver fractions from patient and control liver were used to stimulate Hex A catalyzed hydrolysis of GM_2 ganglioside in the presence of sphingomyelin the stimulation by the patient's enriched liver fraction is completely abolished while that of the control liver is reduced by 20–25% indicating that some nonspecific stimulating activity is present in both liver preparations. However, all of the Hex A stimulation detected in the patient's liver extract is of a nonspecific nature, possibly due to interaction of substrate with a lipid that has surfactant properties released during the heating procedure. The results of this experiment for GM_2 ganglioside hydrolysis are shown in Figure 6. Identical results (not shown) were obtained for asialo GM_2 ganglioside hydrolysis.

In a previous communication (13) we reported that the Hex A-AP did not stimulate enzymic hydrolysis of asialo- GM_2 ganglioside. The discrepancy between our current findings and the pre-

vious observations is explained by the presence of Triton X-100 added to "solubilize" the asialo- GM_2 ganglioside substrate in experiments reported in reference 13. The ability of a wide variety of surfactants to replace the AP has been reported in a subsequent communication (14).

Fig. 4. Stimulation of purified control liver Hex A catalyzed hydrolysis of ganglioside and glycolipid substrates by enriched liver fractions. (A) GM_2 ganglioside hydrolysis and (B) Asialo- GM_2 ganglioside hydrolysis. (Closed circles, solid lines) control liver, (open circles, dashed lines), GM liver.

Fig. 5. Activator protein and Triton X-100 stimulated hydrolysis of Hex A catalyzed hydrolysis of GM_2 ganglioside. (A) Triton X-100 stimulated GM_2 ganglioside hydrolysis and (B) activator protein stimulated GM_2 ganglioside hydrolysis. (Solid line, closed circles) no sphingomyelin. (Dashed line, open circles) 350 μM sphingomyelin.

Fig. 6. Effect of sphingomyelin on stimulation of purified control liver Hex A catalyzed hydrolysis of GM_2 ganglioside by enriched liver fractions. (Solid lines) control liver, (dashed lines) GM liver. (Open symbols) reaction mixtures containing 350 μM sphingomyelin. (Closed symbols) reaction mixtures containing no sphingomyelin.

A mutation at the gene locus controlling the expression of AP could produce either a "catalytically defective" protein or could result in a complete absence of AP depending upon whether a substitution mechanism of mutation occurred or whether a frame shift or deletion mutation occurred at this locus. Figure 7 shows an attempt to determine whether the mutation results in absence of the gene product using polyacrylamide gel electrophoresis. The electrophoretic profile of partially purified AP is shown in gel 3. The slower moving, more densely staining band is the major contaminant comprising 60% of the protein and is electrophoretically identical with human serum albumin (gel 4). The rapidly migrating band in AP preparation is also observed in gel 2 which is the electrophoretic profile of the enriched liver fraction prepared from control liver. This band is absent in the enriched fraction prepared from the patient's liver. The results are consistent with a deficiency of AP in the liver of the patient, however a mutation that grossly alters the electrophoretic mobility of AP cannot be ruled out.

DISCUSSION

Patient GM has been shown to be a case of Type AB GM₂ gangliosidosis similar to the case reported by Conzelmann *et al.* (4). These authors, using highly purified preparations of Hexosaminidase A, have reported that kinetic, thermostability and detergent activation properties of this enzyme from liver of patient and controls are identical. The same group subsequently demonstrated a deficiency of Hex A activator protein in extracts of the type AB patient kidney (2). The analysis reported here represents, to our knowledge, the second instance of documentation of an activator deficiency in the AB variant. Reports suggesting such a deficiency in other AB variant cases have been published (6, 9, 18).

The absence of certain compounds stored in liver or excreted in urine suggests that the substrate specificity of the Hex A AP is probably quite restricted. The absence of globoside which is known to be stored in the tissues of Sandhoff Disease (30) is consistent with the recent finding by Conzelmann and Sandhoff (3) that the AP has little effect on *in vitro* hydrolysis of globoside by Hex A. Similarly the storage of N-acetyl glucosamine containing oligosaccharides in the livers of Sandhoff Disease patients (25, 26)

and their absence in the liver of the AP deficient patients argues that the AP is probably not required for the enzymatic hydrolysis of oligosaccharide substrates.

The results presented here underline the importance of using "natural" substrates for diagnostic purposes rather than relying upon synthetic substrates which provide more convenient assays but whose hydrolysis is not affected by activators [hydrolysis of 4MU β glucopyranoside by glucocerebrosidase appears to be an exception to this (15)]. With respect to GM₂ gangliosidosis, optimum conditions have been reported for determination of Hex A catalyzed hydrolysis of GM₂ ganglioside in fibroblasts (27) and leucocytes (35).

Although many sphingolipid storage diseases have been unambiguously associated with deficiency of specific lysosomal hydrolases, there has also been emerging evidence for a subgroup of these inborn errors of metabolism which are due to a deficiency of specific activator proteins. Thus a case of metachromatic leucodystrophy with near normal levels of arylsulfatase A (11, 31) has recently been described. The cerebroside sulfate storage defect in cultured fibroblasts can be corrected (32) by exogenously supplied cerebroside sulfatase activator (7). In a recent report, a thermostable activator without sphingomyelinase activity, extracted from either normal spleen or in even higher concentration from Gaucher spleen, but not from Niemann-Pick type C spleen, stimulated sphingomyelin hydrolysis by cultured normal or Niemann-Pick type C fibroblasts. The activator protein isolated and purified from Gaucher spleen stimulated the sphingomyelinase activity of normal fibroblasts 3-fold and the same activity of Niemann-Pick type C fibroblasts 25–38 fold (1).

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Fig. 7. Polyacrylamide gel electrophoresis of activator enriched liver fractions: (gel 1) enriched GM liver preparation (800 μ g protein); (gel 2) enriched control liver preparation (800 μ g protein, 500 units AP); (gel 3) partially purified activator preparation (15 μ g protein, 150 units AP); and (gel 4) human serum albumin. Direction of electrophoresis is from cathode (top) to anode (bottom). Arrows indicate position of tracking dye Bromphenol Blue.

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 41. Requests for reprints should be addressed to: Dr. Peter Hechtman, MRC Human Genetics Research Group, Montreal Children's Hospital, 2300 Tupper St., Montreal, Quebec, Canada H3H 1P3.
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