Vol. 181, No. 1, 1991
November 27, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 108-115

MOLECULAR BASIS OF AN ADULT FORM OF ß-HEXOSAMINIDASE B DEFICIENCY WITH MOTOR NEURON DISEASE

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Received September 13, 1991

A patient (KL) with progressive motor neuron disease associated with partial Hex A (αß) and no Hex B (ßß) activity, synthesized ß-chains which only associated with α-chains. To identify the molecular basis of this inability of ß-chains to self associate, RNA from cultured fibroblasts was reverse transcribed, the cDNA encoding the ß-chain amplified by polymerase chain reaction, subcloned, and sequenced to reveal two types of single missense mutation. The first mutation, (Type I) ^{619}A→G, was paternally inherited and converted a ^{207}Ile→Val in a highly conserved region believed to be associated with catalytic activity and activator protein binding. Biochemical evidence for impaired activator protein binding was obtained by purifying Hex A from KL urine and demonstrating a greater than 50% reduction of *in vitro* G_{M2} hydrolysis compared to normal urinary Hex A. In other cDNA species (Type II), a maternally inherited ^{1367}A→C mutation converted ^{456}Tyr→Ser in another highly conserved region of the ß-chain and we propose that this mutation leads to the inability of the ß-chains to self associate and thus reach maturity. These same cDNA species contained a second ^{362}A→G mutation which converted ^{121}Lys→Arg, but is apparently a polymorphism since it also occurs in some normal subjects. We propose that the patient is a compound heterozygote in which a combination of no self-association of the mutant ß-chains and impaired activator protein binding to α-$ß_{(mutant)}$ (Hex A) required for G_{M2} hydrolysis result in total ß-Hex B deficiency and slow accumulation of G_{M2} ganglioside, primarily in motor neurons.

Lysosomal N-Acetyl-ß-D-hexosaminidase is a multisubunit enzyme with two major isoforms, Hex B, (ß,ß) and Hex A [α,ß plus an activator protein] (1). Hydrolysis *in vivo* of G_{M2} by ß-Hex A requires an activator protein called G_{M2}-activator (2, 3). In Ashkenazi Jews, 97% of Tay-Sachs disease results from mutations in either exon 11 or 12 of α-chain, which prevents mRNA production (severe infantile form) (1, 4), and 3% results from a point mutation in exon 7 (^{269}Ser→Gly) in combination with an exon 11 or 12 mutation in the other allele (5) to produce a later onset form of the disease. Many other α-chain mutations have been described (5) but of particular interest are those involving either ^{170}Arg or ^{178}Arg (1, 6), which result in normal activity towards synthetic substrates but failure to hydrolyze G_{M2} (1). Moreover, introduction of the "B1 Variant" mutation, ^{178}Arg→His, into the ß subunit was found to produce ß-hexosaminidase B without catalytic activity (7). Therefore, it is expected that the ^{170}Arg and ^{178}Arg containing region on the α subunit and the homologous regions on the ß subunit directly or indirectly affect catalytic activity of the A and B isoenzymes, respectively. Approximately 25% of infantile Sandhoff disease results from a deletion of half the ß-subunit

0006-291X/91 $1.50

gene (exons 1-5) (8) and the absence of ß-chains renders α-chains unstable, producing a total deficiency of ß-Hex (1). Mutations have been reported for juvenile Sandhoff disease, a 24 bp insertion between exons 12 and 13, leading to abnormal splicing (9), and intron insertions in mRNA which lead to elongated ß-subunits and unstable enzyme (10). A further type of ß-Hex deficiency is illustrated by patient KL (11, 12), with some Hex A activity, but no Hex B activity when assayed with synthetic substrates, and reduced ability to hydrolyze G_{M2} ganglioside. The patient was presented clinically as juvenile muscular atrophy with progressive lower motor neuron disease, and we report here the existence of two deleterious alleles in the patient's ß-chain gene.

MATERIALS AND METHODS

Commercial sources were as follows: radioisotopes (Amersham), restriction enzymes and DNA modifying enzymes (Boehringer Mannheim), pBluescript vector (STRATAGENE), 4MUß-GlcNAc and 4MUß-GlcNAc-6-sulfate (Koch Light), polymerase chain reaction (PCR) kits (Perkin Elmer), "Sequenase" sequencing kit (U.S. Biochemicals) electrophoresis standards, acrylamide and agarose (BRL), and zetaprobe membranes (Bio-Rad). pCD vector containing ß-Hex ß-chain cDNA inserted into a unique Xho I site was a generous gift from Dr. Mahuran, University of Toronto, Canada. The oligonucleotides used were synthesized by the Howard Hughes Medical Inst. (University of Chicago) and fibroblast cells were obtained from the Kennedy Center tissue culture facility (Univ. Chicago).

Isolation of total RNA and genomic DNA were carried out according to standard methods (13). Recombinant, double stranded plasmids were isolated by alkali lysis and further purified by PEG-8000 precipitation (14). Sequencing of the inserts by "Sequenase" sequencing kit was carried out directly from the double stranded plasmid DNA as described by the manufacturer (U.S. Biochemicals).

mRNA was isolated from fibroblasts (15) and reverse-transcribed to obtain cDNA. Synthetic oligonucleotide primers, (5' end starting at base 179, a 19-mer G CTC TCG GTG AAG ATG ACC), and 3' end starting at base pair 1697 a 20-mer GA TTG CTG TGG CCT TTT TCC) were used with Taq polymerase through 25 cycles of PCR (16). A 1.5 kb product was blunt-end ligated into the Sma I site of pBluescript vector (STRATAGENE). The construct obtained was used to transform competent cells (DH5α), and plated on LB agar (plus ampicillin, IPTG and X-Gal). After overnight incubation at 37 °C, recombinants (white colonies) were picked, out of which three were shown to be the expected 1.5 kb after purification and analysis of restriction fragments. The inserts were then sequenced using universal primers, and the synthetic oligonucleotides as primers (Fig.1).

ß-Hex A_1 and A_2 from normal urine and patient (KL) urine were partially purified from ammonium sulfate precipitated urinary proteins using DEAE-Sephadex A-50 columns (17). Tritium labeled G_{M2} ganglioside was prepared by the galactose oxidase and NaB^3H_4 reduction procedure as described by Radin (18) with slight modification (19). G_{M2}-activator was prepared from human liver and KL urine as described previously (20). Hydrolysis of G_{M2} by ß-Hex in the presence of G_{M2}-activator was carried out in a mixture containing the following components in 0.25 ml : ^{3}H-labeled G_{M2} ganglioside (7.5 x 10^3 cpm), 10 nmol; acetate buffer (10 mM), pH 4.6; ß-Hex, 0.1 unit and 5 µg of G_{M2}-activator. The mixture was incubated at 37 °C for 1 to 16 h. The reaction was terminated by heating the mixture at 100 °C for 3 min, and the liberated radioactive GalNAc was determined by the dialysis method (5). After dialysis, the dialysate was analyzed for the liberated radioactive GalNAc, and the retentate, for G_{M3} ganglioside by thin layer chromatography (5).

Patient (KL) and control fibroblast samples were radiolabeled for 16 h with [^{35}S] methionine 100 µCi/ml, cells washed three times with cold (4 °C) 10 mM phosphate buffered saline and then lysed by sonication in 10 mM NaH_2PO_4-NaOH, pH 6.0 (0.5 ml/confluent plate) at 4 °C. The lysates obtained were centrifuged at 10, 000 x g for 15 min (4 °C) and the supernatants were loaded (400 µg protein/column) on 2-ml DE 52 columns preequilibrated with 10 mM NaH_2PO_4-NaOH (pH 6.0). Following absorption of proteins to each closed column for 1 h at 4 °C, the unabsorbed proteins (Hex B) were washed out using 10 ml of equilibration

buffer and the absorbed proteins (Hex A) were eluted using 6 ml of 0.25 M NaCl in the equilibration buffer. Hex A and Hex B fractions were dialyzed against deionized water, lyophilized and the residue obtained was reconstituted in 10 mM Tris. HCl (pH 7.2), 125 mM NaCl, 1% Triton X-100, 0.05% Nonidet P-40 (0.5 ml/sample). The separated ß-Hex A and B proteins were immunoprecipitated using polyclonal Hex B antibody and Pansorbin, and the immunoprecipitates subjected to SDS-PAGE and autoradiography.

In order to trace the parental origin of the mutations in patient (KL) DNA, cDNA was prepared from K.L., her parents and a sibling and relevant portions were PCR amplified employing oligonucleotides 2 and 3, and 6 and 1, respectively (Fig. 1). PCR products amplified with oligonucleotides 2 and 3 were probed with radiolabeled oligonucleotide 11 to identify the $^{619}A \rightarrow G$ ($^{207}Ile \rightarrow Val$) mutation. The region amplified with oligonucleotides 6 and 1 was probed with ^{32}P-labeled oligonucleotide 12 in order to detect the $^{1367}A \rightarrow C$ ($^{456}Tyr \rightarrow Ser$) mutation. Amplified material was applied to a zetaprobe membrane employing the Bio-Rad Bio-Slot apparatus as per manufacturer's instructions. Oligonucleotide probes were radiolabeled with γ-^{32}P-ATP using T_4 polynucleotide kinase (13). Membrane bound PCR products were hybridized in 0.5 M sodium phosphate buffer (pH 7.2) containing 7% SDS as described before (13). Oligonucleotide hybridizations were carried out as follows: for oligonucletide 11, overnight at 52 °C, and for oligonucleotide 12, overnight at 58 °C. Membranes were washed with 40 mM sodium phosphate buffer (pH 7.2) containing 1% SDS for 5 min at room temperature (~23 °C) and then at 42 °C for 15 min, and subsequently, autoradiography was carried out at -70 °C with a DuPont Lightning Plus intensifying screen.

RESULTS AND DISCUSSION

Incubation of cultured skin fibroblast homogenates with 4MUß-GlcNAc showed total ß-Hex activity in KL (11, 12) to be 30-35% of normal (over 97% of it thermolabile, i.e., Hex A). Using 4MUß-GlcNAc-6-sulfate as substrate we observed Hex A levels of 21-23% of control

Synthetic Oligonucleotides used

1) GA TTG CTG TGG CCT TTT TCC (3' end)

2) G CTC TCG GTG AAG ATG ACC (5' end)

3) TCG AAT TCC TCG TAA TCT G

4) C AGA TAA CGA GGA ATT CGA

5) ACC ATC AAT GAA TCC ACC A

6) GG AAA GAC AGC GCA TAT CCT G

7) C AGG ATA TGC GCT GTC TTT CC

8) AGA GGA ATT TTG GTT GAT ACA ($^{619}A \rightarrow G$, $^{207}Ile \rightarrow Val$)

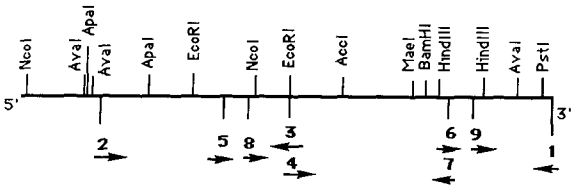


Fig. 1. Oligonucleotides used for PCR-amplification, sequencing and oligonucleotide hybridization.

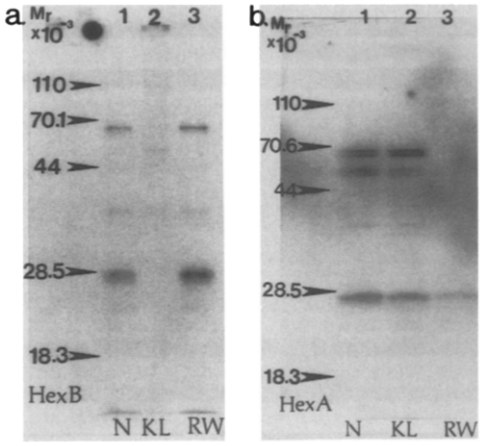
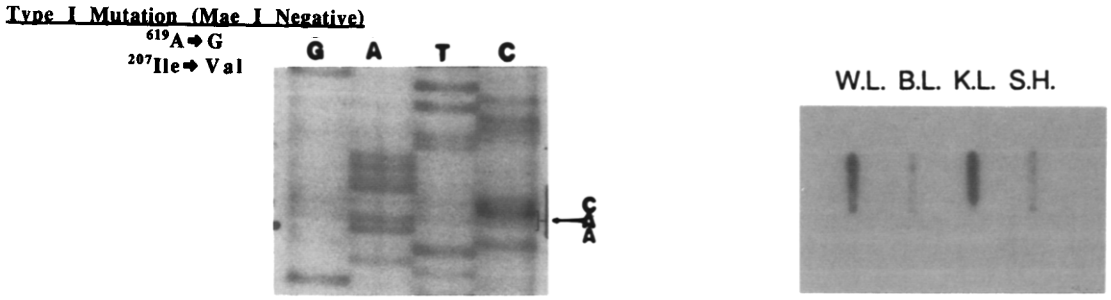


Fig. 2. Immunoprecipitation of Hex B and Hex A proteins. Fibroblast samples from a normal subject, a Tay Sachs patient (RW) and KL were labeled with [^{35}S] methionine, lysed, and Hex A and Hex B protein fractions separated by ion-exchange chromatography and immunoprecipitated as described in the text. (a) The mature (28.5 kDa) and precursor (67 kDa) forms of ß-chain in Hex B (ßß) protein fractions: normal (lane 1), KL (lane 2, note the absence of either form) and Tay Sachs patient (RW) (lane 3, note the increase). (b) The mature and precursor forms of ß-chain in Hex A (αß) protein fractions: normal (lane 1), KL (lane 2, note the presence of both forms) and Tay Sachs patient (RW) (lane 3, note the virtual absence of Hex A).

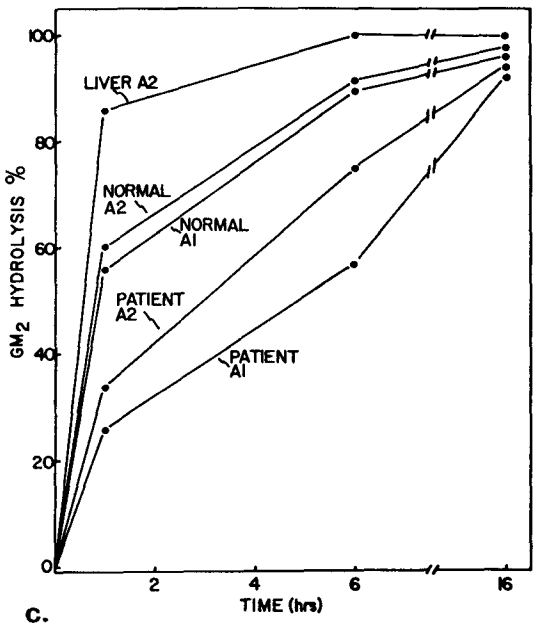
and pulse-chase labelling studies showed normal synthesis of α-chains and deficient ß-chain synthesis (15). Immunoprecipitation of anti-Hex B specific, ^{35}S-labeled proteins from DEAE-52 ion-exchange column fractions showed the total absence of Hex B protein (Fig. 2a) and presence of Hex A protein (Fig. 2b) in KL fibroblast lysates. Since proper subunit-association is a prerequisite for the normal pI of either Hex A or B and their respective maturation in cells, we can infer that all the mutant ß-chains synthesized in KL associate only with the normal α subunits in order to produce only Hex A and no Hex B. To identify the mutation(s) on the ß-chain which impair ß-ß association (Hex B) preferentially over α-ß (Hex A) association, we sequenced the ß-chain encoding cDNA. We have previously shown by Southern blot analysis that there were no major insertions or deletions in ß-chain in either allele (15) and no evidence of reduced amounts or abnormally sized mRNA in patient KL (15). The cDNA made from fibroblast mRNA, following amplification by the polymerase chain reaction, showed three point mutations resulting in three new amino acids as compared to the published normal sequence. Two were found in what we have designated Type II cDNA and another in a separate cDNA (designated Type I). In Type I we found a single A➡G mutation at base pair 619 (Fig.3a) which should result in coding for a Val (GTT) rather than Ile (ATT) at residue 207 in a region which is highly conserved between α- and ß- chains as well as between species(1). Further, from allele specific oligonucleotide hybridization experiments as described in the methods (Fig. 3b), this ^{619}A➡G mutation was also found in the father of KL, who we have previously shown to be completely normal in the ability to hydrolyze the synthetic substrate 4MUß-GlcNAc (12).

We believe that this mutation results in impaired activator protein binding since other mutations in the corresponding region of the highly homologous α-chain can give rise to a Hex A protein which can hydrolyse synthetic 4MUß-GlcNAc, but not G_{M2} (for example; ^{533}G➡A, ^{178}Arg➡His; ^{532}G➡T, ^{178}Arg➡Cys, and ^{509}G➡A, ^{170}Arg➡Gln) (1, 5, 6, 21). Expression of



a. The (-) strand shows the AAT➡AAC mutation (^{619}ATT➡GTT in the (+) strand i.e. ^{207}Ile➡Val).

b.



c.

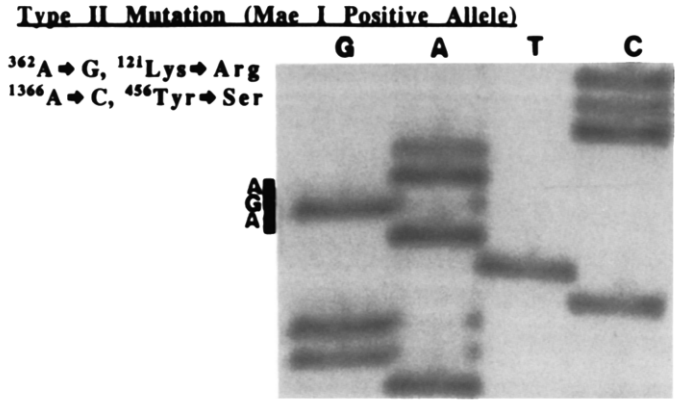
Fig. 3. The Type I mutation. (a) Sequencing gel pattern of the (-) strand showing the AAT➡AAC [i.e. AAT➡GTT in the (+) strand, ^{207}Ile➡Val]; (b) Hybridization experiments using probe 8 confirmed that the ^{207}Ile➡Val mutation was present in the father (WL) and in KL, but not present in the mother (BL) and a sibling (SH). (c) Hydrolysis of G_{M2} in the presence of normal G_{M2}-activator by ß-Hex A1 and A2 isolated from human liver, normal urine and patient KL urine. The data are expressed as % of hydrolysis in comparison with hydrolysis by the same unit (0.1 unit) of ß-Hex isolated from normal human liver. G_{M2} hydrolyses were determined as described in the text. Liver A2, ß-Hex A2 isolated from normal human liver; normal A1 and A2, ß-Hex A1 and A2 isolated from the urine specimen of a normal subject; patient A1 and A2, ß-Hex A1 and A2 isolated from the urine specimen of patient KL. A second experiment, run in the presence of G_{M2}-activator isolated from KL urine, produced identical results.

the ^{178}Arg➡His mutation in the ß-chain (^{211}Arg➡His) produces a ß-hexosaminidase B devoid of catalytic activity (7) suggesting that this region **(-Pro-^{170}Arg-Gly-Leu**(Ileu (ß))**Leu-Leu** (Ileu(ß))-**Asp-Thr-Ser-^{178}Arg-His-Tyr-Leu-Pro-)** is involved in the catalytic activity of both isoenzymes. During the hydrolysis of G_{M2} by ß-hexosaminidase A, the G_{M2}-activator protein binds closely to the catalytic center, which is believed to be flanked by the catalytic domains of the α- and the ß- chains (22), and therefore mutations in

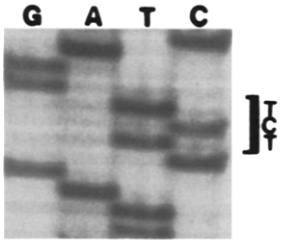
the catalytic region of the ß subunit could also affect activator protein binding. The interior packing and hydrophobicity of this region could be essential for the stability and biological activity of both chains of ß-Hex and would be compromised by such a mutation. For example, in bacteriophage f1 V-protein, mutating -Leu-Ile-Asp into -Leu-Val-Asp-(23) produced a free energy change ($\Delta G°$) of 2.4 kcal/mol, sufficient to cause changes in protein conformation or stability. The rather unexpectedly large destabilization caused by Ile➡Val was attributed to both reduced hydrophobicity (Val is less apolar), but more importantly to packing differences, the creation of cavities where the extra methyl group previously resided (24). The hypothesis of a conformational change leading to impaired G_{M2} hydrolysis, was supported by the finding that *in vitro* hydrolysis of [^{3}H] G_{M2} (in the presence of either normal or KL activator protein) by Hex A purified from KL urine (20) had initial G_{M2} hydrolysis rates of approximately 50% of normal (Fig. 3c). Further, feeding [^{3}H] G_{M2} to cultured fibroblasts and chasing the label for 10 days to allow catabolism, showed about 35% catabolism compared to controls (unpublished data). The heat inactivation profile of Hex A mediated and G_{M2}-activator independant 4MUß-GlcNAc hydrolytic activity in KL fibroblasts was also found to be normal (11), thus negating the possibility of impaired hydrolysis of G_{M2} due to a "less active and unstable αß complex". The Type I mutation therefore most likely inhibits G_{M2}-activator protein binding and results in impaired G_{M2} catabolism.

In Type II we found two differences from the published sequence (25, 26) of ß-Hex ß-chain, a single A➡C mutation at ^{1367}A, which converts ^{456}Tyr➡Ser and a single A ➡G mutation at ^{362}A which converts AAA (^{121}Lys)➡AGA (Arg) (Fig. 4). It is believed (1) that the carboxy terminal regions of the α– and ß- chains are important for subunit association, trafficking and processing of the A and B isoenzymes. An α-chain mutation in an (^{504}Arg➡His) has been shown to impair subunit association (21) and another (^{482}Glu➡Lys) has been shown to impair trafficking and maturation of ß-hexosaminidase A (27). Thus, it is likely that the ^{456}Tyr➡Ser mutation, which is in a highly homologous region near the carboxy terminal, could affect self-association and maturation of ß-chains thus leading to the absence of ß-hexosaminidase activity in the patient. Further, a Chou-Fassman analysis of a dodecapeptide stretch of the ß-chain centered around the ^{456}Tyr➡Ser mutation revealed high probability of a major change in conformation and protein folding due to this mutation (unpublished data). The mother of KL, who only expresses 50% of Hex B activity in cultured skin fibroblasts (12), presumably has this mutation on one allele while her other allele is normal.

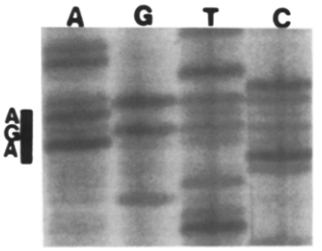
The other mutation, resulting in ^{121}Lys➡Arg, produces no change in net charge (both are basic), but introduces an additional -NH_2 group. However, this appears to be a common polymorphism since Wakamatsu and coworkers (28, and unpublished data) have shown it to produce normal ß-chain when expressed in COS cells. The region surrounding ^{121}Lys is not homologous in either human α- and ß-chains (-**Arg-His-Thr-Leu-**in α-versus **-Ala-Lys-Thr-Gln** in ß-), or in mouse ß-Hex and therefore is less likely to be of functional importance. This mutation introduced a new Mae I restriction endonuclease site (CTAG) which generated fragments of 360, 780, and 630 bp in the PCR-amplified cDNA of Type II ß-chains as compared to 1140 and 630 in the wild type ß-chain and also in the Type I allele. It allowed



a) The ^{362}A➡G (AAA➡AGA, Lys➡Arg) mutation in the (+) strand generates a Mae I site (C/TAG).



b) The ^{1366}A➡C (TAT➡TCT, Tyr➡Ser) mutation shown in the (+) strand of one Mae I positive clone.



c) The ^{1366}A➡C (T➡G in the (-) strand i.e. ATA➡AGA) shown in the (-) strand of a different Mae I positive clone.

Fig. 4. Sequencing gels demonstrating the presence of Type II mutations in the second allele.

us to verify that this was not a PCR artifact since it was seen together with the ^{1367}G➡A (^{456}Tyr➡Ser) mutation in PCR-products which were independently amplified from KL cDNA.

In summary, we have identified two novel and apparently deleterious mutant alleles in a patient with ß-Hex B deficiency and inherited progressive motor neuron disease. In one allele, inherited from the father, we predict that the the mutation allows formation of ß-chain, but the resulting ß-Hex A [α ß] hydrolyses G_{M2} at a reduced rate because of impaired activator protein binding. The other allelic mutation is presumably maternally inherited and results in the lack of ß-chain self association, and thus premature degradation of ß-chains which are not bound to α-chains. We believe that this explains why we only find approximately 50% of the normal amount of mature ß-chain in KL cells.

ACKNOWLEDGMENTS

The excellent technical assistance of Mr. Jonathan Korach is gratefully acknowledged. This work was supported by USPHS Grants HD-06426 and NS-09626.

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