Normal Transcription of the β-Hexosaminidase α-Chain Gene in the Ashkenazi Tay-Sachs Mutation*

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Tay-Sachs disease is a biochemically heterogeneous lysosomal storage disorder caused by lack of the A isoenzyme of β-hexosaminidase; the underlying defect is a mutation in the gene encoding the α-chain. It has been shown that fibroblasts isolated from Tay-Sachs patients of Ashkenazi Jewish origin contain no α-chain mRNA detectable on Northern blots. We now have compared run-on transcription in nuclei isolated from three strains of Ashkenazi Tay-Sachs fibroblasts and from a strain of normal (IMR90) cells. Using α-chain and β-chain cDNAs as probes, we found no difference in the relative amount of [32P]ribonucleotide added to nascent transcripts; the average ratio of α/β hybridizable radioactivity was 1.3 and 1.4 for mutant and normal cells, respectively. The identity of the Tay-Sachs α-chain transcript was confirmed by competition hybridization with excess α-chain mRNA. The results indicate that the Ashkenazi Tay-Sachs mutation permits a normal level of transcription of the α-chain gene and points to a posttranscriptional defect, such as RNA processing, transport, or stability.

The Tay-Sachs disease is an autosomal recessive disorder caused by mutation of the gene encoding the α-chain of the lysosomal enzyme, β-hexosaminidase1 (reviewed in Refs. 1 and 2). The resulting deficiency of the A isoenzyme of β-hexosaminidase, an α,β-dimer, causes pathologic accumulation of GM2 ganglioside in the nervous system. The neurologic disease displays a spectrum of clinical severity, ranging from the "classic" infantile Tay-Sachs disease, fatal in early childhood, through juvenile to milder chronic and adult forms (1-4). The severity of the disease is inversely correlated with the level of residual β-hexosaminidase A activity towards its natural substrate, Gd3 ganglioside (5). The classic form of Tay-Sachs disease is best known for its previously high incidence in Ashkenazi Jews, among whom the gene frequency is 0.015; however, carrier detection programs have nearly eliminated the disease from this population. Such is also the case for clinically classic Tay-Sachs disease among French-Canadians from Eastern Quebec (6).

Recent studies have shown molecular heterogeneity within Tay-Sachs disease (7). Biosynthetic labeling of cultured fibroblasts allows the sorting of Tay-Sachs mutations into two groups: those that allow production of immunoprecipitable α-chain polypeptide (cross-reactive material-positive) and those that do not (cross-reactive material-negative). Cells from a number of clinically classic Tay-Sachs patients are in the cross-reactive material-negative category. In some instances, the altered α-chain gives rise to β-hexosaminidase A with defective catalytic activity or stability (8), whereas altered substrate specificity has been found in a variant called "B1" (9). In cells from two clinically classic Tay-Sachs patients, both of Italian descent but from unrelated kindreds, distinct α-chain mutations result in altered polypeptides that appear not to exit from the endoplasmic reticulum (10, 11). Fibroblasts from a number of patients with juvenile, chronic, and adult forms synthesize α-chains that do not associate with β-chains to form β-hexosaminidase A (12).

On the other hand, fibroblasts from clinically classic Tay-Sachs patients of Ashkenazi Jewish origin are cross-reactive material-negative (10, 13). They lack functional α-chain mRNA for cell-free translation (10) and hybridizable poly(A)* RNA in Northern blot analysis (14-16). However, Southern blot analysis has not shown any obvious alteration in genomic DNA (Refs. 16 and 17 and Footnote 3), in contrast to the French-Canadian Tay-Sachs α-chain gene which has a deletion at the 5′ terminus (17). A gene dosage effect (reduced β-hexosaminidase A activity in heterozygotes (1)) and failure to complement the defect in cells with a structural α-chain gene mutation (12) show the locus of Ashkenazi classic Tay-Sachs mutation to be in the α-chain gene rather than in a gene that would be trans-acting.

To determine whether the deficiency of α-chain mRNA is due to lack of transcription of the α-chain gene or to posttranscriptional events, we have assayed run-on transcription in nuclei isolated from Ashkenazi Tay-Sachs fibroblasts. The technique relies on elongation of nascent transcripts that had been initiated while the cells were intact and allows an estimate of transcription that is not affected by subsequent RNA processing or stability (18, 19).

EXPERIMENTAL PROCEDURES

Materials—Restriction endonuclease enzymes were purchased from International Biotechnologies Inc. (New Haven, CT). Ribonuclease T1, RNase A, RNase T2, DNaSE I, and agarose were obtained from Pharmacia LKB Biotechnology Inc. Bovine serum albumin, proteinase K, yeast tRNA, and nick-translation kits were purchased from Bethesda Research Laboratories. Creatine kinase and phosphocreatine were obtained from Boehringer-Mannheim. [α-32P]JCTP

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(3000 Ci/mmol), α-32PdCTP (3000 Ci/mmol), and Hybrid-mAP messenger RNA affinity paper were obtained from Amersham Corp. A dot-blot manifold and BA-85 (0.45-μm pore size) nitrocellulose filters were purchased from Schleicher & Schuell. Riboprobe transcription kits and SP6 RNA-polymerase were purchased from Promega Biotech (Madison, WI).

Cell Strain Conditions—A normal human fibroblast strain of fetal lung origin (IMR90) was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Ashkenazi Tay-Sachs fibroblasts of fetal skin origin (designated Ash-F and Ash-L) were provided by Dr. M. M. Kaback (University of California, San Diego, CA). A third Ashkenazi Tay-Sachs fetal skin fibroblast strain (designated Ash-S) was from Dr. E. E. Grether (Jefferson Medical College, Philadelphia, PA). Skin fibroblasts from a Sandhoff patient (WG98) were obtained from the Repository of Mutant Human Cell Strains (Montreal, Canada). Cells were cultured in Eagle's minimal essential medium, supplemented with nonessential amino acids, penicillin and streptomycin, sodium pyruvate, and 15% fetal bovine serum, at 35 °C and in 5% CO2. All tissue culture reagents were provided by Gibco.

Recombinant Plasmids—Complementary DNA encoding the entire human β-hexosaminidase α-subunit, pSH05, was provided by Dr. R. Myerowitz (NIH, Bethesda, MD) and was recloned into an SP6 vector as described elsewhere (18, 20). The β-2 kb insert was recovered by PsiI and Xhol double digestion. Complementary DNA encoding the entire β-hexosaminidase β-subunit cloned in pUC18 (pUC21) and in pSP64 (pSP64-β) was obtained from Dr. R. Proia (NIH, Bethesda, MD). The plasmid pUC21 was linearized by KpnI; the β-insert was rescued by PsiI digestion of pSP64-β. Human immunodeficiency α-chain cDNA (pTD-HIV-α3511-1516) (20) was provided by Dr. N. Brown (UCLA School of Medicine, Los Angeles, CA), and its insert was recovered by PsiI digestion. Untransformed 3T3 regions of human β-actin (p33'UT) and γ-actin (pγ3'UT) actin cDNA plasmids were provided by Dr. L. Kedes (Stanford University, Stanford, CA) (21). Escherichia coli HB101 strains carrying recombinant plasmids were grown in Luria-broth under antibiotic selection. Plasmid isolation was performed as described by Maniatis et al. (22). After appropriate digestion with restriction endonucleases, recombinant cDNA inserts were purified by preparative agarose gel electrophoresis and electroelution (22).

RNA Preparation and Northern Blot Analysis—Total RNA was isolated by a modification of the procedure of Chirgwin et al. (23). Confluent fibroblasts grown in 10 15-cm2 flasks were trypsinized, washed in phosphate-buffered saline, and stored as frozen pellets at -70 °C. Frozen cell pellets were disrupted in a baked Dounce homogenizer in the presence of guanidinium isothiocyanate lysis buffer (24); poly(A)+ RNA was purified by anion-exchange chromatography from hydrophobic gel (24). RNA transfer onto nitrocellulose filter, blot prehybridization, and hybridization were performed according to published procedures (25, 26). Nick-translated β-hexosaminidase cDNA inserts were used as hybridization probes (specific activity 5 106 cpn/μg). Blot filters were washed in 1 5 mM sodium citrate, pH 7, 15 mM NaCl, 0.1% sodium dodecyl sulfate at 65 °C. SP6 in Vitro Transcription—The α-subunit cDNA template (pSP64-α) was linearized at the 3′ terminus of the insert by HindIII digestion; the β-subunit cDNA template (pSP64-β) was linearized by Sall digestion. To synthesize 32P-labeled RNA probes, 32P-CTP (0.1 mCi, 3000 Ci/mmol) was added to the Riboprobe transcription reaction mixture to a final concentration of 0.45 μCi/ml. For hybridization experiments, each Riboprobe transcription kit was dispensed as the manufacturer recommended. Isolation of Nuclei and in Vitro Run-on Transcription—For each experiment with Ashkenazi Tay-Sachs cells an identical experiment with normal (IMR90) cells was performed the following day as control for transcription and hybridization. Each Tay-Sachs cell strain was analyzed at least twice. Duplicate cDNA probe filters were used for each hybridization.

For each run-off transcription experiment, fibroblasts were transfected from 5 confluent 15-cm2 flasks into 20 150 25-mm plates and maintained for about 1 week in order to obtain 106 cells. Two days prior to harvest, fresh culture medium was added to the confluent cells. For harvesting, the plates were rapidly chilled on an ice bath, and the cells were scraped and washed twice in cold phosphate-buffered saline. All subsequent steps were processed as fast as possible at 0 or 4 °C.

The transcription experiments were performed by a modification of the procedure developed by Wang and Calame (27), which is in turn a modification of earlier procedures (28, 29). Nuclei were isolated from the cell pellet by gentle lysis in a baked Dounce homogenizer in the presence of buffer containing (30% sucrose, 0.5% Triton X-100, 25 mM Tris, pH 7.5, 25 mM KCl, 7.5 mM MgCl2). To remove detergent, the nuclear pellet was washed in 0.1 M Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl2 and then in wash buffer, 20 mM Tris, pH 8.0, 10 mM MgCl2, 1 mM MnCl2, 14 mM β-mercaptoethanol, 20% glycerol, 0.14 M KCl. The transcription reaction mixture contained the following: wash buffer; 90 ng/ml creatine kinase; 9 mM phosphocreatine; 0.5 mM ATP; 0.25 mM UTP and GTP; 1 mM of (α-32P)CTP (3000 Ci/mmol) and unlabeled CTP to give a final concentration of ~0.45 μM. The mixture was incubated at 30 °C for 20 min. Radiolabeled RNA was purified by serial treatment with DNase I and proteinase K digestion, trichloroacetic acid precipitation, phenol extraction, and ethanol precipitation as described (27).

For hybridization, 5 μg of linearized plasmid probes (pBR322, pUC18, p33'UT, pγ3'UT, pUC31) and cDNA insert probes (pH05, pSP64-β) was denatured with alkali and dotted onto nitrocellulose filters, which were then baked at 80 °C in vacuo for 2 h. Duplicate filters for each probe were prehybridized in 4 10 Denhardt's with 1 mg/ml yeast RNA at 42 °C for at least 4 h (20 10 Denhardt's = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll). Labeled RNA from run-on transcription was ethanol-precipitated, dried, resuspended in -100 μl of 50% deionized formamide, 0.25 M NaCl, 0.02 M Pipes, pH 7.0, 2 mM EDTA, 0.4% sodium dodecyl sulfate, and incubated with the dots for hybridization at 42 °C for 60 h. The filters were washed at 45 °C under conditions identical to those in the published procedure (27). Finally, the filter dots were exposed at -70 °C to Kodak XAR-5 film between two intensifying screens and the radioactivity quantified by scintillation counting after autoradiography.

RESULTS AND DISCUSSION

Absence of α-Chain poly(A)+ RNA in Tay-Sachs Fibroblasts—The absence of β-hexosaminidase α-chain poly(A)+ RNA, previously demonstrated in a number of Ashkenazi cell strains (14–16), was confirmed for the strains used in our study. The poly(A)+ RNA was analyzed by Northern blot hybridization with labeled pH05 insert. As shown previously, there are two α-chain poly(A)+ RNA species (2.1 kb (major) and 2.6 kb (minor)) in normal (IMR90) and Sandhoff cell strains (WG98). However, there was a lack of hybridizable α-chain steady-state mRNA in the three Ashkenazi Tay-Sachs cell strains (Fig. 1, panel A), although a finite amount, <1% absent.
of normal, could be detected after prolonged exposure (not shown). Subsequent rehybridization with a labeled β-chain probe showed that there had been no major RNA degradation in the samples and that β-chain mRNA was present at equivalent levels in the Tay-Sachs and normal cell strains (but not in the Sandhoff disease sample, which has a mutation in the β-chain gene) (Fig. 1, panel B).

**Normal Run-on Transcription in Tay-Sachs Fibroblasts—** Typically, ~6–10 x 10^3 cpm was incorporated into total RNA, of which ~100 cpm was eventually found hybridized to the β-hexosaminidase probes. The low abundance of β-hexosaminidase transcripts is not particularly surprising, since β-hexosaminidase probes. The low abundance of β-hexosaminidase transcripts is not particularly surprising, since β-hexosaminidase transcripts are in the Sandhoff disease sample, which has a mutation in the α-chain gene (Fig. 1, panel E). The top line of each set represents the filters shown in Fig. 2, α-chain nuclear transcripts were labeled in the Tay-Sachs nuclei to the same extent as in the normals. Bacterial plasmids, pBR322 and pUC18, which served as negative controls for transcription, showed no hybridization signal. On the other hand, human β- and γ-actin and β-hexosaminidase β-chain probes, which served as positive controls for transcription, showed hybridizable messages. Quantitation of the radioactivity in the dots by scintillation counting (Table I) confirmed that the extent of α-chain transcription in Ashkenazi Tay-Sachs cells was comparable to that found in normal cells (IMR90). Radioactivity in the α-chain transcripts relative to that in the β-chain transcripts was 1.3 for each of the three Ashkenazi Tay-Sachs strains, compared with 1.1 and 1.7 for the IMR90 strain (Table I).

Because of the 55% homology between α- and β-chain cDNAs (16), we considered whether the β-hexosaminidase nuclear run-on transcripts hybridizing to the α-chain cDNA probe might have been transcripts of the β-chain gene. To test this possibility, 32P-labeled α- and β-chain mRNAs were synthesized in the SP6 expression system, then hybridized to probes and washed under conditions identical to those used for the nuclear transcripts. The substitution of mRNAs for nuclear transcripts to test the specificity of cDNA probes appears valid since only matching exon sequences of the transcripts would remain on the cDNA filter after digestion with RNases. As shown in Table II, the labeled α-chain mRNA hybridized to the α-chain probe without any hybridization to the β-chain or unrelated probes; conversely, labeled β-chain mRNA hybridized only to the β-chain probe. The specificity of this hybridization assay indicates that the radioactivity hybridizing to the α-chain cDNA filter (Fig. 2) was a result of transcription from the α-chain gene.

![Table I](#)

**Table I**

<table>
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<tr>
<th>Probe</th>
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**Table II**

**Specificity of the hybridization assay**

Radiolabeled α- or β-chain mRNA synthesized by the SP6 RNA polymerase expression system, 5 x 10^4 cpm each, were hybridized to the indicated probes and washed under conditions identical to those used for nuclear transcripts. The radioactivity bound to the filter was quantified by scintillation counting. The small amount of β-chain hybridization to the pBR322 and β-actin (pβ3'UT) plasmid probes may be due to a short pBR322 sequence in the pSP64-β, construct which was used as template to generate β-chain mRNA.

**Fig. 3**

**Competition hybridization to α-chain cDNA by pre-saturation with α-chain mRNA.** Nuclear run-on transcripts were synthesized in Ashkenazi Tay-Sachs nuclei (Ash-S) as described in the text. Probe dot filters were preincubated for ~16 h with either yeast tRNA (−competitor) or with β-hexosaminidase α-chain mRNA (+competitor) prior to addition of labeled nuclear run-on transcripts (1 x 10^5 cpm).
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Finally, the identity of the α-chain transcripts synthesized by the Tay-Sachs nuclei was further confirmed by competition hybridization with excess unlabeled α-chain mRNA (30). Filter probes were prehybridized with α-chain mRNA for 4 h, followed by a 16-h prehybridization with unlabeled α-chain mRNA (~70 μg, synthesized from an SP6 expression system) prior to the addition of labeled nuclear transcripts. The addition of this unlabeled RNA competitor markedly diminished the amount of labeled nuclear RNA hybridizing to the α-chain probe (Fig. 3). Quantitation of the radioactivity in the dots by scintillation counting showed an α/β ratio of 0.4 in the presence of competing α-chain mRNA, compared with 1.6 in the control. There was no difference in hybridization of the actin or β-chain transcripts under the two conditions. Thus, at least three quarters of the radioactivity hybridizing to the α-chain cDNA has been identified as α-chain transcript. Whether the residual uncompetited hybridization is due to incomplete saturation of the filters or to presence of some different (e.g. antisense) transcript is not known.

Normal transcription of the β-hexosaminidase α-chain gene rules out a promoter mutation as the cause of mRNA deficiency and implicates a defect in some posttranscriptional process. The mutation might affect RNA processing, nuclear to cytoplasmic transport, mRNA stability, or some combination thereof. The thalassemias provide numerous examples of nucleotide changes that prevent normal RNA splicing and lead to absence of mRNA (reviewed in Refs. 3 and 32). They also provide instances of mRNA deficiency associated with a premature termination codon; the mechanism by which mutations in the coding region affect the level of mRNA is not known, but impaired transport through nucleopores has been suggested (33, 34). Recent mapping of the β-hexosaminidase α-chain gene has shown it to contain >40 kb and 14 exons (35); this large structure must offer many opportunities for mutational error.

In our terminology and discussion, we have made the usual assumption that there is only one mutation in the Ashkenazi population that gives rise to classic Tay-Sachs disease. This assumption is reasonable but not proven. The same population harbors one or more rare alleles that give rise to the milder variants of Tay-Sachs disease (4, 36). The number of mutations associated with Ashkenazi classic Tay-Sachs disease will become apparent as the identification of the defect becomes more precise.

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