THE GENETIC DEFECTS IN GANGLIOSIDE STORAGE DISEASES

PROEFSCHRIFT

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Waarom ter wereld
hebben ze groene rupsen
horens gegeven ?

Taniguchi Buson (1715-1783)

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CHAPTER I

GENERAL INTRODUCTION

I.1. General approaches

The first clinical reports of patients suffering from a sphingolipid storage disease appeared shortly before the turn of the present century (for instance Tay,1881; Gaucher, 1882; Sachs,1887). Most authors noted the familial occurrence, the retardation in motor and mental development and the rapidly progressive course of these disorders. More specific clinical symptoms are hepatosplenomegaly and bony deformities (Gaucher's disease; G_{M1}-gangliosidosis), a cherry-red spot in the macula (most prominent in Tay-Sachs disease but also present in some other lipidoses), blindness and deafness (Krabbe's disease; G_{M2}-gangliosidosis; Metachromatic Leukodystrophy) and vascular lesions such as angiokeratomata (Fabry's disease; variants of G_{M1}-gangliosidosis). In the classical forms of the sphingolipidoses the onset of the symptoms usually occurs in the first year(s) of life, and most patients die in early childhood. Most of the disorders are panethnic, but in some of them the incidence is higher among certain populations. The incidence of Tay-Sachs disease, for instance, is as high as 1:3600 newborns among Ashkenazy Jews, whereas among Caucasians the incidence is less than 1 in 100 000 liveborns. The incidence of most sphingolipidoses is low.

During the last decades an increasing number of variants has been described; they often showed different clinical features, the onset of symptoms might be later and the course of the disease milder. Delineation on the basis of

clinical features alone has therefore been difficult in many cases. Studies on the pathological and histological manifestations of these disorders contributed considerably to the distinction of the various disorders. For example, Pick (1927) provided clear evidence of histological differences between what now is called Niemann-Pick disease and Gaucher's disease. In 1924, the typical globoid cells in the white matter of brain from a patient with Krabbe's disease have been described (Collier and Greenfield,1924) and this abnormality has for a long time been the main parameter for the definitive diagnosis of Krabbe's disease. The same was true for the metachromatic staining of the myelin in the nervous system of patients with Metachromatic Leukodystrophy (Alzheimer,1910).

Ultrastructural investigations of cells from most patients suffering from a sphingolipidosis revealed concentrically laminated, myelin-like figures with a periodicity of 50Å. In both Gaucher's disease and Krabbe's disease tubular arrangements are present, whereas in Metachromatic Leukodystrophy the presence of lamellar leaflets with a herringbone pattern has been shown (for reviews on ultrastructural abnormalities in sphingolipidoses see in Hers and van Hoof,1973).

The first disorder in which the chemistry of the accumulated lipid was correctly identified was Gaucher's disease. Aghion (1934) and Halliday et al.(1940) showed that in the organs and tissues of patients with Gaucher's disease the sphingolipid glucosylceramide accumulates. Glucosylceramide is composed of sphingosine, a long-chain fatty acid and a terminal molecule of glucose. The fatty acid and sphingosine form a complex called ceramide. Subsequently, the storage products in the other inborn errors of sphingolipid meta-

Table I.1. The sphingolipidoses

disease	storage product	enzyme deficiency
Niemann-Pick disease	Sphingomyelin	sphingomyelinase
Gaucher's disease	Glucosylceramide	glucocerebrosidase
Krabbe's disease or Globoid Cell Leuko-dystrophy	Galactosylceramide	galactosylceramide -β-galactosidase
Metachromatic Leuko-dystrophy	Galactosyl-3-sulfate-ceramide	arylsulfatase A
Fabry's disease	Globotriaosyl-ceramide	α-galactosidase A
G_{M1}-gangliosidosis	G_{M1}-ganglioside	G_{M1}-β-galactosidase
G_{M2}-gangliosidosis	G_{M2}-ganglioside	β-N-Acetyl-hexo-saminidase
Farber disease	Ceramide	ceramidase

bolism have been identified (for reviews see in: Hers and van Hoof,1973; Svennerholm,1976; in: Stanbury, Wijngaarden and Frederickson,1978). Ceramide was shown to be common to all sphingolipids that accumulate in the lipid storage diseases, but the composition of the terminal residue differed. (Table I.1.). The elucidation of the chemical composition of the storage products formed the basis for the identification of the responsible genetic enzyme deficiencies.

Gaucher's disease was also the first sphingolipid storage disease in which the nature of the underlying metabolic abnormality was established. Investigations on the biosynthesis of glucosylceramide indicated an essentially normal rate of the formation of this substance in spleen tissue obtained from patients with Gaucher's disease (Trams and Brady,1960). Some five years later, the deficiency of a catabolic enzyme

required for the hydrolysis of the β-glucosidic bond of glucosylceramide was demonstrated (Brady et al.,1965,1966). Based on the nature of the deficient enzyme in Gaucher's disease, the metabolic defect in other sphingolipidoses was postulated to be the lack of catabolic enzymes, involved in the degradation of the accumulating lipids. In the following years, deficiencies of catabolic enzymes were shown in all sphingolipid storage diseases (Table I.1.).

In 1955, de Duve et al. introduced the term "lysosome" to indicate membrane-surrounded cytoplasmic vesicles. With the use of differential centrifugation and with histochemical staining techniques, it was shown that lysosomes contain acid hydrolases (de Duve,1955,1965; Novikoff,1961). These enzymes can degrade proteins, oligosaccharides, nucleic acids and lipid material, exhibit maximal activity at acid pH, and are usually specific for one type of linkage (for review see de Duve and Wattiaux,1966). The concept of lysosomal storage diseases was first described by Hers (1965), who postulated that the two major criteria for such inborn errors of metabolism are the genetic deficiency of one of the acid hydrolases that are normally localized within the lysosome, and the accumulation of undigested material within single membrane bound organelles. The deficiency of acid α1,4- glucosidase in the glycogen storage disease, glycogenosis II (Pompe's disease) was the first example of such a lysosomal storage disease (Hers,1963; Baudhuin et al.,1964). In the sphingolipidoses, the lysosomal localization of the storage products could also be established. The enzymes involved in the degradation of the sphingolipids were shown to be localized in the lysosomes as well, and this characterized the

sphingolipidoses as lysosomal storage diseases.

Since the enzymic defects responsible for the sphingolipidoses are known, the biochemical assay has become an important tool in diagnosis. The enzyme defects are expressed in a variety of cells, tissues and body fluids, and the demonstration of an enzyme deficiency allows a diagnosis even before any clinical features are present. Enzyme assays in leucocytes or cultured fibroblasts sometimes enable the detection of heterozygotes, and enzyme analysis of cultured amniotic fluid cells allows prenatal detection of patients and thereby prevention if selective abortion is carried out (for review see Galjaard,1979).

In some genetic metabolic diseases the mutation can be demonstrated on the level of the DNA itself. With the use of restriction enzymes and DNA hybridization techniques Wong et al. (1978) were able to demonstrate a deletion in the globin gene of a patient with α-thallassaemia. Recently, Kan and Dozy (1978) performed a prenatal diagnosis of sickle cell anaemia by DNA analysis of amniotic fluid cells by taking advantage of a polymorphism very near to the β-globin gene. The great potential of this approach is that expression of the (enzyme)protein that is involved in the genetic defect is not required and any type of DNA-containing cell in principle can be used for (prenatal) diagnosis. As far as the diagnosis of sphingolipidoses is concerned recombinant DNA methods can be used only if specific messenger RNA's can be isolated.

A combination of clinical examiniation, pathological studies and biochemical assays of the responsible enzyme

defect has facilitated the characterization of patients with various types of sphingolipidoses. Moreover, one of the consequences of the increasing knowledge is that, what at first seemed to be a single clinical entity on closer scrunity appeared to be a heterogeneous group of conditions with varying clinical manifestations and/or biochemical characteristics. Clinical heterogeneity, expressed by the occurrence of progressive infantile forms, later onset juvenile forms and milder adult variants, is for example observed in Niemann-Pick disease, G_{M1}-gangliosidosis and Gaucher's disease, whereas biochemical heterogeneity has for instance been found in G_{M2}-gangliosidosis (for review see in: Stanbury et al., 1978; Galjaard,1979). The occurrence of this heterogeneity raises questions about the nature of the underlying gene defect, its correlation with the residual enzyme activity and the impairment of cellular functions.

As a first approach, the presence of different gene mutations in clinical or biochemical variants of the "same" disorder can be established by complementation analysis. This analysis is based on the principle that two mononuclear cells, each carrying an independent mutation affecting the same function, are brought together in a bikaryon using cell fusion techniques; if restoration of the affected functions (complementation) occurs in the heterokaryons this is a proof that two different gene mutations are involved in "the same" metabolic defect. De Weerd-Kastelein et al. (1972) were the first to perform complementation studies on cells from different clinical variants of the genetic skin disease xeroderma pigmentosum. They found restoration of DNA repair in binuclear heterokaryons, and similar complementation studies have since shown the presence of different gene

Table I.2. Diseases for which the presence of multiple complementation groups has been demonstrated.

Disease	Deficient enzyme or cellular function	Authors	no. of complementation groups
Xeroderma Pigmentosum	DNA repair	de Weerd-Kastelein et al.,1972; 1974 Bootsma and Galjaard,1979	7
Maple Syrup Urine Disease	decarboxylase of branched chain keto acids	Lyons et al.,1973	2
G_{M2}-gangliosidosis	β-N-Acetyl-hexosaminidase	Thomas et al.,1974 Galjaard et al.,1974a	2
Methylmalonic-acidemia	methyl malonyl-CoA mutase	Gravel et al.,1975 Willard et al.,1978,1979	6
G_{M1}-gangliosidosis	β-galactosidase	Galjaard et al.,1975	2
Propionyl-CoA carboxylase deficiency	propionyl-CoA carboxylase	Gravel et al.,1977 Wolf et al.,1978	2
Niemann-Pick disease	sphingomyelinase	Besley et al., to be published	2
Mucolipidoses (Sialidoses)	neuraminidase	Hoogeveen et al.,to be published d'Azzo et al.,1979	3

mutations in a number of variants of other genetic metabolic diseases (for review see Ruddle and Creagan,1975; Ringertz and Savage,1976; Bootsma and Galjaard,1979). These studies are summarized in table I.2.

Although the occurrence of genetic complementation indicates that different gene mutations are involved, the absence of restoration of enzyme activity in heterokaryons does not necessarily mean that different clinical or biochemical variants are caused by allelic mutations. No complementation has for instance been found after fusion of cells from patients with the infantile and adult form of Pompe's disease (Reuser et al.,1978) nor after fusion of cells from patients with the Hurler and Scheie syndrome, both with an α-L-iduronidase deficiency, or after hybridization of cells with different types of arylsulfatase deficiencies. Although the absence of multiple complementation groups may be the result of the molecular structure of the (iso)enzymes involved, it may also be due to some disadvantages inherent to the eukaryotic system. Heterokaryons after fusion of human fibroblasts have a limited life span and this might be too short for the necessary intracellular and biochemical events that are required before restoration of the particular metabolic function can be demonstrated. Also, the molecules which have to interact may be present in separate cell compartments. The problem of analyzing heterokaryons in the presence of homokaryons and unfused parental cells can be avoided by using microchemical analysis of single binuclear cells (Galjaard et al.,1974b,1975), or by separation of different cell populations after labelling with fluorescent beads using a FACS II Cell Sorter (Herzenberg et al.,1976).

Initially, complementation studies were described for micro-organisms (for review see Fincham, 1966). In his classical studies on bacteriophage genetics, Benzer (1957) devised a cis-trans test of genetic function. In this test, two haploid genomes, each carrying an independent mutation affecting the same metabolic function, are brought together in the same cell. If the two mutations are located in two different genes, restoration of the enzyme activity and/or the metabolic function will occur to the level of the wild type. This phenomenon is called intergenic complementation. On the other hand, if the two mutations are localized in the same gene, no enzyme at all or a much lower amount of active enzyme is produced. If a low amount of active enzyme is produced, this is the result of intragenic or interallelic complementation. Two similar polypeptides, which are defective in a different way, may associate and correction of the mutation by the tertiary structure of the associated subunits may lead to partial restoration of enzyme activity. This phenomenon will only occur in enzymes, which contain two or more identical subunits (Crick and Orgel,1964; for reviews see Fincham, 1966; Ratner and Rodin,1976).

Intragenic complementation has been described for Escherichia coli β-galactosidase. Both in vivo and in vitro experiments, using mutant strains carrying point mutations in the z-gene of the Lac-operon, resulted in restoration of β-galactosidase activity. Intragenic complementation was also achieved by using fragments of the E.coli β-galactosidase protein (α- and ω-complementation). In all these experiments it was demonstrated that the complemented enzyme was different in structure from the wild type enzyme (Zabin and Villarejo,1975).

In theory, both intergenic and intragenic complementation mechanisms could occur in eukaryotic systems as well. If intergenic complementation occurs, the mutation might be present in:

- two different structural genes coding for enzymes involved in the same metabolic function,
- two different structural genes coding for different subunits of a particular enzyme,
- a structural gene coding for the enzyme involved and in a gene coding for a cofactor or regulatory process which is needed for the enzyme to attain its full enzymatic activity and proper intracellular compartmentalization,
- two different genes coding for factors or processes needed for a normal functioning of the enzyme, in which case the structural genes coding for (the subunits of) the particular enzyme are normal.

Intergenic complementation is involved in the restoration of normal metabolic activity after fusion of fibroblasts from different types of methylmalonic acidemia. The six complementation groups most likely represent two loci involved in the production of the methylmalonyl-CoA mutase protein, and four loci involved in the production of its coenzyme adenosylcobalamin (Gravel et al.,1975; Willard et al.,1978, 1979). The mechanism of complementation of the other disorders listed in Table I.2. was still unknown at the moment that the experimental work described in this thesis, was started.

Our experiments were aimed at an understanding of the nature of the gene mutations involved in different variants of G_{M1}- and G_{M2}-gangliosidosis and of the mechanism of the observed complementation after fusion of fibroblasts from

patients with different variants of G_{M1}- and G_{M2}-gangliosidosis, respectively. This required knowledge of the molecular structure and physico-chemical characteristics of:

- the normal β-galactosidase and N-acetyl-β-hexosaminidase isoenzymes,
- the mutant β-galactosidase and β-hexosaminidase isoenzymes present in patients with G_{M1}- and G_{M2}-gangliosidosis respectively,
- the restored enzyme activity which is present in heterokaryons after fusion of certain combinations of variants of G_{M1}- and G_{M2}-gangliosidosis.

I.2. G_{M2}-gangliosidosis

G_{M2}-gangliosidoses involve different autosomal recessive diseases, characterized by the accumulation of G_{M2}-gangliosides in the lysosomes. The most common form of G_{M2}-gangliosidosis is Tay-Sachs disease or type 1 G_{M2}-gangliosidosis. Another form is called Sandhoff's disease or type 2 G_{M2}-gangliosidosis (Sandhoff,1968; for reviews see O'Brien,1978a and Sandhoff and Christomanou,1979). In addition to these most common forms there are a number of variants which differ in the onset and properties of the clinical features (for review see Galjaard and Reuser,1977; O'Brien,1978a,1978b).

The clinical manifestations of Tay-Sachs disease and Sandhoff's disease are similar. The first symptoms occur a few months after birth; the children are apathetic, and unable to sit at the usual time. After six months a motor weakness develops and unusual limb movements occur. Vision is impaired , and due to macular degeneration a cherry-red spot

can be detected. After one year motor and mental deterioration progresses rapidly. By the age of two years the cranial measurements become larger than normal as a result of proliferation of glial cells in the brain and after a period of progressive deafness, blindness, spasticity and a state of decerebrate rigidity most patients die from bronchopneumonia by the age of two to four years.

The most striking pathological alterations in Tay-Sachs disease and Sandhoff's disease occur in the brain. There is a loss of ganglion cells and Purkinje cells, which is accompanied by a proliferation of glial cells. The cytoplasm of the remaining ganglion cells in the cerebral cortex and Purkinje cells are packed with lysosomes in which lipid material is accumulated as concentrical membranes. Demyelination of the axons is consistently found (Adachi and Volk,1975). Distinct from Tay-Sachs disease, accumulation of glycolipid material in other tissues than the brain is more common in patients with Sandhoff's disease (Sandhoff et al.,1968).

Klenk (1939) was first to discover that the material stored in Tay-Sachs disease was a neuraminic acid containing sphingolipid which he called ganglioside. The amount of G_{M2}-ganglioside in brain of Tay-Sachs patients was found to be 100 to 300 times higher than normal (Svennerholm,1962,1963). Asialo-G_{M2}-ganglioside accumulates to a lesser extent and is 20-50 times higher than control values. Most of the accumulated G_{M2}-gangliosides are located in the concentrical lamellar structures in the lysosomes (O'Brien,1973). In brain tissue of patients with Sandhoff's disease the storage of asialo-G_{M2}-ganglioside is more pronounced than in Tay-Sachs disease. In Sandhoff's disease the visceral organs accumulate globoside in addition to G_{M2}- and asialo-G_{M2}-ganglioside (Schneck,1975).

Studies on the chemical nature of the storage products in G_{M2}-gangliosidosis led to the prediction that the disease would be caused by a defective activity of N-acetyl-β-D-galactosaminidase (Svennerholm,1957,1966), but no decreased hexosaminidase activity could be demonstrated. Robinson and Stirling (1968) then showed the presence of two major N-acetyl-β-D- hexosaminidase isoenzymes, which they called hexosaminidase A (hexA) and hexosaminidase B (hexB). Shortly thereafter, Okada and O'Brien (1969) were able to show that Tay-Sachs disease is caused by a deficiency of hexA, whereas increased activity of hexB is present in these patients. In patients with Sandhoff's disease both hexA and hexB were found to be deficient (Sandhoff et al.,1968). HexA catalyzes the removal of the terminal β-N-acetylgalactosamine from (asialo-)G_{M2}-ganglioside and globoside. HexB has activity against the asialo-G_{M2}-ganglioside and globoside substrates. Assays for hexosaminidase are usually carried out with the artificial substrates p-nitrophenyl-β-D-N-acetylglucosaminide (or -galactosaminide) or 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (or -galactopyranoside). Based on clinical and biochemical studies using both artificial and natural substrates, in the last decade a number of variants of G_{M2}-gangliosidosis have been described. These will be discussed in Chapter III.1.

Although the clinical expression of Tay-Sachs disease and Sandhoff's disease is very similar, both disorders differ in the nature of their residual hexosaminidase activity. To investigate the genetic basis of this biochemical heterogeneity, complementation studies were carried out (Thomas et al.,1974; Galjaard et al.,1974a). In these studies, fibro-

blasts from a patient with Tay-Sachs disease were fused with fibroblasts from a patient with Sandhoff's disease. In the resulting heterokaryons, restoration of hexA activity could be demonstrated, which proved that two different gene mutations are responsible for Tay-Sachs and Sandhoff's disease. Co-cultivation of both types of mutant fibroblasts did not result in reappearance of hexA activity, nor did mixing of cell homogenates from both cell strains.

For an understanding of the mechanism of this complementation knowledge is required about the molecular structure of the hexosaminidase isoenzymes in normal human cells and in cells from patients with Tay-Sachs and Sandhoff's disease. Gene localization studies using electrophoretic and immunological methods were carried out to determine the number and function of genes involved in the expression of the hexosaminidases. The result of these studies will be discussed in Chapter II.2.

I.3. G_{M1}-gangliosidosis

In 1964, Landing et al. reported on eight patients with a lysosomal storage disease, which they called "familial neurovisceral lipidosis". This disorder, which has subsequently been designated as "generalized gangliosidosis", "Landing's disease" and "G_{M1}-gangliosidosis" shows an autosomal recessive inheritance and is characterized by retarded psychomotor development from birth on. Progressive deterioration of motor and mental functions and deafness and blindness occur. Cherry-red spots in the retina, as present in Tay-Sachs disease, are present in some patients, but not in all. Furthermore severe bone deformities and hepatosplenomegaly are present, and the patients usually expire between

one and two years of age, due to bronchopneumonia (for reviews O'Brien,1972,1978a).

The cytoplasm of neurons of all cortices and deep gray matter is filled with enlarged lysosomes. Most of these consist of concentrically arranged lamellae surrounding an inner amorphic core, similar to those seen in Tay-Sachs disease. The cerebral and cerebrellar white matter shows demyelination, and axonal loss (Adachi and Volk,1975). Foamy histiocytes are reported in various visceral organs. Electron microscopy of the lysosomal inclusions in the viscera shows vacuoles containing amorphous or fine-granular material, mixed with membranous lamellae (for review see O'Brien, 1978a).

In the brain and visceral organs from G_{M1}-gangliosidosis patients there are increased levels of G_{M1}-ganglioside and its asialo-derivative. The amount of G_{M1}-ganglioside is approximately 10 times higher than in controls in the gray matter and 20-50 times in liver. The visceral histiocytosis is not due to ganglioside storage, but to storage of keratan-sulfate-like mucopolysaccharides (Suzuki et al.,1971).

In 1968, Okada and O'Brien showed that G_{M1}-gangliosidosis is caused by a defective lysosomal enzyme, acid β-galactosidase. This β-galactosidase deficiency can be demonstrated in cerebral tissue, visceral organs, body fluids, leucocytes and cultured skin fibroblasts. The enzyme can be assayed either with its natural substrate, G_{M1}-ganglioside, or with the artificial substrates p-nitrophenyl-β-D-galactopyranoside and 4-methylumbelliferyl-β-D-galactopyranoside.

Several clinical forms of G_{M1}-gangliosidosis have been described. In addition to the classical type described by Landing et al. (1964) which is referred to as the infantile

type or type 1 G_{M1}-gangliosidosis, Derry et al. (1968) described two siblings with a juvenile form of the disease or type 2 G_{M1}-gangliosidosis. In these patients the onset of symptoms is later and the course of the disease is milder. Mental and motor development is usually normal during the first year of life and early symptoms are loss of speech and generalized muscular weakness. Mental and motor deterioration progresses during the following years and death usually occurs at the age of 3-10 years of life. Bony deformaties are mild or absent and the same is true for hepatosplenomegaly. Histopathology does not enable a distinction between the infantile and juvenile form, nor does the analysis of the storage products or the level of the residual β-galactosidase activity, which is less than 1% in both type 1 and type 2 (O'Brien, 1972,1978a; Galjaard and Reuser,1977).

During the last years a number of other clinical variants with a β-galactosidase deficiency have been described. These patients vary in age from 1½ year (Andria et al.,1978) to 41 years (Stevenson et al.,1978). Mild skeletal abnormalities are present in nearly all patients (Suzuki et al.,1977). Mental retardation is apparent in some patients (Goldberg et al.,1971; Orii et al.,1972; Lowden et al.,1974; Loonen et al., 1974; Yamamoto et al,1974; Stevenson et al.,1978), but in others the intelligence may be (nearly) normal (Pinsky et al., 1974; Wenger et al.,1974; O'Brien,1976; Andria et al.,1978). Other clinical features which may be associated with a β-galactosidase deficiency are myoclonus, cherry-red spot in the macula, ataxia and visceromegaly. In the various patients different combinations of clinical manifestations occur.

In order to investigate the genetic background of this clinical heterogeneity, complementation studies have been performed (Galjaard et al.,1975; Bootsma and Galjaard,1979). Fibroblasts from different patients were fused using inactivated Sendai virus. The β-galactosidase activity was assayed in individual binuclear cells, using a microchemical assay procedure (Galjaard et al.,1974b). An advantage of this approach is that quantitative information is obtained about the restored β-galactosidase activity in heterokaryons. In some of the fusion combinations nearly complete restoration of β-galactosidase activity occurred, comparable with the activity in normal mononuclear fibroblasts. Another approach has been to measure the β-galactosidase activity in a mixed population of heterokaryons, homokaryons and non-fused cells and to compare the analytical data with those of the two parental cell strains measured separately or after co-cultivation.

Based on these two approaches the patients with different forms of β-galactosidase deficiency could be classified in two complementation groups (Table I.3.). In no instance co-culti-

Table I.3. Genetic classification of variants with a β-galactosidase deficiency

Complementation group A	Complementation group B
Infantile (type 1) G_{M1}-gangliosidosis	Variant described by Pinsky et al. (1974)
Juvenile (type 2) G_{M1}-gangliosidosis	Variant described by Loonen et al. (1974)
Variant described by Lowden et al. (1974)	Variant described by Andria et al. (1978)
Variant described by Norden and O'Brien (1975)	Variant described by Kleyer et al. (1979)
Adult variants described by Suzuki et al. (1977)	
Variant of Wenger	
Variant of Svennerholm	
Morquio syndrome with β-GAL deficiency (Krins et al.,1979)	

vation of different mutant cell strains or mixture of two cell homogenates resulted in a restoration of β-galactosidase activity. These data indicate that at least two different gene mutations are involved in the various clinical forms of β-galactosidase deficiency. To obtain insight in the mechanism of complementation, gel filtration studies as well as gene localization studies using electrophoretic and immunological techniques have been performed. These will be discussed in Chapter II.3.

CHAPTER II.
INTRODUCTION AND DISCUSSION OF THE EXPERIMENTAL WORK

II.1. Introduction

For a correct interpretation of both the absence and occurrence of complementation the following three aspects are important:

a. the molecular structure and the processing of the normal enzymes
b. the properties of the mutated enzymes
c. the characteristics of the restored enzyme activity after fusion of two variants with "the same" disorder.

Paigen (1971) used the term "enzyme realization" to indicate all the processes required for the production of a catalytically active enzyme. According to de Duve and Wattiaux (1966) for lysosomal enzymes these processes include transcription of the structural genes involved, production of the protein parts of the enzyme at the rough endoplasmatic reticulum, their transfer to the Golgi system and finally their release within vesicles detached from this system, called primary lysosomes (De Duve and Wattiaux,1966). In some cell types there exists near the Golgi apparatus a relatively direct transport of acid hydrolases from rough endoplasmatic reticulum to lysosomes. This complex system is indicated as the GERL system (Golgi Endoplasmatic Reticulum Lysosomes) (Novikoff,1973). The sugar parts of the acid hydrolases, which are glycoproteins, are added by glycosyltransferases of the Golgi apparatus. There is some evidence that this process already starts in the endoplasmatic reticulum

(for review see Holzman, 1976). In the secretion-recapture model postulated by Hickman and Neufeld (1972) the lysosomal enzymes are first secreted, followed by a receptor-mediated uptake by the same or by adjacent cells to perform their enzymatic functions (for review see Neufeld et al.,1977). In human fibroblasts the phosphorylation of the carbohydrate moiety of precursors of the lysosomal enzymes is critically involved in their recognition (Hasilik et al.,1979). Sly et al. (1979) postulated that the recognition marker functions mainly as an "intra cellular traffic signal" to prevent enzyme secretion and to deliver receptor-bound acid hydrolases to lysosomes, where the marker is removed and the enzyme can exhibit its full enzymatic activity.

In some cases, the naturally occurring substrates for the lysosomal enzymes have to be activated. In vivo, lipids are incorporated into membranes, where they are hardly accessible. Activation of (sphingo)lipids in the membranes might be accomplished by non enzymic proteins, which have recently been demonstrated for a number of acid hydrolases (Li and Li, 1976; Mraz et al.,1976; Hechtman and leBlanc,1977; Fischer and Jatzkewitz,1978; Sandhoff and Conzelmann,1979). The activator proteins are thought to bind to the lipid substrates, thus solubilizing it, the resulting complex being the true substrate for the degrading enzyme.
realization of an enzyme. If structural or regulatory genes, which control the rate of transcription of a structural gene, are involved, only deficiency of one enzyme is expected. If more general functions are mutated, deficiency of a number of enzymes may occur. This is the case in I-cell disease, where due to an altered structure of the common recognition marker of lysosomal enzymes, a severe intracellular deficiency of

these enzymes occur (Mc Kusick et al.,1978).

Several techniques are available for the characterization of the genes and geneproducts. A first approach is the determination of the physico-chemical, kinetic and biochemical properties of the enzyme, such as pH optimum, heat inactivation pattern, K_m and V_{max}, electrophoretic mobility and molecular weight. The probability that a mutation in a structural gene would be reflected in one of these parameters can be estimated. Empirically 65% of the aminoacid substitutions were found to alter the heat lability of E.coli βgalactosidase (Langridge,1968) and 67% of the variants of glucose - 6-phosphate dehydrogenase showed an altered K_m for substrate (Yoshida,1973). Approximately 30% of the aminoacid substitutions will produce a protein with altered electrophoretic mobility (Felton et al.,1974; Neel,1978).

The use of immunological techniques is a second way of studying normal and mutant isoenzymes (for review see Nagradova and Grozdova,1977). Specific antibodies can be raised to purified human enzymes and the amount of cross reacting material in cells and tissues from normal individuals and patients can be established. If an altered amount of antigen per unit enzyme activity is demonstrated, this is an indication for the presence of a structurally altered enzyme in the cells of the tissue tested. The absence of cross reactive material or the presence of reduced amounts of enzyme molecules with a normal activity per unit antigen may be the result of decreased synthesis of the protein, an increased rate of degradation, or a mutation interfering with the binding of the antibody.

A third approach to the characterization of gene(s) and

geneproducts is the analysis of the number of genes that is involved in the expression of a particular protein by studying the (iso)enzyme pattern in interspecies hybrids. For such gene localization studies human fibroblasts or white blood cells are fused with rodent cells. In the resulting heterokaryons nuclear fusion may occur and with the use of selective media (Littlefield,1964) proliferating hybrid cell lines can be isolated. In these hybrids a preferential loss of human chromosomes occurs. The recognition of individual chromosomes or parts of these is essential and this has become possible since the work of Caspersson et al. (1970) who have shown that each human chromosome possesses an unique banding pattern after staining with the fluorochrome quinacrine. During the last years, many other differential staining techniques have been developed (for reviews see Schwarzacher and Wolf,1974; Dutrillaux and Lejeune,1975).

By relating the presence or absence of certain (enzyme) proteins to the presence or absence of specific human chromosomes or parts of these, it has been possible to assign more than 200 gene loci to the various human chromosomes (see Proceedings of the Human Gene Mapping Conferences 1 to 5, 1973-1979).

Electrophoretic studies have been used most often to characterize the presence or absence of human gene products in hybrid clones (Ruddle and Creagan,1975). In some instances however, the use of specific antisera may be required to distinguish the human and rodent gene products; this is especially important if heteropolymeric man-rodent molecules are formed, which may have an electrophoretic mobility that is similar to one of the isoenzymes of both species. Electrophoretic and immunological studies after fusion of normal

rodent cells with fibroblasts or lymphocytes from a patient with a genetic protein defect may contribute to the localization of the gene that carries the mutation.

In our studies, cultured human fibroblasts are used as a model system for the ganglioside storage diseases. A prerequisite for this is the expression of the (deficient) isoenzymes in these cells, which is the case for quite a number of lysosomal enzymes. Fibroblasts are a homogeneous population of cells, and their use is not limited to the patients life. The disadvantages only relying on cultured fibroblasts concern the limited amounts of material, which hamper extensive purification of enzymes. Furthermore, in the gangliosidosis most storage products are found in the nervous tissue, whereas connective tissue is not affected. In fact, cultured fibroblasts from patients with G_{M2}-gangliosidosis do not accumulate gangliosides (Kolodny et al., 1973; Hoffman et al.,1976). Although Svennerholm (personal communication) could not detect any storage of gangliosides in fibroblasts from a G_{M1}-gangliosidosis patient, Suzuki et al., (1978) reported an increase of G_{M1}-ganglioside in cells from similar patients.

All three approaches mentioned in this introduction have been used in our experimental work on the elucidation of the nature of the genetic defect in G_{M2}- and G_{M1}-gangliosidosis, as will be apparent from the next two sections.

II.2. G_{M2}-gangliosidosis

II.2.1. The molecular structure of the β-hexosaminidase isoenzymes

The major acidic β-D-N-acetylhexosaminidase (E.C.3.2.1.30) isoenzymes designated hexA and hexB (Robinson and Stirling, 1968) have been demonstrated in a number of human tissues. They can be separated by electrophoresis, iso-electric focusing and ion-exchange column chromatography (Srivastava et al.,1974a). HexA has a faster electrophoretic mobility (anodal), which is thought to be due to significant amounts of neuraminic acid residues (Freeze et al.,1979). Also differences in heat stability are used for the separate measurement of the activities of both enzymes: 5-10 minutes at 50°C destroys hexA activity whereas hexB is stable (for review see Barrett and Heath,1977). HexA and hexB have a similar molecular weight, 100 000-140 000, depending on the methods used, and similar pH optima and K_m values (Srivastava et al.,1974b; Beutler et al.,1976). Both isoenzymes are glycoproteins (Freeze et al.,1979) and are composed of multiple subunits. The molecular weight of the subunits varies from 17 000-35 000, depending on the methods used (Robinson et al.,1973; Srivastava et al.,1974b; Tallman et al.,1974a; Beutler et al.,1976). Antibodies raised to purified hexA or hexB are reactive to both isoenzymes (Srivastava and Beutler,1973).

These data suggest a structural relationship between hexA and hexB, and several models have been proposed. Robinson and Stirling (1968) originally suggested that hexA and hexB share the same protein part, only differing in neuraminic acid

content. Later Robinson and Caroll (1972) and Desnick et al. (1972) suggested that both hexA and hexB are composed of multiple subunits, one of which is common to both forms. The deficient activity of hexA and hexB in patients with G_{M2}-gangliodosis type 2 (Sandhoff's disease) and the deficiency of hexA in patients with Tay-Sachs disease (G_{M2}-gangliosidosis type 1) also suggests the presence of a common subunit in hexA and hexB (Ropers and Schwantes,1973). A third hypothesis about the molecular structure of the normal hexosaminidases was proposed by Tallman et al.(1974a) who suggested that the two acidic hexosaminidases represent different conformational changes of the same enzyme.

Gene localization studies on the segregation of the hexosaminidase isoenzymes in man-rodent somatic cell hybrids should in principle enable discrimination between some of the models proposed. The data of the first gene localization studies were however contradictory. Gilbert et al. (1975) and van Someren and Beyersbergen van Henegouwen (1973) reported an independent segregation of hexA and hexB whereas Tedesco et al. (1973) and Lalley et al. (1974) found that hexA activity was only expressed in hybrid clones if hexB activity was present as well. The purpose of the experimental work described in Appendix paper I was to investigate the reasons for this discrepancy and to characterize the hexosaminidase isoenzymes in man-Chinese hamster hybrids by means of electrophoretic and immunological methods. With specific antisera against hexA, hexB and an anti-Chinese hamster hexosaminidase antiserum it could be demonstrated that the discrepancies mentioned above were due to misinterpretation of one of the bands of activity on electrophoresis. This band, with an

electrophoretic mobility nearly identical to human hexA was shown to contain a heteropolymeric molecule, with Chinese hamster subunits and subunits specific for human hexA. The expression of normal hexA in hybrid clones turned out to be dependent of the expression of hexB, which fits the two locus subunit model in which hexA=$(\alpha\beta)_n$ and hexB=$(\beta\beta)_n$.

Similar conclusions, based on the application of a number of different techniques, had been drawn by others (Van Cong, 1975; Lalley and Shows,1976; Chern et al.,1976; Swallow et al. 1977). The gene coding for hexB (β-subunit) has been assigned to chromosome 5 (Gilbert et al.,1975; Boedecker et al.,1975), whereas the gene coding for the α-subunit of hexA has been assigned to chromosome 15 (van Heyningen et al.,1975; Westerveld et al.,1975; see also Appendix paper I).

In addition to hexA and hexB a third isoenzyme of β-hexosaminidase (hexC) with an electrophoretic mobility anodal to hexA and hexB was descibed by Hooghwinkel et al.(1972). HexC has a neutral pH optimum, a cytoplasmic localization and it is immunologically distinct from hexA and hexB. The loss of this isoenzyme after freezing of cells and tissues is probably responsible for the fact that it has remained undetected for a considerable period of time. Several investigators have suggested that the genetic origin of hexC is different from that of hexA and hexB (Braidman et al.,1974; Penton et al., 1975; Swallow et al.,1976), but the molecular structure of hexC is still unknown.

Another minor isoenzyme with an electrophoretic mobility similar to that of hexC is the acidic form hexS, which was first detected by Ikonne and Desnick (1974), and more fully described by Ikonne et al. (1975) and Beutler et al. (1975).

The similar electrophoretic mobility of hexS and hexC has led to confusion about the characteristics of both isoenzymes and about their presence or absence in interspecies cell hybrids (Van Cong et al.,1975) and in cells from patients with Tay-Sachs disease and Sandhoff's disease (Ropers and Schwantes, 1973). Methods have been developed to investigate hexS and hexC separately (Swallow et al.,1976; Reuser and Galjaard, 1976), and this has enabled the conclusion that hexC is present in patients with Tay Sachs disease, whereas hexS and hexC are present in patients with Sandhoff's disease. No major differences were found between hexS and hexA regarding thermo-stability, pH optimum and kinetic properties (Ikonne et al., 1975). HexS reacted with an anti hexA antiserum, but not with anti hexB antibodies (Beutler et al.,1975). This suggests a common subunit in hexA and hexS.

Definite evidence for the subunit structure of the acid hexosaminidases came from a series of in vitro experiments by Beutler and Kuhl (1975). By freezing and thawing they were able to dissociate purified hexA and to reassociate the subunits to form hexB and hexS. In a similar way, dissociation and reassociation of hexB and hexS led to the formation of hexA. These results are compatible with the following model of the acidic hexosaminidase isoenzymes (Beutler et al.,1976; Geiger and Arnon,1976) : hexB = $(\beta\beta)_2$; hexA = $(\alpha\beta)_2$ and hex S = $(\alpha\alpha)_2$.

In addition to these three acidic hexosaminidase isoenzymes, intermediate forms I_1 and I_2 have been identified with an electrophoretic mobility in between hexA and hexB (Price and Dance,1972). The intermediate I_2 form is identical to the

P-form, which is the main isoenzyme of hexosaminidase in serum during pregnancy (Stirling,1972). Biochemical and immunological data suggest that hexP or $hexI_2$ is a hexB-like enzyme with a higher degree of glycosylation, or that it is a hexamer of β-chains ($\beta 6$) (Geiger et al.,1978). Heterogeneity of the carbohydrate part of the β-chains may also account for the formation of $hexI_1$ (Beutler and Kuhl,1975).

The relationship between the various hexosaminidase isoenzymes as determined with artificial methylumbelliferyl substrate and those acting on natural G_{M2}-ganglioside is not yet clear. Loss of activity towards G_{M2}-ganglioside substrate occurs upon purification of hexA. Li et al. (1973), Srivastava et al.(1974a), and Hechtman (1977) indicated that this loss of activity is due to the removal of an activating factor (see page 26). Sandhoff and Conzelmann (1979) showed that certain variants of G_{M2}-gangliosidosis are due to the genetic deficiency of an activating protein. These will be discussed in chapter III.1.

The hydrolysis of G_{M2}-ganglioside by hexA can also be stimulated by sodium taurocholate. Neither the activating protein nor sodium taurocholate can increase the barely significant hydrolysis of G_{M2}-ganglioside by hexB. The activating factor has been purified and characterized as a heat-stable, non-enzymatic protein, with a molecular weight of 36 000 (Hechtman and leBlanc,1977; Sandhoff and Conzelmann, 1979; for review see Sandhoff and Christomanou,1979).

II.2.2. The nature of the mutation in Tay-Sachs disease and in Sandhoff's disease

Tay-Sachs disease is characterized by a deficiency of both hexA and hexS and increased activity of hexB (Okada and O'Brien,1968; Reuser and Galjaard,1976; O'Brien, 1978a). In Sandhoff's disease hexA and hexB are deficient and the hexS activity increased (Sandhoff et al.,1968; Beutler et al.,1975). Initially in Tay-Sachs disease no cross reacting material could be shown with anti-hexA antiserum (Srivastava and Beutler,1973) but recently Srivastava and Ansari (1978) found cross reacting material in livers from eight unrelated Tay-Sachs patients, using a monospecific anti-cross linked hex-α antiserum. These data indicate that Tay-Sachs disease is caused by a mutation in the α-subunit of hexosaminidase, as had been suggested earlier by Ropers and Schwantes (1973), Srivastava and Beutler (1974) and Galjaard et al. (1974a).

Sandhoff's disease is thought to be due to a mutation in the β-subunit (Ropers and Schwantes,1973; Galjaard et al.1974a; Beutler et al.,1975). Studies on the presence of enzymatically inactive but antigenically reactive enzyme in Sandhoff patients have been inconclusive (Srivastava and Beutler,1973; Braidman,1974 (personal communication).

A further investigation on the nature of the mutation and on the properties of the residual activity in Sandhoff's disease was performed by analyzing proliferating hybrids between Sandhoff fibroblasts and Chinese hamster cells, as described in Appendix paper II.In the hybrid clones, the formation of heteropolymeric hexosaminidase molecules consisting of Chinese hamster subunits and human hex-α subunits could be demonstrated. Electrophoretic and immunological

studies showed that the properties of the human hex-α subunit were similar to those in hybrids between normal human fibroblasts and Chinese hamster cells. The heteropolymeric isoenzyme is not present in hybrids between fibroblasts from a patient with Tay Sachs disease and Chinese hamster cells (Van Cong et al.,1977). From these data it can be concluded that Sandhoff cells contain an α-subunit of hexosaminidase with normal characteristics, and Sandhoff's disease is therefore likely to be caused by a mutation in the β-subunit of hexosaminidase.

II.2.3. On the mechanism of complementation

After fusion of Tay-Sachs and Sandhoff fibroblasts, heterokaryons show a restoration of hexA activity as revealed by heat inactivation studies and electrophoresis (Thomas et al., 1974; Galjaard et al.,1974a). In view of the data available on the molecular structure of the acid β-hexosaminidases and on the nature of the different gene mutations it is most likely that complementation is the result of combination of normal hex-β chains coded for by the Tay-Sachs genome, and normal α-chains coded for by the Sandhoff genome.

When the protein synthesis is inhibited by cycloheximide no restoration of hexA activity occurs in the heterokaryons which suggests the necessity of de novo protein synthesis. This could explain why co-cultivation of Tay-Sachs and Sandhoff fibroblasts or mixtures of cell homogenates of these two mutant cell types does not lead to reappearance of hexA activity.

II.3. G_{M1}-gangliosidosis

II.3.1.The isoenzymes of normal β-galactosidase

Different forms of β-galactosidase (E.C.3.2.1.23) exist in various human tissues. Acid β-galactosidase is a sialoglycoprotein and can be separated into two isoenzymes A and B on the basis of electrophoretic mobility and molecular weight (Ho et al.,1973; Norden and O'Brien,1973). Both isoenzymes are heat labile, stimulated and stabilized by chloride ions (Ho and O'Brien, 1971) and antibodies to purified βgalA and βgalB are reactive to both isoenzymes (Norden et al, 1974). Acid β-galactosidase is able to hydrolyse the terminal galactose from G_{M1}-ganglioside, asialo-G_{M1}-ganglioside, asialofetuin, keratan sulphate and from artificial β-galactopyranosides (for review see O'Brien,1975; Sandhoff and Christomanou,1979).

Neutral β-galactosidase (M.W.45 000) has a higher pH optimum than acid β-galactosidase. It does not absorb to ConA sepharose and is presumably not a glycoprotein. Monospecific antibodies to acid βgalA do not precipitate neutral β-galactosidase (Norden et al.,1974). Two neutral isoenzymes are present which are heat stabile and one of the isoenzymes is inhibited by chloride ions (Cheetam and Dance,1976). Neutral β-galactosidase cleaves aryl-β-D-galactoside and aryl-β-D-glucoside linkages, but does not cleave galactose from ganglioside G_{M1} (Ho et al.,1973). These data indicate that the acid and neutral β-galactosidases are genetically independent.

In human tissues there is yet another acidic β-galactosidase, which specifically cleaves galactosylceramide. This

enzyme is deficient in Krabbe's disease (Suzuki and Suzuki, 1970; Tanaka and Suzuki,1977; for review see Suzuki and Suzuki 1978). After initial controversy, both galactosylceramide-β-galactosidase and G_{M1}-β-galactosidase were shown to hydrolyse galactose from lactosylceramide (Wenger et al,1974; Suzuki and Suzuki,1974; Tanaka and Suzuki,1975). G_{M1}-β-galactosidase does not cleave galactosylceramide nor does galactosylceramide-β- galactosidase hydrolyse G_{M1}-ganglioside.

The major acidic G_{M1}-β- galactosidase isoenzyme, βgalA, has been purified from human liver and was found to be a monomer of molecular weight 72 000 (Norden et al., 1974) The minor acidic β-galactosidase, βgalB, has a molecular weight of about ten times than that of βgalA (700 000). Because of the immunological relationship between βgalA and βgalB it was suggested that βgalB is a multimeric aggregate of monomeric βgalA (O'Brien,1975).

To characterize the β-galactosidase isoenzymes of human fibroblasts gel filtration experiments were performed. In Appendix paper III it is shown that fibroblasts, under conditions of low ionic strength and neutral pH, show an identical gel filtration pattern as is present in human liver: a major monomeric form of about 70 000 M.W. and a minor multimeric β-galactosidase of 700 000 M.W. If, however, gel filtration was performed in the buffer that is used for the enzyme assay (high ionic strenght acid pH) mainly dimeric β-galactosidase was found. The transitions between monomeric and dimeric β-galactosidase were shown to be reversible. Apparently, dimeric β-galactosidase is the enzymatically active isoenzyme. Hence it follows that kinetic and biochemical parameters cannot be determined separately for βgalA and

βgalB. Dimeric β-galactosidase has also been demonstrated in human liver (Hultberg and Ockermann,1972; Cheetham and Dance, 1976) and in human small intestine (Asp,1971).

Gene localization studies were performed to determine the number of genes which are involved in the expression of the acid β-galactosidase isoenzymes. As is described in Appendix paper IV, human white blood cells from a patient with an X/22 translocation were fused with Chinese hamster cells. The isolated hybrid clones were analysed for chromosome content and enzyme markers for each of the human chromosomes. In none of the hybrid clones, even if these contained the complete human genome was β-galactosidase activity found with an electrophoretic mobility similar to the human parental cell strain. To identify the slower migrating band of β-galactosidase activity in between normal human and Chinese hamster bands, immunological characterization was performed with an anti-human acid β-galactosidase antiserum. Human determinants were shown to be present in this "marker band", which could thus be used for the localization of structural genes coding for β-galactosidase.

A structural gene for acid β-galactosidase could be located on chromosome 22 distal to the breakpoint in q.1.3. These data were contradictory to observations by other investigators. Bruns et al. (1977,1979) and Shows et al. (1977,1979) localized a gene for β-galactosidase on chromosome 3, whereas Rushton and Dawson (1977a) located a β-galactosidase gene on chromosome 12. We have therefore reinvestigated our previous hybrid panel, and isolated a new series of hybrids using normal human white blood cells and Chinese hamster cells. As is described in Appendix paper V, in these hybrids the presence

of two structural genes for β-galactosidase on the human chromosomes 3 and 22 could be demonstrated. The products of the two genes differ in stability and in sensitivity to neuraminidase treatment. Chromosome 3 has to be present in more than 20% of the metaphases in a hybrid clone before the β-galactosidase activity can be demonstrated on electrophoresis. This is probably the reason that we missed the β-galactosidase gene on chromosome 3 in studies on the first hybrid panel, reported in Appendix paper IV. In these hybrids all clones containing chromosome 3 and no chromosome 22 the proportion of metaphases with chromosome 3 varied from 10 to 20%.

Hybrids containing both chromosomes 3 and 22 show on electrophoresis an additional band of activity, which migrates to a position similar to βgalB in the normal human cells. This band is most pronounced after neuraminidase treatment. These studies suggest that there are two genes that code for closely related β-galactosidase subunits. As is shown in Appendix paper VI, which will be discussed later, the β-galactosidase involved is G_{M1}-β-galactosidase.

In view of the available data on the molecular structure of β-galactosidase that is thought to consist of one type of polypeptide, there is no obvious role for two structural G_{M1}-β-galactosidase genes. As discussed earlier dimeric and multimeric aggregationforms of the monomeric β-galactosidase protein are present. One possibility is that the monomeric protein in fact is a dimer, consisting of two polypeptides, which has been suggested by C.Wynn (personal communication). Another possibility is that one gene codes for the monomeric protein whereas a second gene codes for a protein that is part of the multimer. Recently, additional subunits of β-galactosidase have been demonstrated in human urine (Kress

and Miller, 1978), in human liver (Frost et al., 1978) and in human placenta (Lo et al., 1979).

II.3.2. Properties of the residual β-galactosidase activity; nature of the mutation in G_{M1}-gangliosidosis

Tissues and cells from patients with the infantile and juvenile types of G_{M1}-gangliosidosis show a profound β-galactosidase deficiency. Measured with both artificial substrate and with natural G_{M1}-ganglioside substrate the residual β-galactosidase activity is less than 1% of control values. For all patients with the infantile or juvenile form of G_{M1}-gangliosidosis tested, the presence of cross-reacting material to an anti-human β-galactosidase antiserum has been demonstrated (Meisler and Rattazzi,1974; O'Brien,1975,1978a; Ben Yoseph et al.,1977). Our studies described in Appendix paper III showed that the aggregation forms of β-galactosidase in fibroblasts from patients with the infantile or juvenile form differ from normal. In cells from the infantile form only multimeric β-galactosidase was found whereas cells from a patient with the juvenile form only showed the monomeric β-galactosidase. Fusion of both cell types did not result in complementation (Galjaard et al.,1974a) and it has been suggested that the infantile and juvenile types of G_{M1}-gangliosidosis are based on allelic mutations.

To study the nature of the gene mutation, gene localization studies were performed using fibroblasts from a patient with infantile G_{M1}-gangliosidosis (type 1) and Chinese hamster cells (Appendix paper VI). The hybrid cell lines isolated were analysed with electrophoretic and immunological methods.

The presence of two structural β-galactosidase genes on chromosome 3 and 22 could be confirmed. In type 1 X Chinese hamster hybrids a band of activity was shown on electrophoresis which contained human determinants similar as has been described for hybrids between normal human cells and Chinese hamster cells ("control hybrids"). Since the residual β-galactosidase activity in the patient's fibroblasts is below the detectionlevel on electrophoresis, it is likely that the human determinants occur in man-Chinese hamster heteropolymeric molecules. The additional band of activity which is present in "control hybrids" containing both chromosomes 3 and 22, is absent in type 1 X Chinese hamster hybrids. The heteropolymeric isoenzyme present in hybrids containing chromosome 3 but no chromosome 22 was shown to be very labile and sensitive to neuraminidase treatment, which was not the case in "control hybrids". From these observations it is concluded that the infantile type 1 G_{M1}-gangliosidosis is caused by a mutation in the structural G_{M1}-β-galactosidase gene on chromosome 3.

During the last five years a number of patients have been described who are characterized by a later onset of symptoms, milder physical and mental handicaps and survival into adult age. Other variants are still in their youth but have milder clinical features than is the case in the juvenile form of G_{M1}-gangliosidosis. The fibroblasts of all these patients have a higher residual β-galactosidase activity than is present in infantile and juvenile G_{M1}-gangliosidosis. Both with artificial and natural substrates, the β-galactosidase activity in the variants is 10-20% of normal (Pinsky et al.,1974; Galjaard et al.,1974a; Koster et al.1976; Suzuki et al.,1977;

Andria et al.,1978). The residual β-galactosidase activity from three of these clinical variants (Loonen et al.,1974; Pinsky et al.,1974; Andria et al.,1978), belonging to the same complementation group (see Table I.3, page 23) have been investigated in more detail. The kinetic properties, pH optima and heat stabilities of the residual β-galactosidase activities in the variants were found to be the same as the β-galactosidase from normal cultured fibroblasts (Galjaard and Reuser, 1977; Reuser et al.,1979). As described in Appendix paper III, the β-galactosidase in the variants is present in only the monomeric form (M.W. 70 000) under conditions of low ionic strength and pH 7.0. Under the same circumstances β-galactosidase from normal human fibroblasts is present both in monomeric and multimeric forms. Immunological studies revealed the presence of normal amounts of cross reacting material to anti-human β-galactosidase antiserum (O'Brien and Norden,1977; O'Brien,1978a; Reuser et al.,1979). These data indicate the presence of structurally altered β-galactosidase in these clinical variants, with a disturbance in the formation of multimeric aggregates.

Hybrid cell lines were isolated following fusion of fibroblasts from the adult variant of β-galactosidase deficiency (type 4) (Loonen et al.,1974; Galjaard et al.,1975) and Chinese hamster cells. As described in Appendix paper VI these hybrids were analysed with electrophoretic and immunological methods. No differences were observed between these hybrids and "control hybrids", thus no indication for a structural β-galactosidase mutation in this adult type of β-galactosidase deficiency was obtained.

II.3.3. On the mechanism of complementation

The restored β-galactosidase activity after fusion of fibroblasts from patients with the infantile form of G_{M1}-gangliosidosis and patients with a(n) (adult) variant of β-galactosidase deficiency has been further investigated. The kinetic properties, pH optimum and heat stability of this β-galactosidase activity are very similar to the β-galactosidase of normal human fibroblasts. Appendix paper III shows that the restoration of enzyme activity is mainly due to an increase in the fractions corresponding to the monomeric β-galactosidase. In addition, the aggregation to the high molecular weight multimers is restored. Mixing experiments with cell-free extracts ruled out the possibility that the formation of dimeric β-galactosidase would be required for complementation.

Based on these observations a model was suggested involving two different genes: A structural gene responsible for the synthesis of the protein subunit, and a second gene which is involved in a post-translational modification process. The mutation in the infantile type G_{M1}-gangliosidosis is in the structural β-galactosidase gene on chromosome 3. In the (adult) variant forms of β-galactosidase deficiency, the β-galactosidase genes are not mutated, but instead a mutation would be present in a gene, affecting the post-translational modification of β-galactosidase.

In 1978, Wenger et al. found a profound deficiency of N-acetyl-neuraminidase in cells from a 12-year old patient with cherry red spots, intellectual impairment and myoclonus, symptoms which are very similar to those in the variants described above. In the patient described by Wenger et al.,(1978)

the β-galactosidase was deficient in leucocytes and in cultured skin fibroblasts. Based on normal β-galactosidase values in the leucocytes of the patient's parents, Wenger et al. (1978) concluded that the β-galactosidase deficiency was secondary to a primary defect of neuraminidase. In our laboratory a neuraminidase deficiency was also shown in fibroblasts from the adult variant of β-galactosidase deficiency (Loonen et al.,1974) and from patients described by Pinsky et al. (1974) and by Andria et al. (1978).

The complementation observed for β-galactosidase after fusion of fibroblasts from patients with different types of β-galactosidase deficiency may be explained by the fact that normal neuraminidase is supplied by cells from the infantile type G_{M1}-gangliosidosis, which somehow corrects the low β-galactosidase activity present in cells from the β-galactosidase deficient variant. Fusion experiments after enucleation (de Wit-Verbeek et al.,1978) showed that the cytoplasts of fibroblasts from the infantile type G_{M1}-gangliosidosis could complement cells from the adult variant. This indicates that the turnover time of neuraminidase must be sufficient to allow cytoplasts to complement the cells from the adult variant.

It still remains to be explained how a neuraminidase deficiency may lead to a deficiency of β-galactosidase, whilst the activities of the other lysosomal enzymes are normal. The relation between neuraminidase deficiency and β-galactosidase deficiency will be discussed further in chapter III.2.

CHAPTER III.
GENERAL DISCUSSION

III.1. Variants of G_{M2}-gangliosidosis

Increasing knowledge about the genetic defect in G_{M2}-gangliosidosis and about the molecular structure of the hexosaminidases has led to the identification of a number of clinical and biochemical variants, in addition to the classical types Tay-Sachs disease and Sandhoff's disease.

Different types of G_{M2}-gangliosidosis with a varying degree of hexA deficiency and with normal or elevated hexB activity are listed in table III.1. Clinically, later onset and/or less progressive juvenile and adult variants of Tay-Sachs disease have been reported. Among these patients a profound deficiency of hexA as well as a relatively high residual hexA activity was found. Part of this variation is probably due to the fact that in various studies different cells and tissues have been used for hexosaminidase assays. So far, no correlation could be demonstrated between the time of onset of the symptoms, the severity of the clinical features and the degree of hexosaminidase deficiency as assayed with artificial substrates. Assays with natural substrate might give a better insight in a possible correlation between residual enzyme activity and clinical features, but the hexA activity towards presently available G_{M2}-gangliosides is very low compared with that towards artificial substrate and reliable assays are therefore still very difficult.

Several normal healthy adults have been described who showed a partial deficiency of hexA both with artificial and natural substrates (see tabel III.1). O'Brien et al. (1978)

TABLE III.1. VARIANTS OF G_{M2}-GANGLIOSIDOSIS type 1

Variants	origin or race	age at onset of symptoms (years)	age at death (years)	% hexA activity 4MU	% hexA activity G_{M2}	hexB activity	source	references
Infantile type of Tay-Sachs disease	Ashk. Jewish	0-6 months	2-4	0-5	0-5	normal or elevated	liver,serum fibroblasts	Okada & O'Brien,1968
Tay-Sachs variant	English	4 months	3	25	6	normal	fibroblasts	O'Brien et al.,1978b
Juvenile type of Tay-Sachs disease	Non-Jewish	2 2	4 9					Bernheimer & Seitelberger,1968
	Puerto-Rican	5	14½	40	10	normal	liver	Suzuki et al.,1970 Suzuki & Suzuki,1970 Zerfowski & Sandhoff 1974
	European	2½		25		normal	fibroblasts	Okada et al.,1970
	Greek	1-9½	4-15	5-31		normal	leucocytes	Brett et al.,1973
Adult type of Tay-Sachs disease	Ashk. Jewish	4	32 *	2,5 48		normal	serum leucocytes	Rapin et al.,1976
		4	29 *	8,5 40		normal	serum leucocytes	
Normal individuals with (profound) deficiency of hexA	Ashk. Jewish			6	79	normal	leucocytes	Navon et al.,1973,1976 Tallman et al.,1974b
	Non-Jewish			0 15 50		normal	serum leucocytes fibroblasts	Vidgoff et al.,1973, 1974
	Non-Jewish			20	50-60	normal	fibroblasts	Kelly et al.,1976
	Ashk. Jewish			8	41	normal	fibroblasts	O'Brien et al.,1978

reported on a child with Tay-Sachs disease whose fibroblasts showed a profound deficiency of hexA with natural substrate and 25% residual activity with methylumbelliferyl substrate. It is possible that different gene mutations affect the affinity of hexosaminidase A for natural and artificial substrates in a different way. So far, we have never found any restoration of hexA activity after fusion of cells from different variants of G_{M2}-gangliosidosis type 1, which might indicate that these are allelic mutations (Galjaard, personal communication).

Variants in which the total hexosaminidase activity is decreased are listed in table III.2. In addition to the classical type of Sandhoff's disease there is a juvenile form of the disease, and cells from these patients were found to have at least twice the activity of the clinical infantile form of Sandhoff's disease. Furthermore a normal individual has been reported whose leucocytes show a total hexosaminidase activity of about 10% of control values. When assayed with the natural G_{M2}-ganglioside substrate the hexosaminidase activity was found to be about 50%, indicating that also this mutation affects the activities towards natural and artificial substrates in a different way. The same is true for two patients with the clinical features of classical G_{M2}-gangliosidosis but with normal or elevated activities of hexosaminidase, as assayed with artificial substrates. With G_{M2}-ganglioside as a substrate activities of 10% of normal were found (table III.2). Finally there is one patient with an elevated hexosaminidase activity, in whom clinical symptoms became apparent at the age of 18 years (table III.2.).

Studies on the molecular structure of hexosaminidase have indicated that two structural genes are involved in the ex-

TABLE III.2. VARIANTS OF G_{M2}-GANGLIOSIDOSIS type 2

Variants	origin or race	age at onset of symptoms (years)	age at death (years)	total hex activity (% of controls) 4-MU	G_{M2}	% of the total hex activity 4-MU hexA+S	4-MU hexB	source	references
Sandhoff's disease	non-Jewish	0-6 months	2-4	0-5		50	50	liver serum fibroblasts	Sandhoff et al.,1971 Okada et al.,1972
Juvenile type of Sandhoff's disease	Ukranian-Welsh	5	10 ✱	5 15		86	14	fibroblasts leucocytes	McLeod et al.,1977 Wood and McDougall,1976
	Irish	3	4½ ✱	13 2,5		90 85	10 15	fibroblasts leucocytes	Johnson et al.,1977
Normal with deficiency of hexA and hexB	French			10	50			leucocytes	Dreyfus et al.,1975 Dreyfus et al.,1977
Variant AB^+ of G_{M2}-gangliosidosis with normal hexA and hexB		0-6 months	2-4	120	10	42	58	liver	Sandhoff et al.,1971 Conzelmann et al.,1978
	Negro	6 months	14 ✱ months	140		52	48	leucocytes	de Baeque et al.,1975
Adult type of G_{M2}-gangliosidosis	non-Jewish	18	22	185		60	40	brain	O'Neill et al.,1978

✱ still alive

pression of the acid hexosaminidase isoenzymes. The α-subunit is coded for by a locus on chromosome 15, whereas the gene for the β-subunit is located on chromosome 5 (hexA=$(\alpha\beta)_2$; hexB=$(\beta\beta)_2$; see chapter II.2.1.). An activator protein is thought to be involved in the stimulation of hexA to cleave G_{M2}-ganglioside, by solubilization this lipid substrate (Li et al.,1973; Hechtman and leBlanc,1977; Sandhoff and Conzelmann,1979). A genetic defect of the locus coding for this protein is likely to be responsible for the AB^+-variant (see table III.2).

The clinical and biochemical variants described in table III.1 most likely are all variants due to a mutation in the α-subunit, whereas those listed in table III.2 are thought to be mutated in the β-subunit or in the activator protein. The number of different alleles involved, and the possible genotypes of the variants described so far are listed in table III.3.

O'Brien (1978b) considered one of the mutations in the normal individual described by Navon et al. (1973,1976) as a distinct mutation (allele α_6), which would lead to a reduction in hexA synthesis. Tallman et al. (1974b), however, found that in contrast to the 6% residual hexA activity and 5-7% cross-reacting material, 79% G_{M2}-ganglioside hexosaminidase activity is present. It is therefore possible that this normal individual has both an allele as involved in the infantile type of Tay-Sachs disease (α_2) and an allele coding for hexosaminidase with activity against natural but not against synthetic substrates (α_5). The $\alpha_2\alpha_5$ genotype is also thought to be present in the individuals described by Vidgoff et al. (1973), Kelly et al. (1976) and O'Brien et al. (1978). The same phenomenon has been suggested for a mutation

TABLE III.3. THE POSSIBLE GENETIC AND MOLECULAR BACKGROUND OF DIFFERENT VARIANTS OF G_{M2}-GANGLIOSIDOSIS (according to O'Brien, 1978b)

ALLELES	GENOTYPE		TYPE OF INDIVIDUAL
Hex-α locus			
$hex\alpha_1$	$hex\alpha_1$ $hex\alpha_1$		normal
$hex\alpha_2$	$hex\alpha_2$ $hex\alpha_2$		Tay-Sachs disease
$hex\alpha_3$	$hex\alpha_3$ $hex\alpha_3$	$hex\beta_1$ $hex\beta_1$	juvenile Tay-Sachs disease
$hex\alpha_7$	$hex\alpha_7$ $hex\alpha_7$	activator protein	adult Tay-Sachs disease
$hex\alpha_4$	$hex\alpha_2$ $hex\alpha_4$	X_1X_1	α4: allele which codes for hexA with activity towards synthetic substrates but not towards natural substrates
$hex\alpha_5$	$hex\alpha_2$ $hex\alpha_5$		α5: allele which codes for hexA with activity towards natural substrates, but not towards synthetic substrates
Hex-β locus			
$hex\beta_1$	$hex\alpha_1$ $hex\alpha_1$	$hex\beta_1$ $hex\beta_1$	normal
$hex\beta_2$	activator	$hex\beta_2$ $hex\beta_2$	Sandhoff's disease
$hex\beta_4$	protein	$hex\beta_4$ $hex\beta_4$	juvenile Sandhoff's disease
$hex\beta_3$	X_1X_1	$hex\beta_2$ $hex\beta_3$	β3: mutation resulting in hexosaminidase with activity for natural substrate but not for synthetic substrates
Activator protein locus			
X_1	$hex\alpha_1$ $hex\alpha_1$	$hex\beta_1$ $hex\beta_1$	X_1X_1 normal
X_2			X_2X_2 AB^+ variant: Tay-Sachs disease with normal hexA and hexB
X_3			X_3X_3 adult onset G_{M2}-gangliosidosis

at the β-locus in a healthy individual described by Dreyfus et al., (1975,1977). Here, 10% residual hexosaminidase activity was present when measured with artificial substrates, and 10-20% cross-reacting material was found. With the G_{M2}-ganglioside substrate however, 50% residual hexosaminidase activity was measured. This individual was thought to be a compound heterozygote of the Sandhoff allele (β_2) and an anomalous hex-β allele (β_3) with activity towards the natural substrate but not towards synthetic substrates (O'Brien,1978b). Most of these compound heterozygotes are detected because, when married to a carrier of the infantile form of Tay-Sachs disease, they have affected children in their offspring.

Some patients have been reported where the biochemical and clinical features are difficult to classify. Examples are the patients described by Spence et al. (1974) and Lane and Jenkins (1978). In theory many types of compound heterozygotes will occur, and studies of the hexosaminidase activity of the parents and other relatives both with artificial and natural substrates are necessary before a proper classification can be attempted. More data on the molecular structure of the hexosaminidases and on the various steps involved in the intracellular processing of these (iso)enzymes are required before conclusions about the relation between the residual hexosaminidase activity and severity of the clinical features can be made. For an understanding of the pathogenesis of the various types of G_{M2}-gangliosidosis more should be known about the function and the tissue distribution of the hexosaminidases and their natural substrate(s).

III.2. G_{M1}-gangliosidosis

III.2.1. Mouse β-galactosidase

For the study of gene regulation in man much can be learned from experiments on lower organisms where normal gene expression can be perturbed and where crosses between different strains can be carried out to establish the genetic nature of the perturbations. One example of this are the extensive studies on inbred strains of mice where genetic variation exists with regard to their tissue levels of β-galactosidase. The number and nature of the genes involved in the expression of β-galactosidase in these animals may help to understand the variability in the clinical features among patients with different clinical types of G_{M1}-gangliosidosis.

The molecular weight of purified and reduced β-galactosidase in mice is 63 000. The native form of the enzyme appears to be a tetramer of MW 240 000 at pH 5.0. This is reversibly dissociated at alkaline pH to a dimer with an apparent molecular weight of 113 000 (Tomino and Meisler, 1975; Seyedyazdani et al.,1975). Polyacrylamide electrophoresis reveals multiple charge isomers of mouse liver β-galactosidase. It could be demonstrated that these isomers have the same molecular size but they differ in net charge. Treatment of the enzyme with neuraminidase resulted in a reduced electrophoretic mobility of all bands. Sialylation of β-galactosidase is most extensive in liver, less so in brain, spleen and lung, and it does not occur at all in kidney. It is thought that the multiple charge isomers differ in carbohydrate and/or amide content (Tomino and Meisler,1975).

β-galactosidase exhibits a dual subcellular distribution which is tissue-specific. The liver enzyme is almost entirely localized in the lysosomes, whereas in kidney 30-40% of the enzyme is in the microsomal fraction (Lusis et al.,1977).

A number of gene loci determines the level of β-galactosidase activity and the characteristics of β-galactosidase in the different tissues of the mouse. So far, the Bgs, Bgt and Bge loci have been identified and a number of genes involved in the secretion of the enzyme.

The Bgs locus

The Bgs locus determines tissue levels of β-galactosidase in the mouse; enzyme levels in mice carrying the Bgs^h allele are twice as high as in mice carrying the Bgs^d allele. Linkage studies have shown that the Bgs locus is located on chromosome 9 (Lundin and Seyedyazdani,1973; Felton et al., 1974). Immunological studies with antiserum to purified β-galactosidase have indicated that the Bgs locus influences the amount of enzyme protein present in the tissues (Meisler, 1976). After the development of an assay for the in vivo rate of β-galactosidase synthesis, it could be demonstrated that the Bgs locus regulates the β-galactosidase activity by controlling the rate of synthesis of the β-galactosidase molecule (Berger et al.,1978).

The Bgt locus

Closely linked with the Bgs locus on chromosome 9 is the Bgt site, belonging to the temporal class of genes that have been defined as the set of DNA sequences containing the gen-

etic information for the developmental program (Paigen et al., 1976). The Bgt locus determines the developmental expression of β-galactosidase in liver. Adult Bgs^h mice carrying the Bgt^b allele have approximately a two times higher β-galactosidase activity in liver than Bgs^h mice carrying the Bgt^d allele. It was found that this increase in activity is due to an increase in the number of β-galactosidase molecules (Paigen et al.,1976). Berger et al. (1978) showed that the Bgt locus controls the rate of synthesis of β-galactosidase in liver.

The Bge locus

An electrophoretic polymorphism for β-galactosidase has been identified in all tissues of common inbred strains of mice. It is inherited as a single Mendelian trait and is called the Bge locus. This structural β-galactosidase gene is closely linked with the Bgs site on chromosome 9. Two alleles are present, Bge^b with a slow electrophoretic mobility and Bge^a with a fast electrophoretic mobility of β-galactosidase (Breen et al.,1977). Another polymorphism which is expressed in all tissues affects the thermal stability of β-galactosidase. Electrophoretic mobility and thermal stability show concordant strain distributions and cosegregate in genetic crosses (Berger and Lusis,1978). This indicates that a single β-galactosidase structural locus is expressed in all tissues.

Genes involved in secretion

A set of genes has been identified in mice that affects lysosomal enzymes by determining the rate of lysosome excretion. All genes of this type discovered so far also affect

the pigmentation. This is presumably due to abnormal transport of melanosomes, that are structurally related to lysosomes. The beige mutation, located on chromosome 13 results in enlarged lysosomes, diluted pigmentation and abnormal excretion of lysosomal enzymes (Brandt and Schwank, 1976). Animals with the light ear and pale ear mutations, located on the mouse chromosomes 5 and 19 respectively, have a fourfold elevated concentration of kidney β-galactosidase, due to defective urinary excretion of lysosomal enzymes (Meisler, 1978).

Three aspects of β-galactosidase realization, its structure, the regulation of its synthesis and its developmental program are now known to be coded for by genes in close proximity on chromosome 9. This is in agreement with the concept that much of the regulatory information relevant to a particular structural gene is in close association with that gene.

Whether this is also the case for the human genome is still unknown, and so far there is no experimental proof of mutations involving a regulatory process in man. Gene localization studies are not a suitable tool to map regulator genes. In man-rodent somatic cell hybrids the regulators of rodent origin may take over the function of a deficient regulator of human origin, that thus will not come to expression. As far as structural genes in man are concerned, it has been found for different enzymes that genes coding for isoenzymes hydrolysing the same substrate are located on different chromosomes. Even human genes, coding for different subunits of the same isoenzyme can be located on different chromosomes as has been described in this thesis for the α- and β-subunit of hexosaminidase A. It is evident that much experimental work has to

be done before the organization of the human genome is established.

III.2.2. β-Galactosidase and neuraminidase deficiency

After the observation by Wenger et al. (1978) that cells from one of his patients with an adult type of β-galactosidase deficiency showed a deficiency of N-acetyl-neuraminidase as well, in our laboratory a neuraminidase deficiency was also found in some patients who had previously been described as variants of G_{M1}-gangliosidosis (Loonen et al.,1974; Pinsky et al.,1974; Andria et al.,1978). The neuraminidase activities in the parents of one of these patients fall in the heterozygote range, whereas the β-galactosidase activities of these parents showed low control values. This would suggest that the β-galactosidase deficiency is secondary, due to a primary genetic defect in neuraminidase.

In some patients a β-galactosidase deficiency has been found in tissues, whereas the serum activity was normal (Suzuki et al.,1977). Based on this observation it has been suggested that β-galactosidase could be inactivated by neuraminic acid containing storage products. A similar phenomenon has been well documented for some tissues from patients with mucopolysaccharidoses, where certain glycosaminoglycans were found to inhibit the β-galactosidase activity (Ho and O'Brien,1969; Matalon and Dorfman,1972; Kint et al.,1973). Complete inhibition of β-galactosidase activity by heparansulfate and dermatansulfate has also been demonstrated in vitro (Rushton and Dawson,1977b). However, similar experiments with sialyloligosacharides in vitro did not give the same results (Lowden and O'Brien,1979).

Another possibility is that a mutation affects an as yet unknown metabolic process that is required to give both neuraminidase and β-galactosidase their full activity. More basic research is required to elucidate the relationship between β-galactosidase and neuraminidase deficiencies.

In addition to these variants with a combined neuraminidase and β-galactosidase deficiency there are also adult G_{M1}-gangliosidosis patients with a severe β-galactosidase deficiency and normal neuraminidase (Suzuki et al.,1977). Wolfe et al. (1970) defined three biochemical abnormalities which must be present to enable an "unambigous and specific" diagnosis of G_{M1}-gangliosidosis:

- excessive accumulation in the nervous system of (asialo-) G_{M1}-ganglioside
- a profound deficiency of acid β-galactosidase
- accumulation in liver, spleen and kidney of a keratansulfate-like mucopolysaccharide.

Because biopsies of brain and liver are not readily available, the biochemical diagnosis must often be based on the analysis of blood(cells), urine and cultured skin fibroblasts. In these instances accumulation of G_{M1}-gangliosides is usually not possible and hence a certain diagnosis of G_{M1}-gangliosidosis cannot easily be made. It has now become evident that the finding of a β-galactosidase deficiency alone does not allow the extrapolation to the diagnosis G_{M1}-gangliosidosis, but that also the heriditary nature of the β-galactosidase deficiency has to be considered. A practical problem in the establishment of heterozygosity in the patients' parents is the fact that for most lysosomal enzyme activities overlapping values are found between controls and heterozygotes.

There are a number of other diseases in which a neuraminidase deficiency is present (for review see Lowden and O'Brien, 1979). The neuraminidase deficiency in mucolipidosis II ("I-cell" disease) and III, associated with multiple hydrolase deficiencies (Mc Kusick et al.,1978), is not thought to be the primary defect (Spritz et al.,1979). Recently, the group of Neufeld provided evidence that the processing of the lysosomal hydrolases in I-cell disease is affected which leads to a loss of precursor enzymes out of the cell. This should explain the severe intracellular deficiency of a number of lysosomal enzymes, including neuraminidase (Hasilik et al., 1979).

Mucolipidosis I is a neurodegenerative disorder in which neuraminidase deficiency is the only defect so far demonstrated. The activity of β-galactosidase in patients suffering from this disorder is normal (Spranger et al.,1977).

During the last years several patients have been described with a "cherry-red spot-myoclonus syndrome" (O'Brien,1977; Rapin et al.,1978; Thomas et al.,1978; Lowden and O'Brien, 1979). In these young adults an isolated neuraminidase deficiency has been found. Similar clinical features are, however, also found in the patients described by Loonen et al. (1974) and by Wenger et al. (1978) and these patients have both a neuraminidase and a β-galactosidase deficiency. Complementation analysis showed that the neuraminidase deficiencies in mucolipidosis I, I-cell disease and the "cherry-red spot-myoclonus syndrome" with combined β-galactosidase and neuraminidase deficiency are caused by different gene mutations (Hoogeveen et al., to be published). Further studies are required to establish whether the "cherry-red spot-myoclonus syndrome" with isolated neuraminidase defi-

TABLE III.4. DISORDERS WITH A (PRIMARY?) NEURAMINIDASE DEFICIENCY

Disorder	authors	neuraminidase activity	β-galactosidase activity (% of control)
Classical mucolipidosis I	Spranger et al.,1977	not detectable	normal
Adult variant of mucolipidosis I	Durand et al.,1977	" "	normal
Variants of βGAL deficiency previously described as G_{M1}-gangliosidosis	Goldberg et al.,1971	not detectable	10-20
	Pinsky et al.,1974	" "	"
	Loonen et al.,1974	" "	"
	Suzuki et al.,1977	" "	"
	Andria et al.,1978	" "	"
Cherry-red spot-myoclonus syndrome	Wenger et al.,1978	not detectable	10
	Rapin et al.,1978	" "	normal
	Thomas et al.,1978	" "	normal

ciency is a separate clinical entity or that it must be considered as a clinical variant of mucolipidosis I (see also Durand et al.,1977). Table III.4 summarizes the clinical syndromes where a neuraminidase deficiency might be the primary defect.

It is possible that different types of neuraminidases are involved in the various genetic defects. In normal tissues at least two neuraminidase isoenzymes are known (Wenger et al.,1978; for review see Lowden and O'Brien,1979), one active towards sialyloligosaccharides, another towards gangliosides. No human mutants have been reported with a ganglioside (G_{M3} and G_{D1a}) neuraminidase deficiency. The

neuraminidase(s) which act on sialyloligosaccharides hydrolyse both $\alpha 2 \longrightarrow 3$ and $\alpha 2 \longrightarrow 6$ linkages. Thus far no multiple isoenzymes of this neuraminidase have been described, which can partly be due to methodological problems. The recent availability of fluorescent methylumbelliferyl substrate for neuraminidase will be of help in distinguishing various isoenzymes (Potier et al.,1979; Warner and O'Brien,1979).

The heterogeneity of the clinical features among patients with β-galactosidase deficiency is certainly in part determined by different mutations affecting neuraminidase(s) and/or β-galactosidase in various ways. Both of these enzymes seem to follow a "one enzyme - many substrates" model, and a particular gene mutation may only affect the capacity of hydrolysing some of these substrates. As a consequence different storage products may be present in the various organs of patients with different gene mutations thus causing a broad spectrum of clinical symptoms. The molecular structure of the enzymes involved, their intracellular processing and their interaction with the various substrates in vivo have to be understood before complete insight can be obtained in the relation between the nature of the genetic defect and the clinical features in the different variants of a genetic metabolic disease.

SUMMARY

The observed clinical and biochemical heterogeneity in G_{M1}- and G_{M2}-gangliosidosis was shown to have a genetic origin by complementation analysis. The existence of two complementation groups demonstrates that in both lysosomal storage diseases at least two different gene mutations may be involved. The aim of the study described in this thesis was to investigate the nature of the gene mutations involved. For this purpose, the molecular structure of the enzymes β-galactosidase and hexosaminidase, which are deficient in G_{M1}- and G_{M2}-gangliosidosis respectively, has been analysed in fibroblasts from control individuals and patients. In addition, the properties of the restored enzyme activity in heterokaryons after fusion of cells from different variants of G_{M1}-gangliosidosis has been investigated.

G_{M2}- gangliosidosis

Both the classical types of Tay-Sachs disease and Sandhoff's disease are characterized by progressive mental and motor deterioration, a cherry-red spot in the macula and the early death of the patients. Although the clinical features in both diseases are very similar, the nature of the hexosaminidase deficiency is different. The two major hexosaminidase isoenzymes, hexA and hexB, are both deficient in Sandhoff's disease, whereas only hexA is deficient in Tay-Sachs disease. The relation between these hexosaminidase isoenzymes is investigated in Appendix paper I. Gene localization studies, using man-Chinese hamster somatic cell hybrids, indicated that the expression of hexA is dependent on the presence of hexB in the hybrids. This is in agreement with a model in which

hexA and hexB are composed of multiple subunits, one of which is common to both forms (hexA= $(\alpha\beta)_n$; hexB= $(\beta\beta)_n$). The α-subunit was shown to be located on the human chromosome 15, whereas the β-subunit was assigned to chromosome 5. In hybrid clones where hexB is absent, a heteropolymeric isoenzyme is formed, consisting of human hex-α and Chinese hamster subunits $((\alpha\,CH)_n)$.

The characterization of the residual hexosaminidase activity in Sandhoff's disease is described in Appendix paper II. Hybrid cell lines were isolated after fusion of fibroblasts from a patient with Sandhoff's disease and Chinese hamster cells. Allthough both hexA and hexB were absent in the parental Sandhoff fibroblasts, the isolated hybrids expressed the heteropolymeric isoenzyme $((\alpha\,CH)_n)$. The electrophoretic and immunological properties of this heteropolymer were identical to those of the heteropolymer occurring in "control hybrids". It was concluded that in patients with Sandhoff's disease a normal α-subunit is present, which indicates that the β-subunit carries the mutation.

According to the model proposed, Tay-Sachs disease is caused by a mutation in the α-subunit. The complementation occurring after fusion of Sandhoff and Tay-Sachs fibroblasts, can be explained by a combination of normal α-subunits from Sandhoff cells with normal β-subunits present in the Tay-Sachs cells, leading to a restoration of hexA activity.

G_{M1}-gangliosidosis

In addition to the infantile and juvenile forms of G_{M1}-gangliosidosis a number of clinical variants have been described which differ in time of onset of the symptoms, the

involvement of visceral organs, the severity of mental retardation and the level of the residual β-galactosidase activity. Restoration of β-galactosidase activity occurs after fusion of fibroblasts from infantile or juvenile patients with fibroblasts from some of these clinical variants.

As described in Appendix paper III, in control human fibroblasts β-galactosidase exists in dimeric and multimeric aggregation forms probably consisting of one type of polypeptide (Mol. weight 70 000). As the transitions between monomer and dimer are reversible, the dimeric form, which is the predominant form under the conditions of enzyme assay, is thought to be the enzymatically active β-galactosidase isoenzyme.

Gene localization studies were performed to establish the number of genes involved in the expression of β-galactosidase. As described in the Appendix papers IV, V and VI, two structural loci, coding for G_{M1}- β-galactosidase could be assigned to the human chromosomes 3 and 22. The relation between these two gene loci and the monomeric, dimeric and multimeric β-galactosidase still has not yet been established.

All mutant cell strains tested showed abnormal aggregation patterns for β-galactosidase, indicating that structurally altered β-galactosidase is present (Appendix paper III). Furthermore it is shown in Appendix paper VI that the infantile form of G_{M1}-gangliosidosis is caused by a mutation in the structural β-galactosidase gene on chromosome 3. There was no evidence for the presence of a mutation in a structural locus for β-galactosidase in an adult patient with a β-galactosidase deficiency. Since the cells from this patient also showed a neuraminidase deficiency, it is thought that the β-galactosidase deficiency is secondary, due to a primary defect in neuraminidase. As yet, the mechanism of inactivation of β-galactosidase is unknown.

The restoration of β-galactosidase activity after fusion of fibroblasts from patients with the infantile and adult types of β-galactosidase deficiency is mainly due to an increase in monomeric β-galactosidase (M.W. 70 000) (Appendix paper VI). It may very well be that in the heterokaryons the neuraminidase activity of the cells from the infantile type, together with β-galactosidase polypeptides present in the cells of the adult patient, result in restoration of β-galactosidase activity.

SAMENVATTING

In patienten met de ziekte G_{M2}-gangliosidosis is het enzym hexosaminidase geheel of gedeeltelijk afwezig. Er bstaan twee vormen van deze ziekte, de ziekte van Tay-Sachs en de ziekte van Sandhoff, genoemd naar de ontdekkers ervan. Er bestaan ook tenminste twee vormen (iso-enzymen) van het hexosaminidase, die hexA en hexB worden genoemd. Bij de ziekte van Tay-Sachs is alleen hexA afwezig, terwijl bij de ziekte van Sandhoff zowel hexA als hexB afwezig zijn. Dit roept vragen op omtrent de opbouw en relatie van deze iso-enzymen.

In Appendix paper I kon met genlocalisatie technieken aannemelijk worden gemaakt dat hexA en hexB één eiwitketen gemeenschappelijk hebben, de β-subeenheid. Daarnaast bezit hexA nog een eigen, specifieke subeenheid (α-subeenheid). In een model: hexA $= \alpha\beta$; hexB $= \beta\beta$. Eveneens met genlocalisatie technieken kon worden aangetoond, dat patienten met de ziekte van Sandhoff wel in staat zijn een normale α-subeenheid te maken (Appendix paper II). Een defecte β-subeenheid belemmert in deze patienten de vorming van hexA en hexB. De ziekte van Tay-Sachs wordt volgens het model veroorzaakt door een defecte α-subeenheid.

Bij patienten waar het enzym β-galactosidase defect is (G_{M1}-gangliosidosis) kan de ernst en de aard van de ziekte erg verschillen. Sommige patienten overlijden al in hun eerste levensjaar (infantile vorm van de ziekte) terwijl andere de volwassen leeftijd bereiken (volwassen vorm). De oorzaken van deze "klinische heterogeniteit" zijn bestudeerd.

In Appendix paper III zijn de eigenschappen van het normale β-galactosidase onderzocht. De werkzame vorm van het en-

zym bleek een molekule te zijn bestaande uit twee identieke eiwitketens. Onder sommige omstandigheden waren ook grotere molekulen, met meer dan twee identieke eiwitketens mogelijk, terwijl de eiwitketen ook apart kon voorkomen. In patienten waar slechts een geringe hoeveelheid actief β-galactosidase aanwezig was, bleek tevens dat de nog aanwezige eiwitketens van structuur veranderd waren.

Genlocalisatie technieken leerden (Appendix papers IV, V en VI) dat twee genen, gelegen op de menselijke chromosomen 3 en 22, informatie bevatten voor structurele onderdelen van het β-galactosidase. Hoe dit gegeven in overeenstemming gebracht moet worden met de thans bestaande opvattingen over de molekulaire bouw van β-galactosidase is nog onduidelijk.

Tevens kon worden aangetoond (Appendix paper VI) dat de infantile vorm van de ziekte veroorzaakt wordt door een erfelijke verandering in het gen op chromosoom 3. Voor de volwassen vorm konden geen erfelijke veranderingen in beide β-galactosidase genen worden vastgesteld. In dergelijke patienten is echter ook een defect aangetoond van een ander enzym, neuraminidase. Verondersteld wordt nu dat in sommige volwassen varianten het neuraminidase defect verantwoordelijk is voor het onwerkzaam worden van β-galactosidase. Verder onderzoek is nodig om op te helderen hoe de gecombineerde neuraminidase en β-galactosidase deficiënties onderling samenhangen. Wel is nu duidelijk geworden dat de verschillende klinische varianten van G_{M1}-gangliosidosis eigenlijk een ander ziektebeeld betreffen dan de afwijkingen die gepaard gaan met zowel een neuraminidase als een β-galactosidase defect.

Voor belangstellenden is een nadere uitleg van de gebruikte methoden, technieken en verkregen resultaten verkrijgbaar.

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Petty

CURRICULUM VITAE

Op 24 september 1950 werd H.L.Hoeksema te Groningen geboren. Het eindexamen HBS-B werd in 1967 behaald aan het Christelijk Lyceum te Leiden. Een jaar later volgde het diploma leerling-analist (Chemische richting), waarna in 1970 het examen Biochemisch laboratoriumassistent met goed gevolg werd afgelegd. In de periode 1968-1970 was zij werkzaam bij het Medisch Biologisch Laboratorium, destijds van de Rijksverdedigingsorganisatie TNO, te Rijswijk.

In 1970 werd de studie Biologie aangevangen aan de Rijks Universiteit te Groningen. Het kandidaats B4 (Biochemie) werd behaald in december 1973. Voor het doctoraal examen werd onderzoek verricht op de afdelingen Experimentele Fytomorfologie (R.U. Groningen) en Celbiologie en Genetica (Erasmus Universiteit, Rotterdam). In de periode 1975-1976 was zij student-assistent in de celbiologie aan de R.U. Groningen. Februari 1976 werd het doctoraal examen (met onderwijsbevoegdheid) afgelegd.

Na haar doctoraal examen kon zij dankzij financiële steun van de stichting FUNGO van ZWO een promotieonderzoek verrichten op de afdeling Celbiologie en Genetica, onder leiding van Prof. Dr. H. Galjaard en Prof. Dr. D. Bootsma. De resultaten van dit onderzoek zijn in dit proefschrift beschreven. In deze periode is tevens een bijdrage geleverd aan het onderwijs in de Celbiologie, Histologie en Microscopische Anatomie. Sinds maart 1979 is zij voor enkele uren als docent verbonden aan de Stichting Opleiding Fysiotherapie Thim van der Laan te Utrecht.

Characterization of β-D-*N*-Acetylhexosaminidase Isoenzymes in Man-Chinese Hamster Somatic Cell Hybrids

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INTRODUCTION

Several forms of β-D-*N*-acetylhexosaminidase (E.C.3.2.1.30) have been demonstrated in different human tissues [1–3]. Four isoenzymes, characterized by immunological and biochemical techniques [4–8], have been designated β-D-*N*-acetylhexosaminidase A, B, C and S, further to be referred to as hex A, hex B, hex C, and hex S.

Deficient hex A activity and an increased activity of hex B is found in G_{M2} gangliosidosis type 1 or Tay-Sachs disease, whereas G_{M2} gangliosidosis type 2 or Sandhoff disease is associated with a deficiency of both hex A and hex B [9–12].

Biochemical, immunological, and genetical studies suggest a structural relationship between hex A and hex B [1, 5, 10, 13–19]. Robinson and Carroll [19] and Desnick et al. [10] suggested that both isoenzymes are composed of multiple subunits, one of which is common to both forms. Two subunit models have been suggested: (1) the three locus model [10]: hex A = $(\alpha\beta)_n$, hex B = $(\beta\gamma)_n$; and (2) the two locus model: hex A = $(\alpha\beta)_n$, hex B = $(\beta\beta)_n$. Tay-Sachs disease can be explained by a defective α subunit specific for hex A and Sandhoff disease, by a defective common β subunit. Recently, strong evidence in favor of the two locus model has been presented by Beutler and Kuhl [20] in in vitro enzyme hybridization studies.

Studies of the segregation of the hexosaminidase markers in man-rodent somatic cell hybrids can discriminate between the different subunit theories. The results however are contradictory. Lalley et al. [21] found that hex A was never expressed in these hybrids in the absence of hex B, which fits the two locus model. Gilbert et al. [15] and van Someren and Beyersbergen van Henegouwen [14], however, reported an independent loss of hex A and hex B in cell hybrids, which favors the three locus model.

These conflicting results may be due to a misinterpretation of the electrophoretic patterns obtained from hybrid lines, if one relies solely on electrophoretic mobility. The formation of heteropolymeric molecules, especially forms with nearly the same electrophoretic mobility as hex A, might confuse the interpretation of the segregation

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results. Therefore, a further characterization of the hexosaminidase isoenzymes in hybrid lines is needed. It is possible to obtain information about the presence of possible heteropolymeric molecules in hybrid cell lines by using specific antisera against human and Chinese hamster hexosaminidase.

In the present study, a series of man-Chinese hamster hybrids were investigated using an anti-Chinese hamster hexosaminidase serum, a specific anti-human hex A serum, and an anti-human hex B serum. It was found that a hex A-like enzyme present in the hybrid lines consists of both Chinese hamster and specific human hex A moieties. The expression of human hex A was found to be dependent on the presence of hex B, and it was shown that a gene, coding for a specific hex A subunit is syntenic with the mannosephosphate isomerase (*MPI*) and pyruvate kinase (*PK-3*) loci, assigned to chromosome 15.

MATERIALS AND METHODS

Purification of Chinese Hamster Hexosaminidase

Chinese hamster hexosaminidase was purified 290 times from pooled homogenates of liver and kidney. The 20,000 *g* supernatant was applied on a ConA sepharose column, equilibrated with 10 mM sodium-phosphate buffer, pH 7.0, containing 0.1 mM $CaCl_2$, 0.1 mM $MnSO_4$, and 0.5 M NaCl. Hexosaminidase was eluted with 1 M α-methylglucoside in the same buffer solution; pooled fractions were dialyzed and applied to a second affinity column. *N*-acetylglucosamine, bound to CN-Br activated sepharose as described by Lotan et al. [22] was used as affinity ligand. Hexosaminidase was eluted with 10 mM *N*-acetylglucosamine and 0.2 M NaCl in 10 mM sodium-phosphate buffer, pH 6.0. Pooled fractions, containing hexosaminidase were lyophilized and used to raise antisera.

Antisera

Antisera against purified human hex A, hex B, and Chinese hamster hexosaminidase were raised in New Zealand white rabbits by a slight modification of the method described by Carroll and Robinson [23]. Specific human hex A antiserum was prepared by absorption of anti-hex A serum with human liver hex B, according to Bartholomew and Rattazzi [24].

Cell Lines

Normal fibroblasts, hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficient fibroblasts, or normal leukocytes were used as human parental cell lines in the fusion procedure. The Chinese hamster parental cell lines used were thymidine kinase (TK) or HPRT deficient fibroblasts. Details of the fusion procedure and the isolation and propagation of the hybrid cell lines have been described previously [25]. Preparation of cell lysates was carried out as described by Meera Khan [26]. The human controls used were a tetraploid epithelial kidney cell line (T-cell) [27] and a normal fibroblast cell strain.

Electrophoresis

Electrophoresis of hexosaminidase, MPI, and PK-3 was carried out on cellulose acetate gel (Cellogel, Chemetron, Milan, Italy). Hexosaminidase electrophoresis was performed in 0.05 M potassium-phosphate buffer, pH 6.6, for 1 hr at 4°C. The staining procedure for hexosaminidase was carried out according to Okada and O'Brien [11]. The electrophoresis and staining procedures of MPI and PK-3 have been published by van Someren et al. [28].

Immunoprecipitation

Lysates were incubated with antiserum overnight at 0°C. After incubation, the treated lysates were centrifuged at 35,000 *g* for 20 min. The supernatant was used for electrophoresis.

Electrophoresis of lysates of normal human fibroblasts reveals three bands of activity: hex B, hex A and hex C (fig. 1*a*, lane 1). In lysates of Chinese hamster fibroblasts, two bands of activity, described as hex 1 and hex 2 (fig. 1*b*, lane 1), were found.

The specificity of the antisera against Chinese hamster hexosaminidase and against human hex A and hex B was tested with lysates of human and Chinese hamster fibroblasts. The resulting electrophoretic patterns are illustrated in figure 1*a* and 1*b*, lanes 2, 3, and 4. With the concentrations used, the antiserum against Chinese hamster hex 1 does not cross-react with human hex A, hex B, and hex C (fig. 1*a*, lane 2) nor with

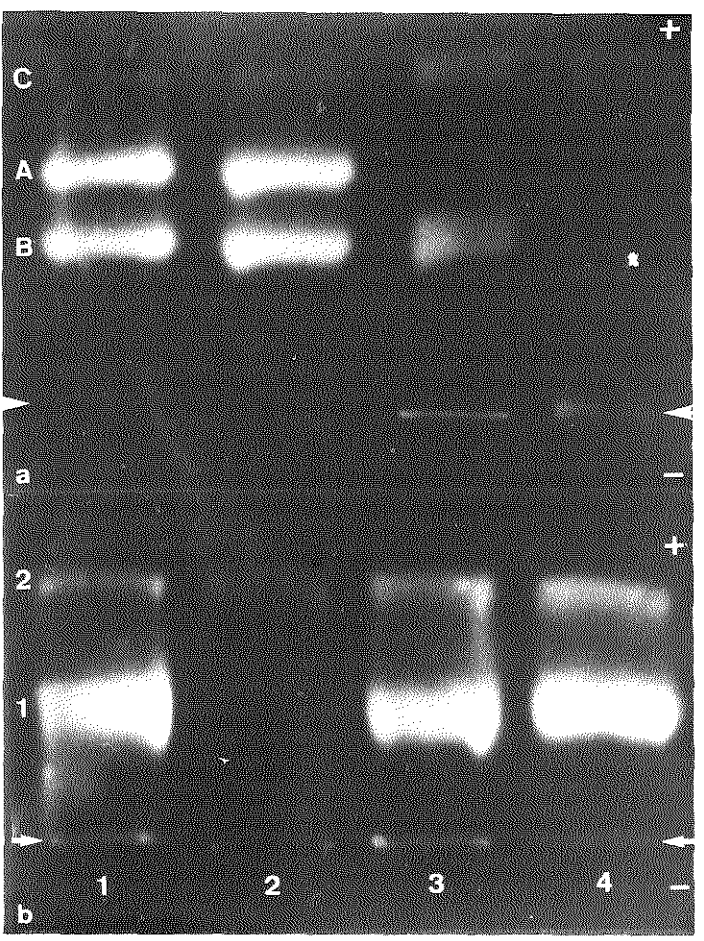

FIG. 1. — Electrophoretic patterns of human (*a*) and Chinese hamster (*b*) fibroblast lysates. *Lane 1*, A, B, C refer to hex A, hex B, and hex C; 1 and 2 to Chinese hamster hex 1 and hex 2: *lane 2*, after treatment with anti-Chinese hamster hexosaminidase serum; *lane 3*, after treatment with specific anti-human hex A serum; *lane 4*, after treatment with anti-human hex B serum. The amount of lysate used in the various lanes depended on the antibody concentrations of the antisera, explaining differences in staining intensity.

the fastmoving Chinese hamster hex 2 band. The specific anti-human hex A serum does not remove hex B and hex C, whereas hex A disappeared (fig. 1*a*, lane 3). The anti-human hex B serum reacts with both hex A and hex B but not with hex C (fig. 1*a*, lane 4). No reaction of specific anti-human hex A and anti-human hex B sera with the Chinese hamster hexosaminidases was observed (fig. 1*b*, lane 3 and 4). These results were confirmed with double immunodiffusion in agarose gels [29] and by antigen-antibody titrations in various combinations of antigens and antisera.

Four types of hybrid lines could be distinguished by electrophoresis of different man-Chinese hamster cell hybrids (fig. 2). They are indicated as hex A′+/hex B+, hex A′+/hex B−, hex A′−/hex B+, hex A′−/hex B−. The band of activity with a mobility comparable to hex A is called hex A′.

Lysates prepared from these four different types of hybrid clones were treated with the various antisera, and the resulting isoenzyme patterns of hexosaminidase were studied by electrophoresis. The results obtained with a hex A′+/hex B− hybrid are shown in figure 3. After treatment with anti-Chinese hamster hex 1 serum, both the Chinese hamster hex 1 and the hex A′ band disappear. The specific anti-human hex A serum removes the hex A′ activity, whereas no reaction with Chinese hamster hexosaminidase was observed. Anti-human hex B serum does not change the electrophoretic pattern. Nine hex A′+/hex B− hybrid clones from five independent fusion experiments were tested in this way; they invariably showed the same immunological characteristics.

Eight hex A′+/hex B+ hybrid cell lines from independent fusion experiments were also studied with antisera (fig. 4). After treatment of the lysates with anti-Chinese hamster hex 1 serum, the hex 1 band disappears along with part of the activity of the hex A′ band (fig. 4, lane 3). Various concentrations of the anti-Chinese hamster hex 1 serum were used, but a complete removal of the activity on the hex A′ position could not be attained. The activity of hex B does not seem to be affected. With specific anti-human hex A serum, all the activity is removed at the hex A′ position, whereas hex B and

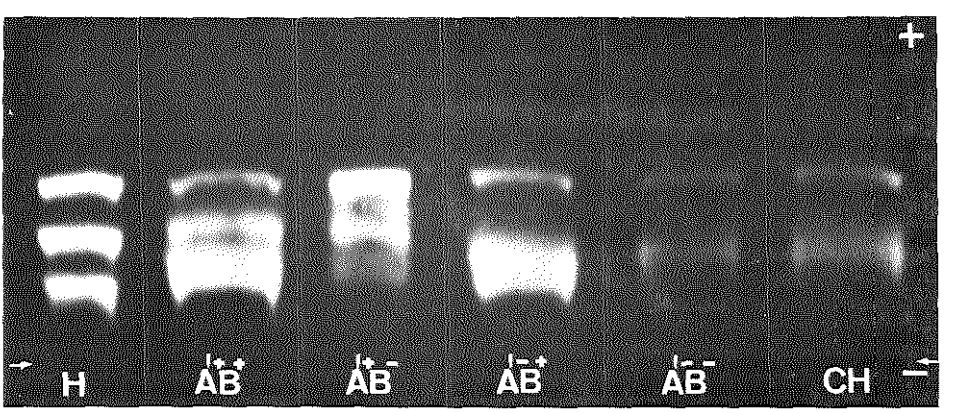

FIG. 2.—Hexosaminidase electrophoretic patterns. *From right to left*: H = human T-cell (Electrophoretic mobility of hex isoenzymes of T-cell and human fibroblast are comparable; the only difference is the intensity of the fastest moving band.) A′+/B+ = hybrid clone with activities at hex A′ and hex B position; A′+/B− = hybrid clone with activity at hex A′ position; A′−/B+ = hybrid clone with activity at hex B position; A′−/B− = hybrid clone having lost the activities at hex A′ and hex B position.

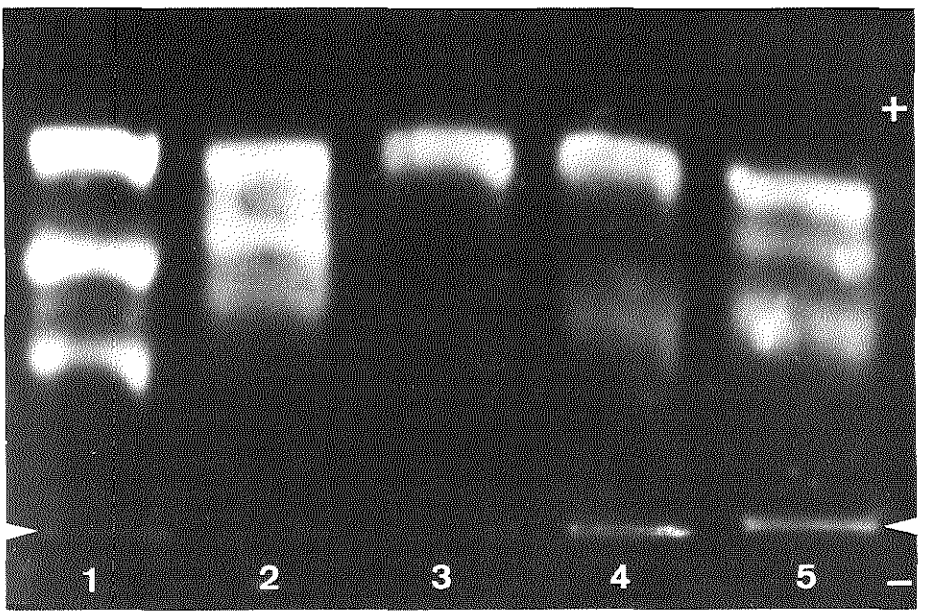

FIG. 3.—Electrophoretic patterns of a hex A′+/hex B− hybrid, following treatment with different antisera. *Lane 1*, human T-cell; *lane 2*, hybrid without antiserum treatment; *lane 3*, hybrid following treatment with the anti-Chinese hamster hexosaminidase serum; *lane 4*, hybrid following treatment with the specific anti-human hex A serum; *lane 5*, hybrid following treatment with the anti-human hex B serum.

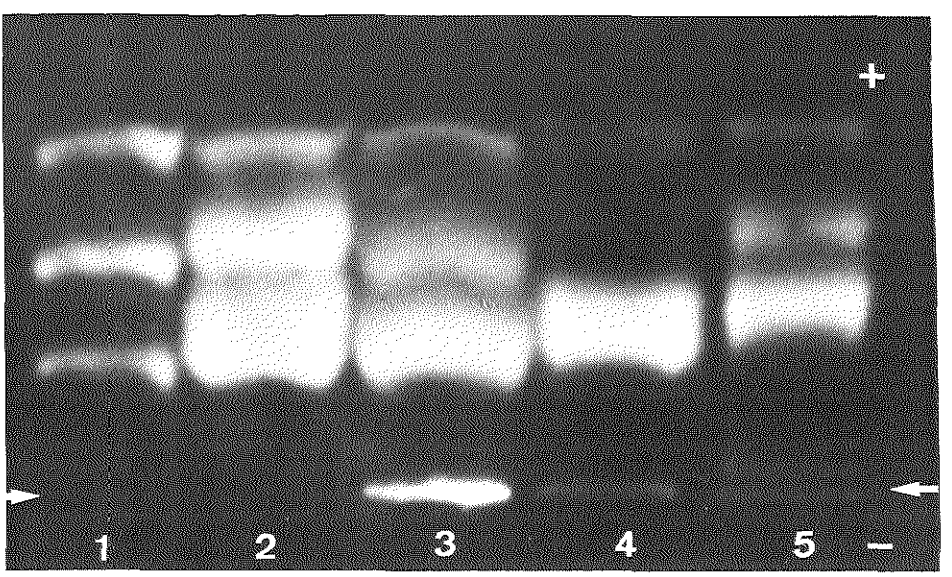

FIG. 4.—Electrophoretic patterns of a hex A′+/hex B+ hybrid, following treatment with different antisera. *Lane 1*, human T-cell; *lane 2*, hybrid without antiserum treatment; *lane 3*, hybrid following treatment with the anti-Chinese hamster hexosaminidase serum; *lane 4*, hybrid following treatment with the specific anti-hex A serum; *lane 5*, hybrid following treatment with the anti-hex B serum.

Chinese hamster hex 1 activities remain unchanged. When treated with anti-human hex B serum, part of the activity at the hex A′ position remains, and a normal Chinese hamster activity was detected. Also in this case, various dilutions of the antiserum were tested, and always residual activity at the hex A′ position remained present. Figure 5 illustrates the typical electrophoretic patterns of a hex A′−/hex B+ hybrid after incubation with the three antisera. Seven clones were tested. Hex B could be removed only by the anti-human hex B serum. The Chinese hamster hex 1 band cross-reacts with the anti-Chinese hamster hex 1 serum. In hex A′−/hex B− hybrids, changes in the electrophoretic pattern were observed only after treatment with the anti-Chinese hamster hex 1 serum, which removes the Chinese hamster hex 1 band.

A relationship between the absence and the presence of the hex A′ band, MPI and PK-3 was established, as shown by the data from several fusion experiments (table 1). No syntenic relationship was found with 24 other enzyme markers tested.

DISCUSSION

The discrepancies in the literature between data obtained in various experiments with man-rodent somatic cell hybrids [14, 15, 21] may be due to different interpretations of the hexosaminidase electrophoretic patterns from hybrid cell lines with particular regard to the activity at the human hex A position. The characterization of the isoenzymes in the hybrid cell lines can be greatly improved by the use of anti-Chinese hamster and anti-human hexosaminidase sera, as seen in the present study.

In hex A′+/hex B− hybrid clones, the hex A′ band was shown to interact with both

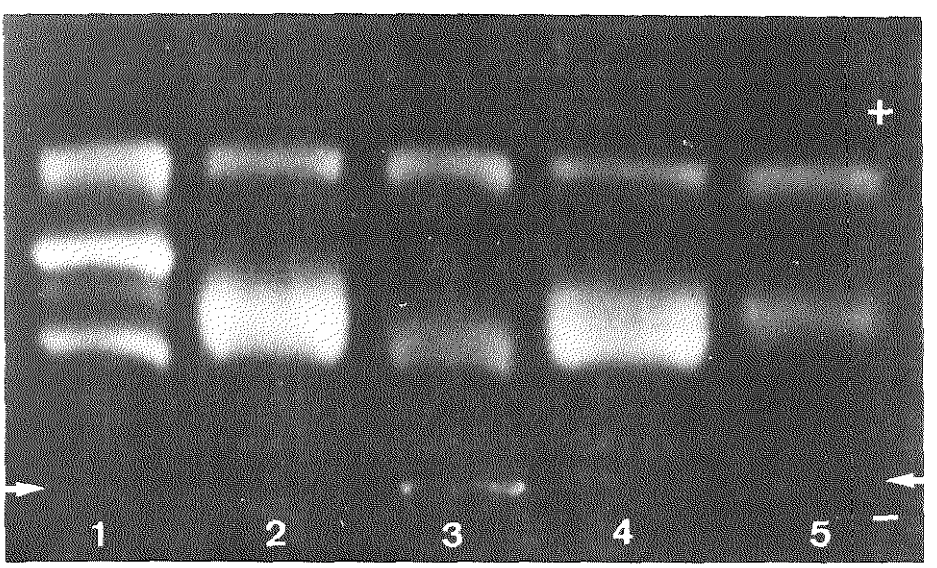

FIG. 5.—Electrophoretic patterns of a hex A′−/hex B+ hybrid, following treatment with different antisera. *Lane 1*, human T-cell. In fresh homogenates an additional band of activity can be found between hex A and hex B. This band corresponds to the intermediate forms I_1, I_2, as seen by DEAE cellulose chromatography [34]. *Lane 2*, hybrid without antiserum treatment; *lane 3*, hybrid following treatment with the anti-Chinese hamster hexosaminidase serum; *lane 4*, hybrid following treatment with the specific anti-hex A serum; *lane 5*, hybrid following treatment with the anti-hex B serum.

TABLE 1

ABSENCE AND PRESENCE OF HEX A′, MPI, AND PK-3 IN PRIMARY AND SECONDARY HYBRID LINES

	MPI		PK-3	
	+	−	+	−
Hex A′:				
+	30	3	27	3
−	0	28	0	35
PK-3:				
+	33	0	. . .	. . .
−	0	29	. . .	. . .

the anti-Chinese hamster hex 1 and the specific anti-human hex A sera, whereas no cross-reaction with the anti-human hex B serum was found. This finding indicates, that in these hybrids the isoenzyme at the hex A′ position is not a normal human hex A, but a heteropolymeric molecule, containing both specific human hex A and Chinese hamster hexosaminidase moieties. The formation of heteropolymeric hexosaminidase molecules in man-Chinese hamster hybrids has been suggested before by Ropers and Schwantes [17], who supposed a heteropolymeric molecule at the hex A position to consist of Chinese hamster and human hex C subunits. In their theory, hex B and hex C are homopolymers, $(\beta\beta)_n$ and $(\alpha\alpha)_n$, respectively, whereas hex A consists of B− and C subunits $(\alpha\beta)_n$. Van Cong et al. [30] described a band of activity comparable with hex A′, which they called "hex A fast." A relationship was demonstrated between the presence and absence of "hex A fast," MPI, and hex C. Following the subunit model of Ropers [16], "hex A fast" was explained as a heteropolymeric molecule with α subunits from human hex C and β-like subunits from Chinese hamster hexosaminidase. Recently it has been shown that at the hex C position two isoenzymes (hex C and hex S) can be detected [3, 20]. In view of these studies, it is likely that the fastest moving hex band described by Van Cong is identical with hex S. Our electrophoresis system does not discriminate between hex S and hex C.

The residual activity in hex A′ +/hex B+ hybrid clones that could be detected at the hex A′ position after treatment with the anti-Chinese hamster hex 1 or anti-human hex B serum indicates the presence of two different isoenzymes at the hex A′ position. Since no activity is left after treatment with the specific anti-human hex A serum, a heteropolymeric molecule is thought to be present, similar to the heteropolymeric molecule found in hex A′ +/hex B− hybrid clones at the hex A′ position, which consists of human hex A and Chinese hamster hex 1 moieties. The other isoenzyme at the hex A′ position cross-reacts with specific anti-human hex A and anti-human B sera and appears to represent a normal human hex A molecule.

From these studies, we conclude that normal human hex A is present only in the presence of hex B, which is in agreement with the results of Lalley et al. [21]. A heteropolymeric isoenzyme can be present independently of human hex B. The results of our present study fits into the two locus subunit model. According to this theory, the heteropolymeric isoenzyme at the hex A′ position has been shown to possess α subunits from human hex A and β-like subunits from Chinese hamster hex 1. The formation of

other heteropolymeric molecules, in particular between the human β subunit and an α-like subunit from the Chinese hamster cannot be excluded. The resolution of our electrophoresis system in the hex 1 and the hex B region is, however, insufficient to distinguish possible heteropolymeric molecules.

Evidence for the syntenic relationship between *hex A*, *MPI*, and *PK-3* [14, 27, 31, 32] and the assignment of *MPI* to chromosome 15 [33] puts this synteny group on chromosome 15. Our results show a relationship between the absence or presence of the isoenzymes on the hex A′ position, and that of MPI and PK-3. With respect to the two locus subunit model, the gene coding for the α subunit of hex A is localized on chromosome 15. Previously, *hex B* has been assigned to chromosome 5 by Gilbert et al. [14]. According to this model, it should be the gene, coding for the β chain which was localized on chromosome 5.

Studies of the expression of hexosaminidases in man-Chinese hamster hybrid cell lines can be a tool for understanding the molecular structure of hexosaminidase, provided that the characterization of human, Chinese hamster, and possible heteropolymeric components does not rely solely on electrophoretic mobilities.

SUMMARY

A series of man-Chinese hamster hybrids were investigated with the use of an anti-Chinese hamster hexosaminidase serum, a specific anti-human hex A serum and an anti-human hex B serum. The expression of human hex A was found to be dependent on the presence of hex B. A heteropolymeric molecule is formed independently of hex B, which consists of Chinese hamster and specific hex A moieties. It has an electrophoretic mobility nearly identical to hex A. A relationship between the absence and presence of the heteropolymeric molecule, mannosephosphate isomerase (MPI), and pyruvate kinase (PK-3), assigned to chromosome 15, was established. With respect to the two locus subunit model, the gene coding for the α subunit, specific for hex A, has been localized on chromosome 15.

ACKNOWLEDGMENTS

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PAPER II

Characterization of Residual Hexosaminidase Activity in Sandhoff's Disease Using Man-Chinese Hamster Cell Hybrids

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Summary. To obtain information about the nature of the residual hexosaminidase activity in Sandhoff's disease, hybrid cell lines between fibroblasts from a patient with Sandhoff's disease and Chinese hamster cells were isolated.

In these hybrid cell lines, a heteropolymeric isoenzyme was detected that is composed of human α- and Chinese hamster hexosaminidase subunits. Due to the electrophoretic and immunological behavior of the heteropolymeric molecules in interspecies hybrids with normal fibroblasts and with cells from a patient with Sandhoff's disease, we conclude that Sandhoff cells contain an α-subunit of hexosaminidase with normal characteristics.

Introduction

Multiple forms of β-D-N-acetylhexosaminidase (E.C. 3.2.1.30) (hex) have been characterized in normal human tissues, the most common forms designated as hex-A, hex-B, hex-C and hex-S (Robinson and Stirling, 1968; Hooghwinkel et al., 1972; Ikonne et al., 1975; Beutler et al., 1975). In man, two lysosomal storage diseases are associated with a deficient activity of acid forms of hexosaminidase. G_{M2} gangliosidosis type 1 (Tay-Sachs disease) is characterized by a deficiency of hex-A (Okada and O'Brien, 1969) and hex-S (Reuser and Galjaard, 1976). In G_{M2} gangliosidosis type 2 (Sandhoff's disease), both hex-A and hex-B are absent, whereas hex-S is present (Desnick et al., 1972). Complementation studies in heterokaryons obtained after fusion of Tay-Sachs and Sandhoff fibroblasts show that these two enzyme defects are based on different gene mutations (Thomas et al., 1974; Galjaard et al., 1974).

Biochemical and immunological data presented by Robinson and Stirling (1968) and by Srivastava and Beutler (1973) suggest a structural relationship between hex-A and hex-B. A two-locus subunit model has been postulated (Desnick et al., 1972; Ropers and Schwantes, 1973) in which hex-A = $(\alpha\beta)_n$; hex-B = $(\beta\beta)_n$. This model was firmly established by the reassociation experiments of Beutler and

Kuhl (1975). Tay-Sachs disease might be explained by a defective α-subunit, whereas Sandhoff's disease would be caused by a mutation in the β-subunit.

Studies with man-rodent somatic cell hybrids also support the two-locus subunit model, since the expression of human hex-A depends on the presence of hex-B (Lalley et al., 1974; Van Cong et al., 1975; Hoeksema et al., 1977). In some hybrid clones, a heteropolymeric isoenzyme consisting of both Chinese hamster and specific human hex-A subunits was produced independently of hex-B (Hoeksema et al., 1977). The genetic information for hex-B: in this model the gene coding for the specific β-subunit could be assigned to chromosome 5 (Gilbert et al., 1975), whereas the gene coding for the specific α-subunit of hex-A was assigned to chromosome 15 (van Heyningen et al., 1975; Hoeksema et al., 1977).

The residual hexosaminidase activity in Sandhoff's disease, which is about 1% of normal controls, consists mainly of hex-S (Beutler et al., 1975). This isoenzyme, with an electrophoretic mobility nearly identical to the neutral form hex-C, is immunologically related to hex-A (Beutler et al., 1975), and reassociations experiments by Beutler and Kuhl (1975) suggest that hex-S is an α-chain homopolymer.

The purpose of the present study was to investigate the nature of the residual hexosaminidase activity in fibroblasts from a patient with Sandhoff's disease. Hybrids between Chinese hamster cells and fibroblasts from a patient were used and the nature of the hexosaminidase isoenzyme pattern was analyzed with electrophoretic and immunological methods.

Materials and Methods

The hybrids were isolated following fusion of fibroblasts from a patient with Sandhoff's disease with thymidine-kinase-deficient Chinese hamster fibroblasts. The fusion procedure, isolation, and propagation of the hybrid clones were described by Westerveld et al. (1971). Lysates were prepared by sonication in 5 mM phosphate buffer pH 6.4 containing 1 mM Na_2EDTA and 1 mM β-mercaptoethanol. The sonicate is centrifuged at 27,000 g for 20 min at 4° C. Chromosome preparations were made on the same batches of cells as the lysates for the enzyme studies. The chromosomes were identified with the trypsin-Giemsa technique described by Seabright (1971).

The methods for enzyme purification and for the preparation of antisera against human liver hex-A and hex-B and Chinese hamster hexosaminidase were published earlier (Hoeksema et al., 1977). Cell homogenates (5 µl) were incubated with 5 µl antiserum overnight at 0° C. After centrifugation at 35,000 g the supernatant was used for hexosaminidase electrophoresis, which was done on Cellulose acetate gel (Cellogel, Chemetron, Milan, Italy) in 50 mM potassium-phosphate buffer pH 6.6 for one hour at 4° C. Staining for hexosaminidase was performed with methylumbelliferyl substrate according to Okada and O'Brien (1969).

The specificity of the antisera was tested with lysates of human and Chinese hamster fibroblasts. Both antihuman hex- ($\alpha\beta$) and antihuman hex- (β) antisera removed human hex-A as well as hex-B, whereas no reaction with Chinese hamster hexosaminidase was observed. Anti-Chinese hamster hexosaminidase antiserum did remove Chinese hamster hexosaminidase (hex-1), but not the most anodal band (hex-2). No reaction with human hex-A and hex-B was observed. These results were already published in more detail by Hoeksema et al. (1977).

Results

After fusion of Sandhoff fibroblasts and Chinese hamster cells, two hybrid cell lines that contained the human chromosomes 5 and 15 were isolated. The

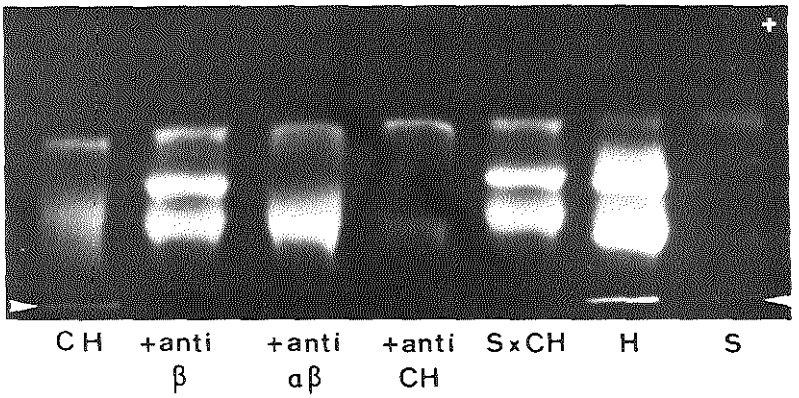

Fig. 1. Hexosaminidase isoenzyme pattern of Chinese hamster cells (*CH*), fibroblasts from normal human (*H*) and from a patient with Sandhoff's disease (*S*). The pattern of a Sandhoff-Chinese hamster hybrid ($S \times CH$) is shown before and after treatment with antihuman hex-(β), antihuman hex-($\alpha\beta$) and anti-Chinese hamster hexosaminidase antisera

hexosaminidase isoenzyme pattern of such a hybrid line, those of both parental cell strains, and the effect of the various antihexosaminidase antisera are shown in Figure 1.

Electrophoresis of control human fibroblasts reveals three bands of activity: starting at the origin hex-B, hex-A, and the most anodal band, which consists of both hex-C and hex-S. The Sandhoff cells express most clearly the hex-C/hex-S activities and weak activities for hex-A and hex-B are present after administration of large amounts of cell homogenate. The isoenzyme pattern of Chinese hamster cells shows two bands: a major band (hex-1) with a mobility intermediate between the human hex-A and hex-B band, and a more anodal band (hex-2) with a mobility comparable to human hex-C/hex-S.

The electrophoretic pattern of a Sandhoff-Chinese hamster hybrid cell line reveals three bands of activity. An isoenzyme is present at the Chinese hamster hex-1 and human hex-B position. The second band of activity has an electrophoretic mobility comparable to hex-A, designated the hex-A′ position. The most anodal band is present at the Chinese hamster hex-2 and human hex-C/hex-S position.

Following treatment of the Sandhoff-Chinese hamster hybrids with the anti-Chinese hamster hexosaminidase antiserum, both the Chinese hamster hex-1 band and the band at the hex-A′ position disappear. A faint activity remains at the human hex-B position. This activity was removed by subsequent incubation of the treated homogenate with antihuman hex- (β) antiserum. The most anodal hex-C/hex-S/hex-2 band is not influenced by the anti-Chinese hamster hexosaminidase antiserum. Treatment of the Sandhoff-Chinese hamster hybrid cell lysates with antihuman hex- ($\alpha\beta$) antiserum removes the band at the hex-A′ position, while the antihuman hex- (β) antiserum does not change the electrophoretic pattern.

Discussion

According to the two-locus subunit model (hex-A $=(\alpha\beta)_n$, hex-B $=(\beta\beta)_n$), human chromosome 5 bears the genetic information for the hex β-subunit, whereas the gene coding for the hex α-subunit is on chromosome 15 (Gilbert et al., 1975; van Heyningen et al., 1975; Hoeksema et al., 1977). Some of the hybrid cell lines, isolated after fusion of normal human fibroblasts and Chinese hamster cells express a band of activity on a position comparable to hex-A (hex-A′ position). This band represents a heteropolymeric molecule containing human α- and Chinese hamster hexosaminidase subunits.

The proliferating Sandhoff-Chinese hamster hybrid cell lines described in the present paper did contain both the human chromosomes 5 and 15, and thus the necessary genetic information for the human α- and β- hexosaminidase subunits. The band at the hex-A′ position was also present in these hybrids. The activity, remaining at the hex-B position in the Sandhoff-Chinese hamster hybrids after treatment with the anti-Chinese hamster hexosaminidase antiserum, was the residual activity of Sandhoff hex-B, due to the presence of chromosome 5 in the hybrid lines. This suggests a structural mutation of the β-subunit of hexosaminidase in Sandhoff's disease.

Van Cong et al. (1975) also found an isoenzyme at the hex-A′ position in Sandhoff-Chinese hamster and Sandhoff-mouse hybrids. However, the interpretation of their data was complicated by the fact that they could not characterize the expressed isoenzyme. In our present studies of Sandhoff-Chinese hamster hybrids, the activity at the hex-A′ position interacted both with anti-Chinese hamster hexosaminidase antiserum and antihuman hex- $(\alpha\beta)$ antiserum, but the antihuman hex- (β) antiserum did not react. We therefore conclude that the isoenzyme at the hex-A′ position is a heteropolymer consisting of human α- and Chinese hamster hexosaminidase subunits. Since the electrophoretic and immunological behavior of this heteropolymeric molecule is similar to that in normal man-Chinese hamster hybrids (Hoeksema et al., 1977), we also conclude that Sandhoff fibroblasts contain an α-subunit with normal characteristics.

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PAPER III

INTERGENIC COMPLEMENTATION AFTER FUSION OF FIBROBLASTS FROM DIFFERENT PATIENTS WITH β-GALACTOSIDASE DEFICIENCY

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Summary

Acid β-galactosidase from human liver consists, after gel filtration at pH 7.0, of a monomeric isoenzyme, β-galactosidase A and small amounts of a multimer, β-galactosidase B (Norden, A.G.W., Tennant, L. and O'Brien, J.S. (1974) J. Biol. Chem. 249, 7969–7976).

Our studies showed identical gel filtration patterns for β-galactosidase from human liver and cultured skin fibroblasts. Gel filtration in the buffer used for enzyme assays (pH 4.5) however, revealed primarily dimeric β-galactosidase, minor amounts of β-galactosidase A but no multimers. As the transitions between monomeric and dimeric β-galactosidase were reversible, dimeric β-galactosidase is apparently the enzymatically active isoenzyme.

The isoenzymes from patients with four different clinical variants of β-galactosidase deficiency showed altered aggregation patterns, although their molecular weights corresponded to the normal molecular weights.

It could be demonstrated that the restoration of β-galactosidase activity, which occurred after cell fusion of certain combinations of different β-galactosidase deficient fibroblasts, is not the result of intragenic complementation. Mixing experiments with cell-free extracts ruled out that the formation of dimeric β-galactosidase is required for complementation.

To explain the complementation a model is suggested involving two different genes: a structural gene responsible for the synthesis of the polypeptide chain and a second gene which is involved in a modification process.

Introduction

A deficiency of the acid forms of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is responsible for the autosomal recessive disease G_{M1}-gangliosidosis, where gangliosides and glycosaminoglycans are accumulated in

various tissues [2]. Several clinical forms of β-galactosidase deficiency have been described which differ in time of onset of the symptoms, the involvement of visceral organs and the presence of mental retardation [3—5].

Galjaard et al. [6] and Reuser et al. [7] showed that the gene mutation in the classical, infantile G_{M1}-gangliosidosis (type 1) and its juvenile form (type 2) differed from the mutations in three other clinical variants of β-galactosidase deficiency (type 3 [8] and adult type 4 [9] and the patient described by Andria et al. [10]). This was shown by genetic complementation analysis in which cultured skin fibroblasts from different patients were fused yielding heterokaryons. The presence of the genetic information of both parental cells strains in these heterokaryons can give rise to restoration of β-galactosidase activity. So far, two complementation groups have been demonstrated.

An understanding of the mechanism of complementation requires knowledge about the molecular structure of β-galactosidase in control and patient's cell strains. In liver, two β-galactosidase isoenzymes have been demonstrated: a monomer, β-galactosidase A, and its multimeric aggregate β-galactosidase B [1,11].

In the present study we have used gel filtration techniques to analyse normal β-galactosidase, the residual activity in cultured skin fibroblasts of patients and the restored activity after genetic complementation. To explain this genetic complementation an intergenic model is proposed.

Materials and Methods

Cell and tissue materials. Skin fibroblasts were cultured in Ham's F10 medium, supplemented with 15% fetal calf serum and antibiotics as described earlier [12]. The cells were free of mycoplasma contamination judged by the method of Chen [13]. The β-galactosidase-deficient cell strains used were from patients with the infantile type 1 [6], the juvenile type 2 [14] or the adult type 4 [9] and from a patient recently described by Andria et al. [10]. Subcultures from early passages were harvested by trypsinization and cell homogenates were prepared by repeated freezing and thawing in the same buffer used for the gelfiltration. After centrifugation for 20 min at 1500 × *g*, the supernatant fractions were used for biochemical analysis.

Cell fusion using 10^6 cells from each β-galactosidase-deficient strain was carried out as described [6].

Liver autopsy tissue was frozen 5—15 h after death and kept at −20°C until use. It was homogenized with a Waring Blendor in ice-cold buffer and centrifuged for 20 min at 1500 × *g*. The supernatant fractions were separated by column chromatography.

Gelfiltration. Packing and elution of Sephacryl S200 superfine and Sepharose 6B (Pharmacia) columns (0.5 × 35 cm) were performed under gravity at 4°C.

Two different buffers were used: buffer I (10 mM sodium phosphate/10 mM NaCl, pH 7.0) and buffer II (0.1 M sodium acetate/0.1 M NaCl, pH 4.5), using the same buffer throughout the whole experimental procedure. Routinely 100 μl lysate, representing 10^6 cells, were applied to the columns and 100-μl fractions (Sephacryl S200) and 150-μl fractions (Sepharose 6B) were collected. The recovery of β-galactosidase activity after gelfiltration was about 50%. The

columns were calibrated by the method of Andrews [15] by using the Combithek calibration protein set from Boehringer (Mannheim). As markers were included: cytochrome *c* (M_r = 12 500), chymotrypsinogen *a* (25 000), albumin from hen egg (45 000), albumin from bovine serum (67 000), aldolase (158 000), catalase (240 000), ferritin (450 000). Dextran blue was used to estimate the void volume.

Interconversion of monomeric, dimeric and multimeric β-galactosidase. After gelfiltration on Sephacryl S200, the fractions containing the various isoenzymes were collected and concentrated. The buffer system was changed and the enzyme was incubated for 30 min at 37°C, followed by 60 min at 4°C. The enzyme activity was then measured and the remaining volume was applied to a Sephacryl S200 column, equilibrated with the other buffer system.

Enzyme assay. β-Galactosidase activity was assayed with 1 mM 4-methylumbelliferyl-β-D-galactopyranoside in buffer II by the method of Galjaard et al. [16].

Results

The most widely used buffer for gel filtration studies of acid β-galactosidase is a low ionic strength buffer at pH 7.0 (buffer I) [11,17]. However, enzyme activity is always measured at low pH, which might influence the aggregation forms of the enzyme present at pH 7.0.

The elution patterns of β-galactosidase in homogenates of normal human skin fibroblasts and normal human liver in buffer I are shown in Fig. 1A. Both patterns are similar and comprise a major peak, β-galactosidase A (M_r 65 000—75 000), and a minor peak, β-galactosidase B (M_r 600 000—800 000 on a

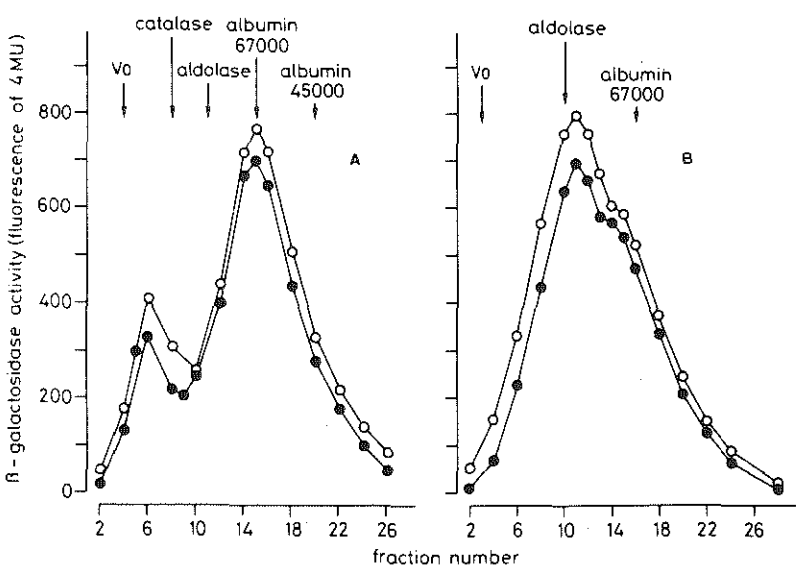

Fig. 1. Gelfiltration pattern of human liver and fibroblasts β-galactosidase, Sephacryl S200 elution patterns of homogenates from normal human liver (○———○) and cultured human skin fibroblasts (●———●). A, eluted in buffer I; B, eluted in buffer II.

Sepharose 6B column). In buffer II, β-galactosidase B has disappeared, and a peak with a molecular weight of 130 000—150 000 becomes prominent (Fig. 1B). The presence of minor quantities of β-galactosidase A is seen by the shoulder in the elution pattern.

To investigate whether the observed differences in the two buffer systems are based on reversible aggregation and dissociation phenomena, fractions isolated in one of the buffer systems were subjected to a second gel filtration in the other buffer (for details see Materials and Methods). The data showed that all of the changes in molecular weight are reversible except the multimeric isoenzyme cannot be generated from the M_r = 140 000 fractions. These findings indicate that we are dealing with the multimeric and dimeric aggregation forms of the monomeric β-galactosidase polypeptide. As transitions between monomer and dimer are reversible, most of the β-galactosidase is in the dimeric from during the enzyme assay at pH 4.5 in buffer II.

The isoenzymes in fibroblasts from patients with the infantile (type 1), juvenile (type 2) or adult (type 4) form of β-galactosidase deficiency had molecular weights indistinguishable from those of the normal isoenzymes. The aggregation patterns in buffer I differed from the normal enzyme (Table I). The aggregation forms in buffer II are similar, except for the infantile type 1.

The restored β-galactosidase activity after complementation between fibroblasts of the infantile or juvenile types and fibroblasts from either a patient with the adult type or the patient described by Andria et al. [10] was analysed by gel filtration. Fig. 2 shows the data for a fusion of infantile type 1 and adult type 4 cells. The heterokaryon homogenate shows after gel filtration in buffer I increased activity in both the fractions corresponding to the monomeric and multimeric β-galactosidase (Fig. 2). In buffer II, the dimeric and monomeric β-galactosidase were increased. Similar results were obtained in fusions between fibroblasts of the patient described by Andria et al. [10] and those of infantile type 1 or juvenile type 2 patients.

To investigate the possibility whether the increase in β-galactosidase activity after cell fusion is due to the formation of dimeric enzyme, studies with mixtures of cell free extracts were performed. The data presented in Table II show

TABLE I

AGGREGATION FORMS OF RESIDUAL β-GALACTOSIDASE ACTIVITY IN FIBROBLASTS FROM PATIENTS WITH G_{M1}-GANGLIOSIDOSIS

Monomer: β-galactosidase with molecular weight (M_r) 65 000—75 000; dimer: β-galactosidase with M_r = 130 000—150 000; multimer; β-galactosidase with M_r = 600 000—800 000.

	10 mM phosphate/10 mM NaCl buffer (pH 7.0)			0.1 M sodium acetate/0.1 M NaCl buffer (pH 4.5)		
	Monomer	Dimer	Multimer	Monomer	Dimer	Multimer
Control	++	—	+	+	++	—
Infantile Type 1	—	—	+	+	++	+
Juvenile Type 2	+	—	—	±	++	—
Type 'Andria'	+	—	—	+	++	—
Adult Type 4	+	—	—	+	++	—

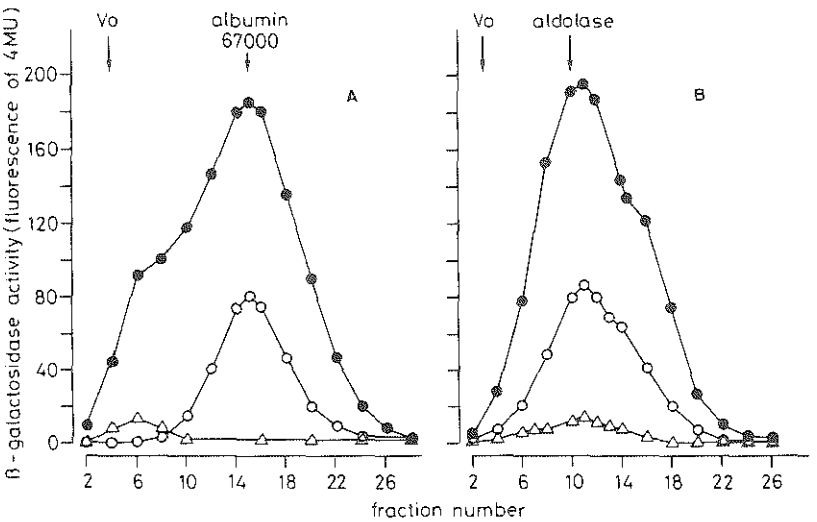

Fig. 2. Gelfiltration pattern of β-galactosidase after genetic complementation. Sephacryl S200 elution patterns of homogenates after fusion of: infantile (type 1) and adult (type 4) fibroblasts (●——●); type 1 and type 1 fibroblasts (△——△); and type 4 and type 4 fibroblasts (○——○). A, eluted in buffer I, pH 7.0; B, eluted in buffer II, pH 4.5.

that mixing of cell-free extracts of type 1 and type 4 fibroblasts, under conditions where monomeric β-galactosidase is prevailing (buffer I) and subsequently allowing dimers to be formed (buffer II) does not lead to increased β-galactosidase activity. Gel filtration of the mixture in buffer II showed that the β-galactosidase indeed exists predominantly in the dimeric form. Similar results were obtained when homogenates of type 2 were mixed with homogenates of type 4 fibroblasts or fibroblasts of the patient described by Andria et al. [10].

TABLE II

ATTEMPTED COMPLEMENTATION IN MIXED CELL HOMOGENATES OF TYPE 1 AND TYPE 4 CELLS

Equal amounts of homogenates of cells from the infantile and adult type were mixed in buffer I. The pH was adjusted to 4.5 with buffer II. After 30 min incubation at 37°C, followed by 60 min at 4°C the mixture was assayed for β-galactosidase activity. The protein content was determined according to Lowry et al. [27]. The aggregation state of the β-galactosidase was determined with a Sephacryl S200 column. 1 X 4: β-galactosidase activity after fusion of infantile (type 1) and adult (type 4) fibroblasts. The heterokaryons and cells used for the mixing experiments have been cultivated for the same period of time at the same cell density. This is essential for a meaningful comparison of the specific activities [28].

Cell strains	β-Galactosidase activity (nmol 4-methylumbelliferone/mg protein per h)
Infantile Type 1	3
Adult Type 4	44
Type 1 X Type 4	190
Type 1 + Type 4	23
Control	493

Discussion

Subunit structure of β-galactosidase

Human acid β-galactosidase in liver consists of a monomeric polypeptide, β-galactosidase A (M_r = 70 000) and a multimeric aggregate of the monomeric form, β-galactosidase B (M_r = 600 000—800 000) [1], under conditions of low ionic strength and neutral pH. Whether this multimeric form also contains additional subunits remaines uncertain.

The enzyme from human fribroblasts shows identical gel filtration patterns compared to that of human liver, indicating that the isoenzymes have the same subunit composition. At high ionic strength and acid pH (buffer II) dimeric β-galactosidase was the predominant form, whereas the multimeric form was absent.

Repeated gel filtration of isolated fractions in both buffers used indicated that dimeric β-galactosidase is the enzymatically active form, irrespective of whether monomers or multimers are isolated initially. In this light, characterization of separated β-galactosidase A and B [3,18] is meaningless.

A dimeric form of β-galactosidase has already been demonstrated in human liver in varying amounts depending on the experimental conditions [17,19] and in human small intestine [20].

The β-galactosidase isoenzymes from all four patients tested have molecular weights which are indistinguishable from those found in normal fibroblasts, suggesting that complete or nearly complete polypeptide chains are synthesized. However, β-galactosidase present in these patients shows aggregation patterns different from normal, indicating that we are dealing with structurally altered β-galactosidase. This is in agreement with immunological studies showing an increased amount of antigen per unit enzyme activity in adult type [21] and in infantile type β-galactosidase deficiency [22]. No difference in heat stability, K_m and pH optimum of β-galactosidase in normal, type 4 and type 1 × type 4 heterokaryons could be demonstrated [4].

Mechanism of complementation

For dimeric enzymes, Crick and Orgel [23] have postulated a model for intragenic complementation, based on interaction of defective subunits. The reversibility in the formation and dissociation of dimeric β-galactosidase in cell-free extracts made an experimental test of this model possible. As no complementation could be demonstrated, the Crick and Orgel model for intragenic complementation is not applicable.

An intragenic mechanism similar to α-complementation described for β-galactosidase deficient strains of *Escherichia coli* [24] is unlikely because of the presence of nearly normal amounts of immunological cross-reacting material in type 1 as well as in type 4 cells [21,22,25] and the identical heat sensitivity of restored β-galactosidase activity in heterokaryons and normal β-galactosidase [4,26].

The intergenic complementation model, proposed by O'Brien and Norden [21] in which they postulated that β-galactosidase B, the multimeric isoenzyme, contains a subunit not shared with β-galactosidase A can be ruled out. This model would predict that the increased β-galactosidase activity represents

exclusively multimeric enzyme; this is not observed.

We propose the following intergenic complementation model which is compatible with the experimental results. In one of the complementation groups, (e.g. in type 4 cells), a normal structural β-galactosidase gene is present, however, the lack of a second gene product results in defective β-galactosidase with abnormal aggregation properties. The β-galactosidase deficiency in the other complementation group, type 1 and type 2 G_{M1}-gangliosidosis, is thought to result from allelic mutations in the structural β-galactosidase gene. After fusion of cells from different complementation groups the modification factor or process can act on the defective β-galactosidase, resulting in a structurally normal β-galactosidase.

Enucleation studies, recently carried out in our laboratory, showed that the presence of the type 4 nucleus is required for complementation, whereas the cytoplasm of type 1 cells is sufficient [26]. These results are compatible with the proposed model for complementation.

Biochemical and gene localization studies are currently underway in our laboratory to establish this model.

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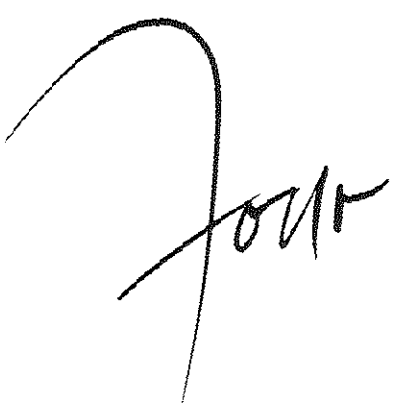

Regional Localization of a β-Galactosidase Locus on Human Chromosome 22

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Abstract—*Human white blood cells with an X/22 translocation [46, XX, t(X; 22)(q23; q13)] were fused with Chinese hamster cells. The isolated hybrids were analyzed for human chromosomes and 21 enzyme markers. An electrophoretic technique for studying the β-galactosidase isoenzymes in man–Chinese hamster hybrid cells was developed. Immunological studies showed that the β-galactosidase marker studied in these hybrids did contain immunological determinants of human origin. Furthermore the results provided evidence that a locus for β-galactosidase is situated on chromosome 22 distal to the breakpoint in q13.*

INTRODUCTION

In human tissues two different acid β-galactosidase (βGAL) isoenzymes can be detected with GM1-ganglioside as well as with 4-methylumbelliferyl-β-D-galactopyranoside as a substrate. These isoenzymes are designated as the A and B form. The βGAL-A isoenzyme, the main form in human liver, is thought to be a monomer, whereas βGAL-B is a polymer with a molecular weight about 10 times higher than that of βGAL-A (1–3). Antisera prepared against βGAL-A react also with βGAL-B indicating that the two isoenzymes have at least one polypeptide in common (3).

Deficiency of βGAL is found in patients with GM1-gangliosidosis. Several clinical variants have been described (4–9), based on time of onset of symptoms, degree of visceral organ and skeletal involvement, occurrence of mental retardation and other clinical features. To study whether different gene mutations are involved, Galjaard et al. (10) assayed the

βGAL activity in heterokaryons after fusion of cultured fibroblasts of different clinical variants of GM1-gangliosidosis. Restoration of βGAL activity in some of the combinations revealed the presence of two complementation groups. Because of the biochemical and immunological relationships between the βGAL isoenzymes, these complementation results might be explained in terms of intragenic as well as intergenic complementation.

Information about the number and localization of genes involved in the expression of βGAL in human cells can be obtained in studies of interspecific somatic cell hybrids. Furthermore the formation of heteropolymeric βGAL molecules in these hybrids could give information about the structure of the βGAL isoenzymes. The man–Chinese hamster hybrids preferentially lose human chromosomes which might result in segregation of genes involved in the expression of the enzyme at least when these genes are located on different chromosomes.

In this paper the βGAL isoenzymes in man–Chinese hamster hybrids were characterized by immunological and electrophoretical methods, and it was shown that a locus for βGAL is located on chromosome 22q13-qter.

MATERIALS AND METHODS

Man–Chinese hamster hybrid cells were isolated following fusion of hypoxanthine phosphoribosyltransferase (HPRT) deficient Chinese hamster cells with human leukocytes. The human leukocytes were obtained from a female carrier of a balanced X/22 translation (X/22 Breda; 46, XX, t(X; 22)(q23; q13)). The intact X-chromosome was the late replicating one (11). The Chinese-hamster HPRT-deficient cell line, E36, is a derivative from the V79 cell line (12). The Chinese hamster cell lines a3 and wg3-h, derived from the DON line, were also used as controls. The Chinese hamster cell lines and the hybrids were isolated and propagated as described elsewhere (13).

In the hybrid and parental cell populations the following enzymes were characterized by means of Cellogel electrophoresis (Cellogel, Chemetron, Milan, Italy): phosphoglucomutases (PGM_1, PGM_3, EC 2.7.5.1; PGM_2, EC 2.7.5.6), malate dehydrogenase (soluble) (MDH_S, EC 1.1.1.37), galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.10), superoxide dismutases (SOD_S, SOD_M, EC 1.15.1.1), β-glucuronidase (βGUS, EC 3.2.1.31), glutathion reductase (GSR, EC 1.6.4.2), adenylate kinase-1 (AK_1, EC 2.7.4.3), glutamate oxaloacetate transaminase (soluble) (GOT_S, EC 2.6.1.1), lactate dehydrogenases (LDH_A, LDH_B, EC 1.1.1.27), nucleoside phosphorylase (NP, EC 2.4.2.1), mannose phosphate isomerase (MPI, EC 5.3.1.8), peptidase-A (PEPA, EC 3.4), glucose phosphate isomerase (GPI, EC 5.3.1.9), adenosine deaminase

(ADA, EC 3.5.4.4), glucose-6-phosphate dehydrogenase (Gd, EC 1.1.1.49), phosphoglycerate kinase (PGK, EC 2.7.2.3).

For the characterization of most of the enzymes we followed the methods already published (14–16). The technique reported by Grzeschik (17) was used for βGUS and for PGM_2, the electrophoretic procedure described by Quick et al. (18) was adapted to the Cellogel system by Meera Khan (personal communication). The lysates were prepared as described by Meera Khan (14) by either sonicating or repeated freezing and thawing of the cells in lysis buffer. For the analysis of the hybrids studied here, the freezing and thawing method was preferred.

In order to study βGAL (E.C. 3.2.1.23) in man–Chinese hamster hybrids an electrophoretic technique was developed using the Cellogel system. Electrophoresis buffer: 0.05 M Na_2-β-glycerophosphate, pH 6.2; running time is 3 hr at 4°C, keeping the amperage constant at an initial voltage of 200 V. The gel was incubated with 0.5 mM 4-methylumbelliferyl-β-D-galactopyranoside in 50 mM acetate buffer, pH 4.8, containing 10 mM NaCl. Following incubation for 30 min at 37°C, the reaction was stopped with 1 M carbonate-bicarbonate buffer pH 10. Fluorescent bands indicating the zones of βGAL activities were visualized under long-wave UV light (350 nM).

For the immunological characterization of the βGAL isoenzymes an antiserum against human βGAL was prepared. Human β-galactosidase was purified 165 times from liver. The liver was homogenized in 10 mM potassium phosphate buffer pH 6.9. The 25,000*g* supernatant was applied on a Con A Sepharose column, equilibrated with 10 mM potassium phosphate buffer, pH 5.6, containing 0.1 mM $CaCl_2$, 0.1 mM $MnSO_4$, 0.1 mM $MgSO_4$, and 0.5 M NaCl. A 1:1 dilution of this buffer containing 1 M α-methylmannoside was used for the elution of β-galactosidase. Pooled fractions were dialyzed against 25 mM acetate buffer, pH 5.6, containing 25 mM NaCl, and applied on a CH-Sepharose column with galactosylamine as an affinity ligand, equilibrated with the same buffer. β-Galactosidase was eluted with the same buffer solution containing 200 mM NaCl and 20 mM galactose. Polyacrylamide gel electrophoresis of the immunogen revealed that next to the major band containing βGAL other lysosomal enzymes were found to be present also. The major contaminating component was β-D-*N*-acetyl hexosaminidase. Antiserum against this human β-galactosidase preparation was raised in New Zealand white rabbits using a slight modification of the method described by Carrol (19). For the immunoprecipitation tests, lysates were incubated with antiserum overnight at 0°C. After the incubation the mixtures were centrifuged at 35,000*g* for 20 min, the supernatant was used for electrophoresis. Double immunodiffusion was carried out in 1% agarose gels in 10 mM potassium phosphate buffer, pH 6.6, allowing diffusion of the antiserum and the

lysates to take place at room temperature for 16 hr in a humidified chamber. The gels were washed for at least 12 hr in 10 mM potassium phosphate buffer, pH 6.6, at 4°C. Precipitation arcs were stained with the 5-bromo-4-chloro-3-indolyl-β-D-galactoside substrate, according to Lake (20).

Chromosome preparations were made on the same batches of cells as the lysates for the enzyme studies. Identification of the chromosomes was achieved using Q-banding (21), trypsin–Giemsa staining (22), and R-banding (23). At least 20 metaphases of each clone were analyzed.

RESULTS

The electrophoretic patterns of βGAL obtained from human liver, cultured human and Chinese hamster fibroblasts are presented in Fig. 1. The human fibroblasts and liver showed a two-banded pattern, channels 1 and 2, respectively. The isozymes of both tissues did have the same electrophoretic mobility. The fast-moving band is designed as βGAL-A, and the more cathodal band is called βGAL-B according to earlier published electrophoretic patterns (24–26). At our electrophoretic conditions the βGAL activity in leukocytes (about 25% of the activity present in fibroblasts) is too low to obtain a clear banded pattern. The Chinese hamster cell line E36 (channel 3) showed a different electrophoretic pattern as compared to wg3-h and a3 cells (channels 4 and 5). A good separation from the human isoenzymes could only be established with the E36 cells and the human fibroblast or human liver lysates. Therefore E36 cells were used as the rodent fusion partner.

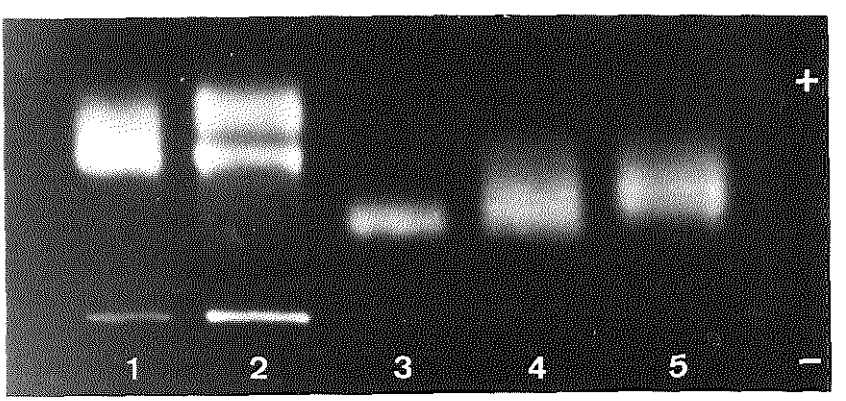

Fig. 1. Electrophoresis of βGAL on Cellogel showing patterns obtained from: human primary fibroblasts (1), human liver (2), Chinese hamster E36 (3), Chinese hamster wg3-h (4), Chinese hamster a3 (5). The lysates of channels 3, 4, and 5 are prepared by sonication. The lysates of channels 1 and 2 are prepared by freezing and thawing.

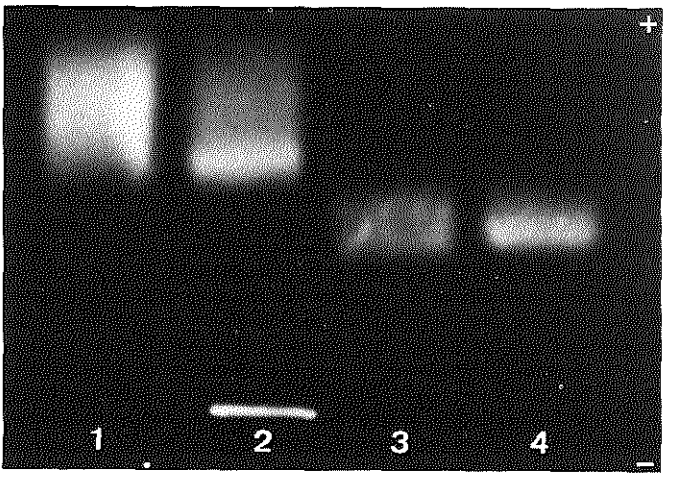

Fig. 2. The effect of different lysis procedures on the electrophoretic mobility of βGAL. Human primary fibroblasts (1 and 2), Chinese hamster E36 (3 and 4). The lysates of channels 2 and 3 are prepared by freezing and thawing, those presented in 1 and 4 by sonication.

It was found that different techniques for preparation of lysates, i.e., sonication or freezing and thawing, yielded different electrophoretic patterns of the βGAL isoenzymes (Fig. 2). Following sonication human fibroblasts showed a diffuse band at the A and B position (channel 1). Most of the activity was found at the A position. When the lysate was prepared by freezing and thawing (channel 2), the main activity was found at the B position. The lysate of Chinese hamster E36 cells showed one band after sonication (channel 4), whereas the presence of two bands is indicated when the lysates were prepared by freezing and thawing (channel 3). In another electrophoretic buffer system (0.05 M Tris-citrate, pH 5.0) the separation between these two Chinese hamster isoenzymes was improved. Unfortunately this buffer system could not be used because it did not allow the electrophoretic separation of the human and Chinese hamster isoenzymes.

With regard to the βGAL isoenzymes, two types of man–Chinese hamster hybrid cell lines were found (Fig. 3): hybrid cell lines which expressed only E36 βGAL activity (channel 2) and hybrid lines showing an additional band of activity with a mobility intermediate to the Chinese hamster and the human B-band (channel 3). This band will be referred to as the test marker. The isoenzyme pattern of 4 hybrid cell lines was characterized with antibodies specific for human β-galactosidase. The resulting electrophoretic pattern of one of these lines is presented in Fig. 4. The antiserum did react with human β-galactosidase, removing all enzyme activity (channel 1). In channel 4 a hybrid with the additional

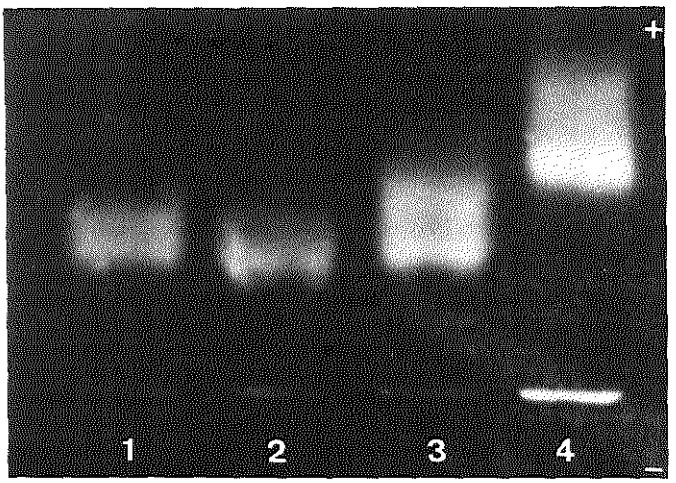

Fig. 3. Electrophoretic pattern of βGAL isoenzymes from man–Chinese hamster cell hybrids: Chinese hamster E36 (1); man–Chinese hamster hybrid without test marker (2); man–Chinese hamster hybrid with test marker (3); human primary fibroblast (4). All the lysates were prepared by freezing and thawing.

band of activity is shown. Following treatment with anti-human βGAL serum, the marker band disappeared whereas the E36 band of activity was not affected (channel 3). No reaction of anti-human βGAL serum with E36 βGAL was observed (channel 5). These results were confirmed with double immunodiffusion in agarose gels using various progressive dilutions of the antiserum (Fig. 5). In all the antiserum concentrations tested

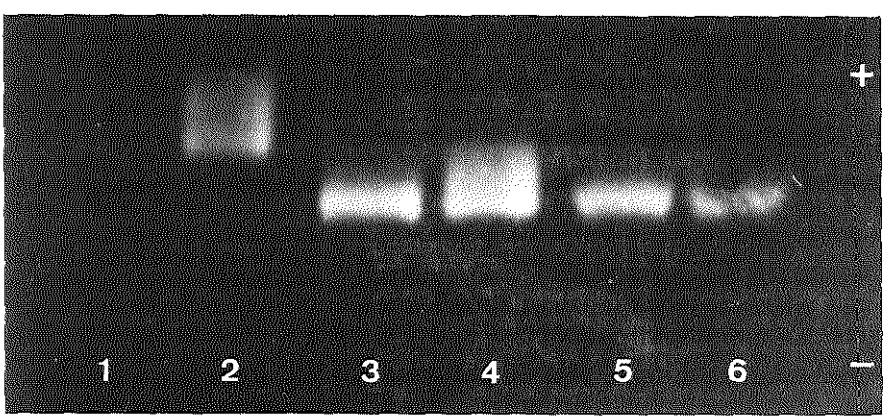

Fig. 4. The effect of anti-human βGAL serum on the electrophoretic pattern of βGAL from primary fibroblasts (1 and 2), man–Chinese hamster hybrid with test marker (3 and 4), and Chinese hamster E36 (5 and 6). The lysates of channel 1, 3, and 5 were treated with antihuman βGAL serum. All the lysates were prepared by freezing and thawing.

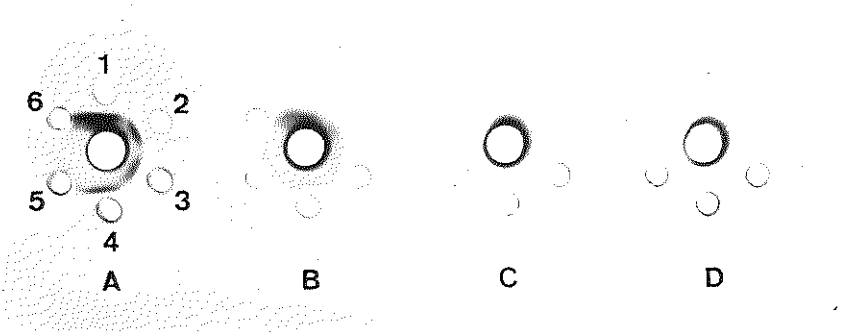

Fig. 5. Double immunodiffusion of anti-human βGAL and lysates of various cell lines on agarose gel. The following dilutions of the antiserum were used 1:0 (1); 1:2 (2); 1:4 (3); 1:8 (4); 1:16 (5); 1:32 (6). The cell lines tested were human fibroblasts (A); hybrid with test marker (B); hybrid without test marker (C); E36 (D).

a precipitation line was observed in the gel containing the human fibroblasts and the man–Chinese hamster hybrids with the test marker band. The E36 and hybrid with the marker band did not show a precipitation line in the gels. Furthermore, antigen–antibody titrations in various combinations of antigen and antiserum showed no reaction of anti-human βGAL serum with Chinese hamster βGAL. At normal experimental conditions, no enzyme activity was found at the human βGAL-A and B position in any of 25 independent primary hybrid clones obtained from two fusion experiments. When overloading the gel with lysate of a hybrid showing the additional βGAL band, sometimes a slight βGAL activity was observed at the βGAL-B position.

The translocation chromosomes, a diagram of the translocation, and the position of the X-chromosomal markers on these chromosomes are represented in Fig. 6. The *PGK* marker is located on the X/22 and the *βGAL-A*, *HPRT*, and *Gd* marker on the 22/X translocation product (see ref. 27). In this reference the X/22 Breda line is reported as X/22 Leiden. The presence of the 22/X derivative could not be established in hybrid cells with certainty because this chromosome does not show a very characteristic banding pattern and moreover it could not be distinguished from Chinese hamster chromosomes of the same size. The data from Table 1 show that the *βGAL* marker is present together with chromosome 22 and/or the X/22 chromosome. Two clones expressed the test marker and *PGK* together with chromosome 22 and the X/22 translocation product. Six clones did contain the X/22 chromosome, *PGK*, and the *βGAL* marker, but no intact chromosome 22. In two clones the *βGAL* marker was found together with chromosome 22, while *PGK* and the X/22

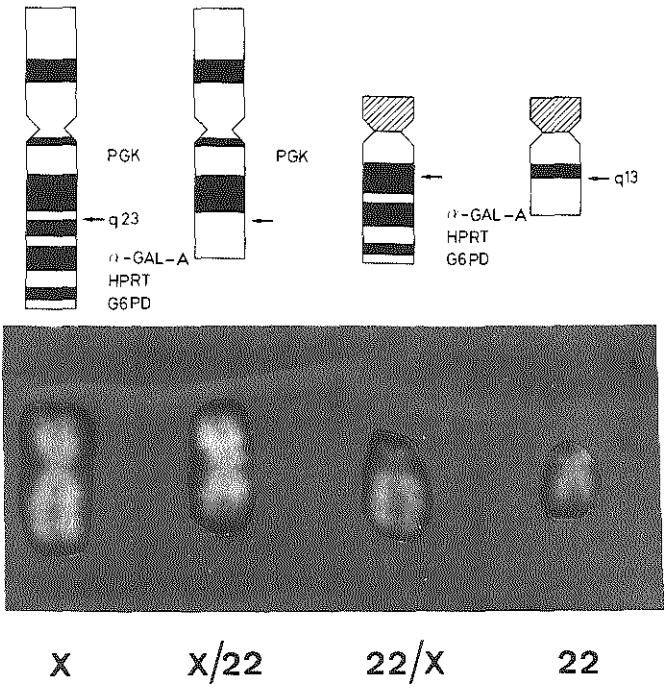

Fig. 6. The X chromosome, chromosome 22, and their translocation products (Q-banding) together with a diagram of this X/22 Breda translocation showing the breakpoints in chromosome X and 22 and the position of the X-chromosomal markers on the translocation products.

translocation chromosome could not be detected. Thirteen clones were negative for the two enzyme markers, the X/22 piece, and chromosome 22. In one clone *PGK* and the *βGAL* marker were present in apparent absence of chromosome 22 and the X/22 translocation product. The chromosome data of the 13 clones being negative for the test marker revealed that all human autosomes, except chromosome 22 and the X/22 translocation product, could be recognized (Table 2). To extend the assignment

Table 1. Distribution of PGK and βGAL and the presence or absence of human chromosome 22 and the X/22 translocation chromosome

Number of clones	PGK	βGAL	X/22	22
2	+	+	+	+
6	+	+	+	−
2	−	+	−	+
13	−	−	−	−
1	+	+	−	−

Table 2. Human chromosome constitution of the hybrid clones negative for human PGK and βGAL

Cell line[a]	Human chromosomes[b]																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	X/22
E16.4a																	+							
E16.13a			+		+	+[c]					+	+	+	+										
E16.14a	+	+	+	+	+		+	+	+	+		+[c]	+	+			+	+	+	+	+			
E16.21a													+							+				
33.9			+								+										+		+	
33.14			+		+						+	+	+	+					+		+		+	
33.15													+					+					+	
33.1.10	+				+				+		+	+	+	+				+	+	+				
33.11.20	+		+		+				+	+	+	+	+	+	+			+			+		+	
33 m.p.	+		+		+					+		+		+		+					+			

[a]Analysis of ten hybrid clones are given: in the other three lines no intact human chromosomes could be recognized. The primary clones are derived from two fusion experiments (the "E16" and "33" series).
[b]The retained chromosomes were found in a frequency of 10% or more.
[c]Frequency of chromosome retention is about 5%.

Table 3. Synteny relationships between βGAL and 19 human enzyme markers

Marker	Chromosome to which marker is assigned[a]	βGal/marker, number of clones[b]			
		++	+−	−+	−−
PGM_1	1	5	6	3	5
MDH_S	2	3	8	4	10
GALT	3	5	2	8	1
PGM_2	4	2	3	5	2
PGM_3	6	0	10	0	9
SOD_M	6	1	5	0	9
βGUS	7	4	2	2	2
GSR	8	6	5	3	10
AK_1	9	4	7	3	10
GOT_S	10	7	4	3	10
LDH_A	11	8	3	5	5
LDH_B	12	5	6	6	4
NP	14	7	4	6	7
MPI	15	3	3	1	3
PEPA	18	6	5	7	5
GPI	19	6	5	4	9
ADA	20	9	2	7	6
SOD_S	21	2	3	3	6
Gd	22/X[c]	11	0	11	2

[a] Confirmed or tentative assignment, see Baltimore Conference on Human Gene Mapping (28).
[b] ++ means both markers are present; +− means βGAL present, but the other absent; −+ means βGAL absent, whereas the other marker is present; −− both markers are absent.
[c] In these particular experiments (27).

data we studied the segregation pattern of the *βGAL* marker together with 19 other enzyme markers, firmly or tentatively assigned to particular chromosomes (see Baltimore Conference on Human Gene Mapping, 1975, 28) (Table 3). No concordant segregation between the test marker and the other enzyme markers was found.

DISCUSSION

Evidence has been presented for a molecular relationship between βGAL-A and βGAL-B, A is thought to be a monomer and B a decamer of A subunits (3, 29). Our data obtained with lysates either prepared by sonication (mainly βGAL activity at the A position) or freeze–thawing, may indicate that βGAL-B can be converted into βGAL-A; they support the hypothesis of a relationship between the two types of βGAL.

In our experimental conditions the electrophoretic bands of human βGAL-A and -B are absent in man–Chinese hamster hybrid cells. The results obtained with a specific anti-human β-galactosidase serum

revealed that a human βGAL component in these hybrids is represented by an intermediate band. It is attractive to postulate that the intermediate band in hybrid cells is a heteropolymer between human and Chinese hamster subunits. In these hybrids the Chinese hamster karyotype is pseudotetraploid, and this could result in an excess of Chinese hamster βGAL subunits. If this is the case, formation of heteropolymers would use up all the human subunits available and explain the absence of human βGAL-A and -B in the hybrid cells. A definite proof of this hypothesis can be obtained by further identification of the marker band using specific antisera against Chinese hamster βGAL. The *βGAL* marker was found to be present or absent together with human chromosome 22 and/or the X/22 translocation chromosome (Table 1). The exceptional clone expressing the enzyme but not the chromosome 22 and 22/X was interpreted as the result of secondary chromosome breakage and rearrangement occurring in these hybrid cells. Ideally to assign a gene to a chromosome all the other human chromosomes should be definitely excluded as candidates for carrying that specific gene. Table 2 demonstrated that all human autosomes except chromosome 22 and the translocation product X/22 are present in at least 10% of the metaphases studied in the hybrids having lost the *βGAL* marker (The normal X chromosome, being inactive, is not considered here). These exclusions were confirmed by the segregation patterns of isoenzyme markers presented in Table 3 since these marker enzymes have been assigned to the following chromosomes: 22/X, 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 14, 15, 18, 19, 20, and 21 (28).

In these experiments a human line carrying a balanced translocation [t(X;22) (q23;q13)] was used. Regional mapping of the *βGAL* locus was therefore possible. In the six clones where the X/22 translocation is present and the chromosome 22 absent (Table 1), *βGAL* is present together with *PGK*. In this particular translocation *PGK* was assigned to the X/22 derivative and the other X markers (*Gd*, *HPRT*, and *αGAL-A*) to the reciprocal 22/X derivate (27). The 22/X translocation chromosome could not be recognized cytologically. But in Table 3 we see that 11 clones are positive for *Gd* (and therefore presumably contain the 22/X derivative) and negative for the *βGAL* marker band.

Combination of all these data demonstrate that a locus for βGAL is located on chromosome 22 and more precisely in the region 22q13-qter.

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material from the patient with the X/22 translocation. The purification of β-galactosidase was carried out in collaboration with Mr. I. Brown, Drs. M. W. Ho and D. Robinson (Dept. of Biochemistry, Queen Elizabeth College, London). This work was supported in part by The Netherlands Foundation for Medical Research FUNGO, and EURATOM, Contract no. 196-76 BION.

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PAPER V

ASSIGNMENT OF STRUCTURAL β-GALACTOSIDASE LOCI TO HUMAN CHROMOSOMES 3 AND 22

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SUMMARY

Hybrid cell lines isolated after fusion between Chinese hamster E36 cells and normal human white blood cells were analyzed for human β-galactosidase isoenzymes and for human chromosomes, especially 3, 12, and 22, the candidates for bearing a β-galactosidase locus. Results of neuraminidase treatment of the cell lysates and immunological studies showed that in man two structural β-galactosidase loci are present and can be assigned to chromosomes 3 and 22. No correlation was found between the expression of human β-galactosidase and the presence of human chromosome 12.

INTRODUCTION

Independent studies on the localization of genes involved in the expression of the lysosomal enzyme β-galactosidase (βGAL; E.C. 3.2.23) have revealed conflicting data. Our own group located a structural βGAL locus on chromosome 22 and excluded the other chromosomes (de Wit et al., 1977). Bruns et al. (1977) and Shows et al. (1977) assigned a βGAL locus on chromosome 3 and excluded the other chromosomes as well. Recently Rushton and Dawson (1977) assigned a βGAL locus on chromosome 12. Different techniques for the detection of βGAL activities have been used in these studies, and it cannot be excluded that these techniques may have different specificity toward different gene products involved in the expression of βGAL. The discrepancies in the literature could therefore indicate the existence of more than one structural βGAL locus. Moreover the use of human cells from only one individual, carrying an X/22 translocation, in the production of the hybrids in our previous study (de Wit et al.,1977) could have affected our data.

In the present study we reinvestigated our previous man-Chinese hamster hybrids with the X/22 translocation by using different electrophoretic techniques, and a new man-Chinese hamster hybrid cell panel was isolated in which βGAL was characterized with electrophoretic and immunological methods.

The data revealed that chromosome 3 and 22 both bear a structural βGAL locus. Assignment of βGAL to chromosome 12 was excluded in the present study.

MATERIALS AND METHODS

Thirty-six primary man-Chinese hamster hybrid cells were isolated following fusion of hypoxanthine phosphoribosyl-transferase (HPRT) deficient Chinese hamster cells (E36) with normal human male leucocytes.

The E36 cell line is a derivative from the V79 cell line (Gillin et al.,1972). The mutant cell line and the isolated hybrids were grown respectively in F10 and F10 HAT (Ham,1973, Littlefield,1964) medium supplemented with 5% fetal calf serum and antibiotics.

The fusion procedure is essentially the same as described previously (Westerveld et al.,1971). The hybrid panel used in our previous study (de Wit et al.,1977) in which a human X/22 translocation (X/22 Breda; 46 XX,t(x;22)(q23;q13)) is involved was reinvestigated.

The following enzymes were characterized by means of Cellogel electrophoresis: β-galactosidase (βGAL; E.C.3.2.23), lactate dehydrogenase (LDH; E.C.1.1.1.27), aconitase (mitochondrial) ($ACON_m$; E.C. 4.2.1.3) and glutathione peroxidase (GPX; E.C. 1.11.1.9). The methods for the characterization of LDH, $ACON_m$ and GPX are described by Meera Kahn (1971, 1977) and Wijnen et al. (1977).

The genes coding for the following enzymes have been firmly or tentatively assigned to particular chromosomes: $\underline{LDH_B}$ to 12 and $\underline{GPX}$ to 3 (Bootsma and Ruddle,1977), $\underline{ACON_m}$ to 22 (Ferguson-Smith and Westerveld,1977).

Two different electrophoretic techniques were applied in order to characterize βGAL. One technique was performed by Cellogel electrophoresis in a 0.05 M Na_2-β-glycero-phosphate buffer pH 6.2 at an initial voltage of 200 volts keeping the amperage constant for 3 hours at 4°C as described by us before (de Wit et al.,1977). The other method has been published by Bruns et al. (1977), and involves Cellogel electrophoresis in a 0.1 M Tris, citric acid buffer, pH 6.5 at 200 volts for 4 hours at 4°C.

The cell sonicates were prepared in 5 x 10^{-3} M phosphate buffer pH 6.4, containing 1 x 10^{-3} M Na_2EDTA, 1 x 10^{-3} M β-mercaptoethanol and 2 x 10^{-3} M NADP as described by Meera Khan (1971) or in 5 x 10^{-3} M potassium phosphate buffer pH 6.5 containing 1 x 10^{-3} M Na_2EDTA as described by Bruns et al. (1977) Treatment of cell sonicates by neuraminidase (Sigma type VI) was performed according to Bruns (1977). Anti-serum against purified acid human βGAL isolated from liver was raised in New Zealand White Rabbits (de Wit et al., 1977).

Lysates were incubated with antiserum overnight at 0°C, the mixtures were then centrifuged at 35,000 g for 20 min. and the supernatant was used for electrophoresis.

Chromosome preparations were made from the same batches of cells as where the lysates for enzyme studies. Identification of the chromosomes was achieved for enzyme studies. Identification of the chromosomes was achieved using Q-banding (Jongsma et al., 1973), trypsin-Giemsa staining (Seabright, 1971), and R-banding (Verma and Lubs, 1975). At least 20 metaphases of each clone were analyzed.

RESULTS

The isolated hybrid cell lines were analyzed for the presence or absence of the human chromosomes 3, 12, and 22 and for the presence of human GPX, LDH_B and $ACON_m$.

The βGAL isoenzyme patterns were investigated of hybrids with chromosome 3 in the absence of 22 (3+22-), hybrids containing chromosome 22 but not chromosome 3 (3-22+), hybrids with both chromosomes 3 and 22 (3+22+), hybrids with chromosome 12 but without the chromosomes 3 and 22 (3-12+22-). Comparable results were obtained with the electrophoretic method previously described by our own group (de Wit et al.,1977) and with that used by Bruns (1977) without the neuraminidase treatment. The best separation of the different isoenzymes was obtained by applying to the gels the lysates prepared according to the method of Bruns et al. (1977) combined with the electrophoretic system developed by us (de Wit et al.,1977). Fig. 1 shows the βGAL

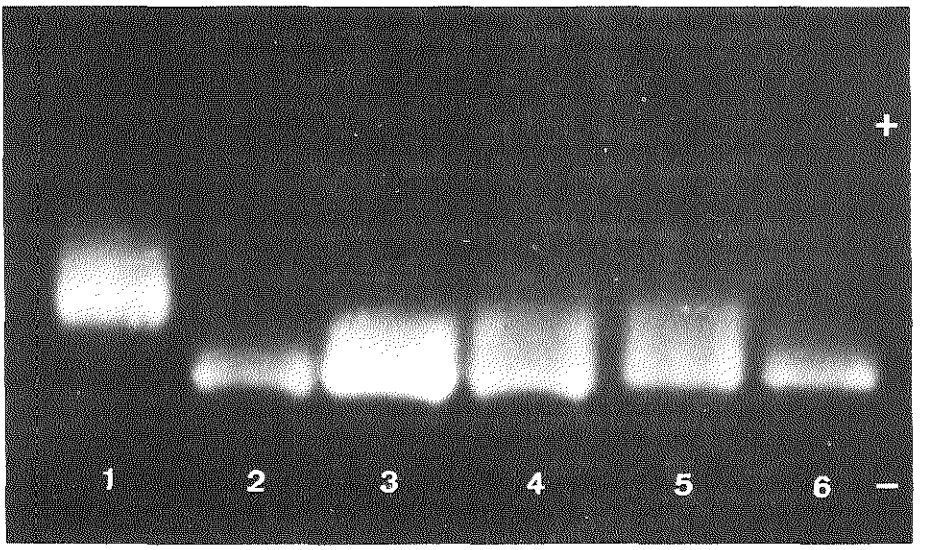

Figure 1. Electrophoretic pattern of βGAL isoenzymes from man-Chinese hamster cell hybrids: human primary fibroblast (1); Chinese hamster E36 (2); man-Chinese hamster hybrid with chromosome 22 present in 65% of the metaphase cells and without 3 (3); hybrid with chromosome 3 present in 87% of the metaphase cells and without 22 (4); hybrid with chromosomes 22 and 3 in 80% and 70% of the metaphase cells, respectively (5); hybrid without chromosomes 22 and 3 and with chromosome 12 present in 60% of the metaphase cells (6).

electrophoretic patterns of the above-mentioned types of hybrids obtained with this method.

Lysates of human fibroblasts yielded two bands, designated βGAL_B and βGAL_A (lane 1, see also Fig.2 lane 1). The Chinese hamster E36 cells show one band of activity (lane 2). A hybrid cell line containing chromosome 22 in the absence of chromosome 3 is presented in lane 3.

In our previous paper (de Wit et al.,1977) the

activity anodal to, but not separated from, the hamster band is referred to as the marker band. Hybrid cell lines, containing chromosome 3 in the absence of chromosome 22 also show this marker band (lane 4).

A very weak band of activity in these hybrids was observed when chromosome 3 was present in about 20% of the cells, indicating that this frequency is the lower limit at which βGAL can be detected. In 3+22+ hybrid cell lines next to the additional activity seen in 3+22- and 3-22+ hybrids, a more anodal band is present at the human βGAL_B position (Fig.1, lane 5, see also Fig.2 and 3, lane 9). In 3-22- hybrids in which the markers GPX and $ACON_m$ were absent but chromosome 12 and LDH_B are present, activity could be detected only at a position comparable to that of the Chinese hamster band (lane 6). The presence or absence of chromosome 12 in hybrids having either chromosome 3 or 22 or both did not influence the electrophoretic patterns, as shown in Fig.1.

To investigate the presence of determinants of human origin in the marker band, the hybrid cell lines were analyzed with an antiserum directed against human βGAL. The results are presented in Fig.2. The human βGAL activity disappeared (lane 2) whereas the anti-human βGAL serum only slightly reduced the Chinese hamster βGAL activity (lane 4). Quantitative measurements showed that the human βGAL activity is reduced by 98%, whereas the Chinese hamster activity is reduced by 9%. The marker band of lysates from 3-22+, 3+22-, and 3+22+ hybrids

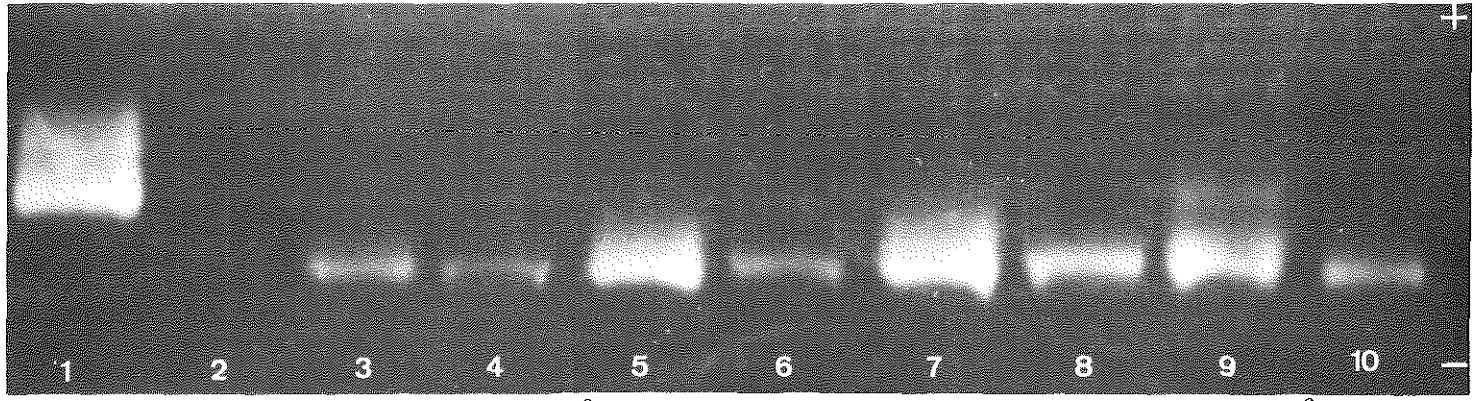

Figure 2. The effect of anti-human βGAL serum on the electrophoretic pattern of βGAL from human fibroblasts (1 and 2); Chinese hamster E36 (3 and 4); 22+3- hybrid (5 and 6)(see text); 3+22- hybrid (7 and 8); 22+3+ hybrid (9 and 10). The lysates of channels 2,4,6,8 and 10 were treated with anti-human βGAL serum

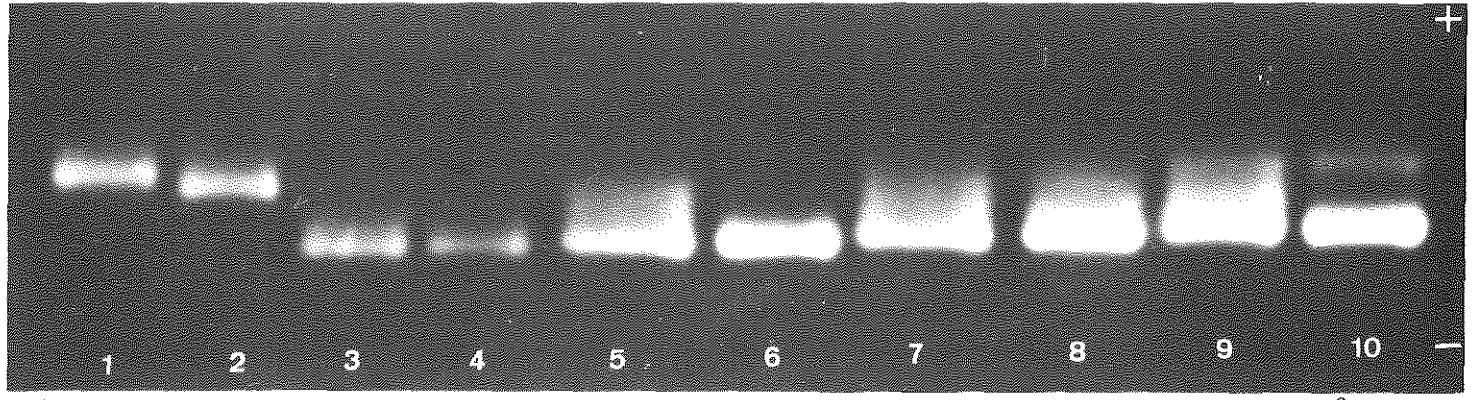

Figure 3. The effect of neuraminidase treatment on the electrophoretic pattern of βGAL from human primary fibroblasts (1 and 2); Chinese hamster E36 (3 and 4); 22+3- hybrid (5 and 6); 3+22- hybrid (7 and 8); 3+22+ hybrid (9 and 10). The lysates of channels 2,4, 6,8 and 10 were treated with neuraminidase

was removed after treatment of the lysates with anti-human βGAL serum (lanes 6,8,and 10).

Thus the electrophoretic mobility and antigenicity of the marker bands in 3-22+ hybrids and 3+22- hybrids were similar. The marker band in 3-22+ hybrids was very labile compared with that in 3+22- hybrids and was found only when freshly prepared lysates were used.

The effect of neuraminidase treatment on human cells, Chinese hamster cells, and the different types of hybrid cells is presented in Fig.3. Neuraminidase treatment of human lysate shifted the position of human βGAL to a slightly more cathodal position (lanes 1 and 2) whereas that of the Chinese hamster βGAL was not affected (lanes 3 and 4). Neuraminidase treatment removed the marker band in 3-22+ hybrids (lanes 5 and 6) but not in 3+22- hybrids (lanes 7 and 8). The more anodal band in 3+22+ hybrids remained after neuraminidase treatment, whereas the intermediate activity disappeared.

The results of the analysis of all 36 primary hybrid cell lines are summarized in Table 1. Fifteen clones did express the βGAL marker band. In six of these clones the marker band disappeared after neuraminidase treatment. These six clones had all lost chromosome 3 and GPX but not chromosome 22 and $ACON_m$.

In nine clones the βGAL marker band was not affected. Four of these clones had retained chromosome 3 and GPX but lost chromosome 22 and $ACON_m$. Three clones had retained chromosomes 3 and 22 as

Table 1. The expression of human βGAL before and after neuraminidase treatment and the presence of GPX, $ACON_m$, and human chromosomes 3 and 22

Number of clones	β-Galactosidase		Chromosomes		GPX	$ACON_m$
	-neur.	+neur.	3	22		
6	+	-	-	+>10%	-	+
4	+	+	+>20%	-	+	-
3	+	+	+>30%	+>25%	+	+
20	-	-	-	-	-	-
1	+	+	-	-	-	-
1	-	-	+ 5%	-	+	-
1	+	+	+50%	+5%	+	-

Table 2. Relationship between βGAL and LDH_B and βGAL and chromosome 12 in the man-Chinese hamster hybrids.

LDH_B \ βGAL	+	-
+	10	11
-	5	10

Chromosome 12

Chromosome 12 \ βGAL	+	-
+	6	4
-	3	1

Table 3. Human βGAL, GPX, $ACON_m$, and PGK activity in the absence or presence of human chromosomes 3, 22, and X/22 in hybrids with an X/22 translocation.

Number of clones	βGAL	Chromosomes			Markers		
		3	22	X/22	GPX	$ACON_m$	PGK
1	+	-	+50%	+ 5%	-	+	+
3	+	-	-	+15- 50%	-	+	+
2	+	+30-80%	-	+50-100%	+	+	+
2	-	-	-	-	-	-	-
4	-	+ 5-10%	-	-	-	-	-
1	+	-	-	-	-	-	+
1	+	-	-	+ 5%	-	-	+

well as GPX and $ACON_m$. In one clone having chromosome 22 in 5% of the cells, $ACON_m$ could not be detected whereas βGAL, chromosome 3, and GPX were present. Only one clone, in which GPX, $ACON_m$, and chromosomes 3 and 22 were lost, did express the marker band, and this band was not affected by neuraminidase. Twenty-one cases had lost the marker band. In twenty of these clones GPX and $ACON_m$ were also lost and chromosomes 3 and 22 could not be detected. One clone that was negative for the βGAL marker band did express GPX, whereas chromosome 3 was present in a low frequency.

The segregation data for βGAL and LDH or chromosome 12 of the primary clones (Table 2) indicate that there is no correlation between the presence or absence of LDH_B or chromosome 12 and βGAL.

Previously we assigned a structural locus for βGAL to chromosome 22 by studying hybrids isolated after fusion of white blood cells from an individual with an X/22 translocation with Chinese hamster cells. Human PGK segregated with the X/22 piece, whereas $\underline{\alpha GAL}_A$, HPRT, and Gd were assigned to the 22/X chromosome (de Wit et al., 1977) These hybrids were reinvestigated with respect to βGAL and chromosome 3 and 22. The results are shown in Table 3: four clones did express βGAL, PGK, and $ACON_m$ and had retained chromosome 22 and or X/22. Two clones with chromosomes 3 and X/22 did contain human βGAL, GPX, $ACON_m$, and PGK, Two other clones had lost chromosomes 3, 22, and the X/22 piece, and no human information for the four markers could be detected

either. Four clones had retained chromosome 3 in a low frequency (5%-10%) and lost chromosomes 22 and X/22. In these clones βGAL,GPX, $ACON_m$, and PGK were also absent. PGK and βGAL were present in two clones. In one of these clones the X/22 piece was present in 5% of the cells, whereas in the other clone chromosomes 3, 22, and X/22 could not be detected. In these two clones GPX and $ACON_m$ were absent.

DISCUSSION

Three human chromosomes, namely 3, 12, and 22, have been proposed as candidates for bearing a βGAL locus. Our previous study (de Wit et al., 1977) as well as the data described in this paper and the data published by Bruns et al. (1977) and Shows et al.(1977) conflict with the assignment of βGAL to chromosome 12 (Rushton and Dawson, 1977). The discrepancy between the data of Rushton and Dawson (1977) and these studies remains unexplained.

Electrophoresis of the 3-22+ hybrid cell lines did show an additional band of activity designated as the "marker band". An anti-human acid-βGAL antiserum precipitates this βGAL activity, thus demonstrating that it contains human βGAL determinants and that a structural βGAL locus is located on chromosome 22. Electrophoresis of lysates from 3+22- hybrid cell lines revealed a

similar marker band. The absence of this band after treatment with the antiserum indicates that a second structural βGAL locus is located on chromosome 3.

The marker bands present in these two types of hybrid cell lines differ in several characteristics. First, the marker band in 3-22+ hybrids is thermolabile compared with the marker band in 3+22- hybrids. After neuraminidase treatment the marker band in 3-22+ hybrids is not detectable whereas the pattern seen in 3+22- hybrids is not affected by this treatment. βGAL activity is observed in 3-22+ hybrids if the chromosome 22 is present in at least about 5% of the cells (see Table 3).

However the frequency of the chromosome 3 in 3+22- hybrids should be higher than 20% before the marker band can be seen on the gel. The discrepancy between our previous results (de Wit et al., 1977) and those presented in this paper may be due to the high frequency of chromosome 3 needed to express the marker band. In none of the hybrid cell lines of the panel previously used was chromosome 3 present in a frequency higher than 20%.

The more anodal band in 3+22+ hybrids remains after neuraminidase treatment. Possibly there is an interaction between the gene products of the two loci that could result in a complete human βGAL, whereas the marker bands in 3+22- and 3-22+ hybrids may represent heteropolymeric βGAL, consisting of human and Chinese hamster subunits (de Wit et al.,1977).

Bruns et al.(1977), who located βGAL on chromo-

some 3, routinely used neuraminidase-treated hybrid cell lysates for their electrophoresis. This fact together with the instability of the marker band in 3-22+ hybrids may be responsible for the absence of this marker band in their studies. Shows et al.(1977), using an anti-human βGAL antiserum, also assigned βGAL to chromosome 3. The discrepancy between their results and those described in this paper remains unexplained.

The assignment of βGAL loci to chromosome 3 and 22 indicates that at least two structural βGAL loci exist in the human genome. At present it cannot be excluded that one of the marker bands contains another human β-galactosidase, for instance the galactosyl-cerebroside βGAL deficient in Krabbe's disease (Suzuki and Suzuki,1974). This possibility would be ruled out by the demonstration of a βGAL isoenzyme composed of the gene products of both loci in 3+22+ hybrids. This will be investigated with a specific anti-Chinese hamster antiserum.

Complementation analysis with fibroblasts from different types of patients with a G_{M1}-βGAL deficiency has indicated the presence of two complementation groups (Galjaard et al.,1975). Analysis of βGAL in human liver has shown that the enzyme consists of a 70,000 mol.wt. monomeric protein and a multimeric aggregate of the same protein (Norden et al.,1974). Analysis of the restored βGAL activity after fusion of fibroblasts with different types of βGAL deficiency (Hoeksema et al.,1979)

and complementation studies with enucleated fibroblasts (de Wit-Verbeek et al.,1978) provided evidence for intergenic complementation, in which only one structural βGAL gene is involved. Cells from patients with type 1 or type 2 G_{M1}-gangliosidosis, belonging to one complementation group, are thought to be mutated in a structural βGAL gene, whereas recently evidence was presented that in cells of patients with the adult type of βGAL deficiency, belonging to the other complementation group, a neuraminidase deficiency was present as well (Wenger et al.,1978). It has been suggested that the βGAL deficiency is due to an inhibition of its βGAL activity by accumulated sialo-oligosaccharides (O'Brien,1978).

Studies are now in progress in which we isolate cell hybrids after fusion of Chinese hamster cells with fibroblasts from different types of patients with a βGAL deficiency. These studies should give more insight into how the two loci are involved in the expression of βGAL and which βGAL gene is mutated in the different types of G_{M1}-gangliodosis.

ACKNOWLEDGEMENTS. This work was supported in part by The Netherlands Foundation for Medical Research FUNGO.

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We wish to thank Dr.H.Galjaard for stimulating discussions and helpful suggestions.

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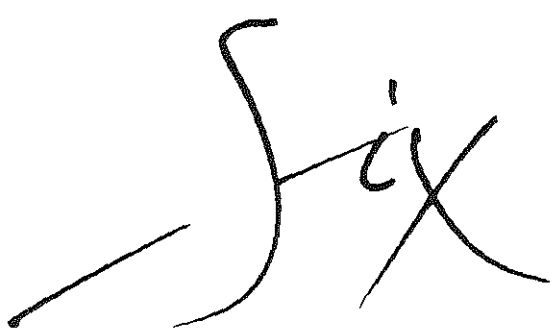

PAPER VI

THE GENETIC DEFECT IN THE VARIOUS TYPES OF HUMAN β-GALACTOSIDASE DEFICIENCY

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SUMMARY

Gene localization studies revealed the presence of two structural β-galactosidase (βGAL) loci, on the human chromosomes 3 and 22 (de Wit et al., in press). To determine the function of these genes, proliferating hybrid cell lines were isolated following fusion of fibroblasts from two different patients with a βGAL deficiency and Chinese hamster cells. The hybrids were analysed electrophoretically and immunologically.

Fibroblasts from a patient with an adult type of βGAL deficiency associated with a neuraminidase deficiency were used for the first fusion. No evidence for a structural βGAL mutation is found in these hybrids. The absence of a structural βGAL mutation is in agreement with a primary defect in neuraminidase in this adult patient.

Fibroblasts from a patient with the infantile type 1 G_{M1}-gangliosidosis were used for the second fusion. It is concluded that the human determinants present in the isolated hybrid lines occur in heteropolymeric man-Chinese hamster molecules.

The heteropolymeric isoenzyme in 3+22- hybrids is very labile and sensitive to neuraminidase treatment. Therefore it is concluded that the infantile type 1 patient is mutated in the structural βGAL gene on chromosome 3. Because this patient has a primary defect in G_{M1}-βGAL the βGAL gene on chromosome 3 is apparently a G_{M1}-βGAL gene. Interaction of the two βGAL loci result in an additional band of βGAL activity on electrophoresis. This suggests that also the gene on chromosome 22 is a structural G_{M1}-βGAL gene.

INTRODUCTION

The autosomal recessive disease G_{M1}-gangliosidosis is characterized by the lysosomal storage of gangliosides and glycosaminoglycans, due to a deficiency of acid G_{M1}-β-galactosidase (β-D-galactoside galactohydrolase E.C.3.2.1.23) (Okada and O'Brien, 1968). Several clinical forms of human acid β-galactosidase deficiency have been described, which differ in time of onset of the symptoms, the involvement of visceral organs and the occurrence of mental retardation (O'Brien, 1975; Galjaard and Reuser, 1977; Suzuki et al., 1977).

Somatic cell hybridization studies by Galjaard et al. (1975) showed that the infantile type 1 and juvenile type 2 G_{M1}-gangliosidosis are based on a different gene mutation than that in a patient with the adult variant of β-galactosidase deficiency (designated as type 4). Complementation studies with enucleated fibroblasts from the different types of patients (de Wit-Verbeek et al.,1978) and gel-filtration studies of human β-galactosidase (Hoeksema et al.,1979) provided evidence for the occurrence of intergenic complementation. Recently, it has been shown that the β-galactosidase deficiency in patients with milder clinical features, including the adult type 4, is associated with a neuraminidase deficiency (Wenger et al.,1978; Lowden and O'Brien, 1979; Hoogeveen et al., to be published).

Immunological studies (Meisler and Rattazzi,1974; O'Brien and Norden,1977) showed the presence of immunologically cross reacting material for β-galac-

tosidase in fibroblasts from patients with the infantile and adult types of β-galactosidase deficiency. Gelfiltration studies (Hoeksema et al.,1979) indicated a disturbance of the aggregation of β-galactosidase monomers to high molecular weight forms. These data indicate the presence of structurally altered β-galactosidase in infantile and adult patients.

Gene localization studies of β-galactosidase (βGAL) have been contradictory. De Wit et al. (1977) located a structural βGAL locus on chromosome 22. On the other hand, Bruns et al. (1977) and Shows et al. (1978) presented evidence for a βGAL locus on chromosome 3. Rushton and Dawson (1977) assigned a βGAL locus on chromosome 12. Recently, analysis of a new series of man-Chinese hamster hybrid cell lines in our laboratory showed the presence of two structural βGAL loci on chromosomes 3 and 22 (de Wit et al., in press).

The purpose of the present study was to investigate which structural genes coding for βGAL are involved in the infantile and adult types of β-galactosidase deficiency. Proliferating cell hybrids were isolated after fusion of fibroblasts from patients with the infantile type 1 or adult type 4 βGAL deficiency and Chinese hamster cells.

Electrophoretic and immunological characterization of the βGAL pattern in these hybrids confirmed the presence of two structural βGAL genes, on the human chromosomes 3 and 22. The data indicate that in the infantile type 1 the mutation is located in the

structural gene for βGAL on chromosome 3. There was no evidence for a structural βGAL mutation in the adult type β-galactosidase deficiency.

MATERIALS AND METHODS

Skin fibroblasts from a patient with the infantile type 1 G_{M1}-gangliosidosis and from a patient with the adult type 4 β-galactosidase deficiency (Loonen et al.,1974; Galjaard et al.,1975) were cultured in Ham's F10 medium supplemented with 15% fetal calf serum and antibiotics. The Chinese hamster cell line used (E36) is a derivate of the V79 cell line (Gillin et al.,1972) and is deficient in hypoxanthine phosphoribosyltransferase. The cells were free of mycoplasma contamination as judged by the method of Chen (1977).

The fusion procedure and isolation of the hybrid cell lines in the HAT selection system (Littlefield, 1964) was essentially the same as described previously (Westerveld et al.,1971). The same batches of cells were used for chromosome analysis and preparation of cell homogenates. Trypsin-Giemsa staining (Seabright,1971) was used for identification of the chromosomes. Cell homogenates were prepared by sonication in 5.10^{-3} M potassium phosphate buffer pH 6.5 containing 1.10^{-3} M Na_2EDTA.

Cellogel electrophoresis for β-galactosidase was performed in a 0.05 M Na_2 β-glycerophosphate buffer pH 6.2 during three hours at 4°C as described before (de Wit et al.,1977). Treatment of the cell homogenates by neuraminidase (Sigma type VI) was performed according to Bruns (1977). Electrophoresis of glutathion peroxidase (GPX; E.C. 1.11.1.9) and aconitase (mitochondrial) (ACONm; E.C. 4.2.1.3) was carried out as described by Wijnen et al. (1977) who assigned GPX to chromosome 3, and Meere Khan (1977) who assigned ACONm to chromosome 22.

Antiserum against partially purified β-galactosidase and N-acetyl-α-galactosaminidase isolated from human liver was raised in rabbits (Schram et al., 1977). Homogenates were incubated with antiserum during three hours at 0°C, the mixtures were then centrifuged at 35.000g for 30 minutes and the supernatant was used for electrophoresis.

β-Galactosidase was assayed quantitatively with 1mM 4-Methylumbelliferyl-β-D-galactopyranoside in 0.05 M sodium-acetate buffer in 0.1 M NaCl, pH 4.2 as described before (Galjaard et al., 1974).

RESULTS

I. Hybrids between human fibroblasts from the adult type β-galactosidase deficiency and Chinese hamster cells

Twenty-eight primary and sixty-seven secondary hybrid clones were isolated following fusion of fibroblasts from a patient with the adult type 4 β-galactosidase deficiency and Chinese hamster cells. The hybrid clones were analysed for the presence and absence of the human chromosomes 3 and 22, and for the presence of human β-GAL, GPX and ACONm. Eighteen primary clones retained all human chromosomes.

The hybrid cell lines isolated could be divided in four categories: hybrids in which chromosome 3 is present in the absence of chromosome 22 (3+22-), hybrids in which chromosome 3 is absent and chromosome 22 is present (3-22+), hybrids in which both chromosomes 3 and 22 are present (3+22+) and hybrids in which both chromosomes 3 and 22 are absent (3-22-).

Figure 1 shows the βGAL electrophoretic patterns of three types of hybrid clones (3+22-; 3-22+; 3+22+) and of the parental cell lines. Fibroblasts from the adult type 4 patient have 10-20% residual βGAL activity. A concentrated cell homogenate is used to visualize this βGAL activity on electrophoresis (figure 1, lane 4). Both 3+22- and 3-22+ hybrids show activity at the position of the hamster-

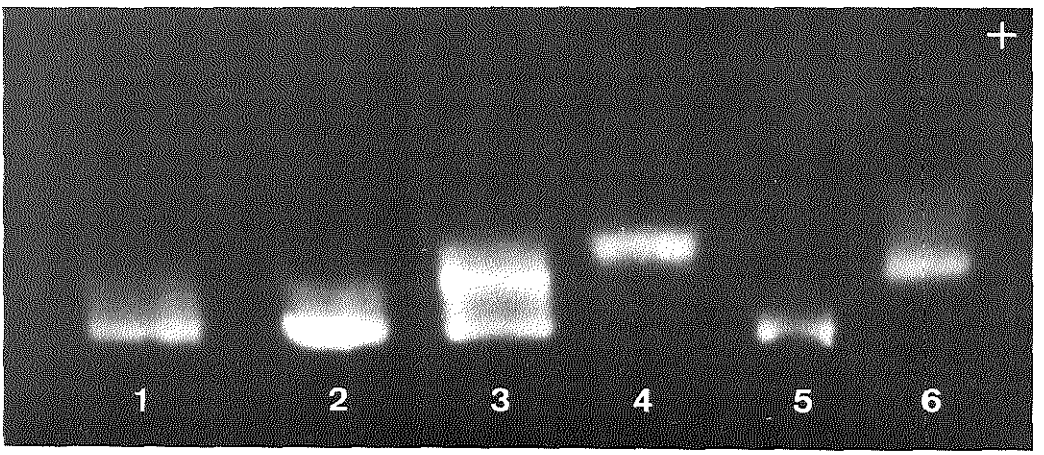

Figure 1. Electrophoresis of β-galactosidase (βGAL) from type 4 x Chinese hamster hybrids. Lane 1: 3+22- hybrid; lane 2: 3-22+ hybrid; lane 3: 3+22+ hybrid; lane 4: fibroblasts from the adult type 4 patient; a concentrated cell homogenate is used to visualize the βGAL activity (4 x the protein concentration used in the other lanes). Lane 5: Chinese hamster E36. Lane 6: normal human fibroblasts. A: βGAL_A; B: βGAL_B.

band (see figure 1, lane 5) and activity anodal but not separated from this band. The more anodal activity is referred to as the markerband. In 3+22+ hybrids (figure 1, lane 3) an additional, anodal band of activity is present at a position comparable to the control human βGAL_B position ("B"-band). Hybrids which lost both chromosomes 3 and 22 (3-22-) show a βGAL electrophoretic pattern comparable to the Chinese hamster pattern.

To investigate the presence of human determinants in the markerbands, the βGAL isoenzyme pattern of

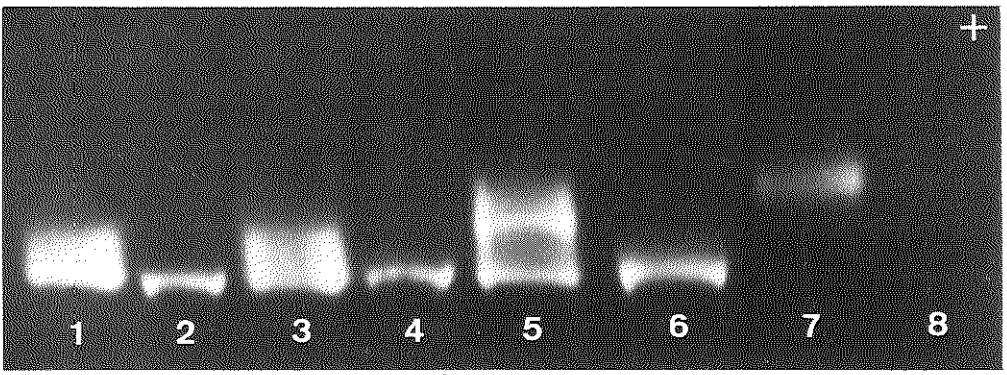

Figure 2. The effect of anti-human βGAL antiserum on the electrophoretic pattern of βGAL from type 4 x Chinese hamster hybrids. Lane 1 and 2: 3+22- hybrid; lanes 3 and 4: 3-22+ hybrid; lanes 5 and 6: 3+22+ hybrid; lanes 7 and 8: fibroblasts from the adult type 4 patient. The cell homogenates of lanes 2,4,6 and 8 were treated with the anti-human βGAL antiserum.

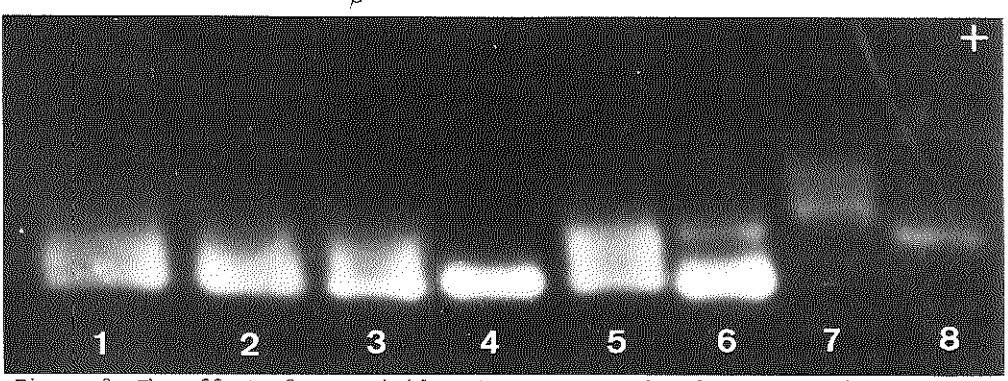

Figure 3. The effect of neuraminidase treatment on the electrophoretic pattern of βGAL from type 4 x Chinese hamster hybrids. Lanes 1 and 2: 3+22- hybrid; lanes 3 and 4: 3-22+ hybrid; lanes 5 and 6: 3+22+ hybrid; lanes 7 and 8: fibroblasts from the adult type 4 patient. Lanes 1,3,5 and 7 show the βGAL patterns after incubation with buffer for 1 hour at 37°C. Lanes 2,4,6 and 8 show the βGAL patterns after treatment with neuraminidase for 1 hour at 37°C.

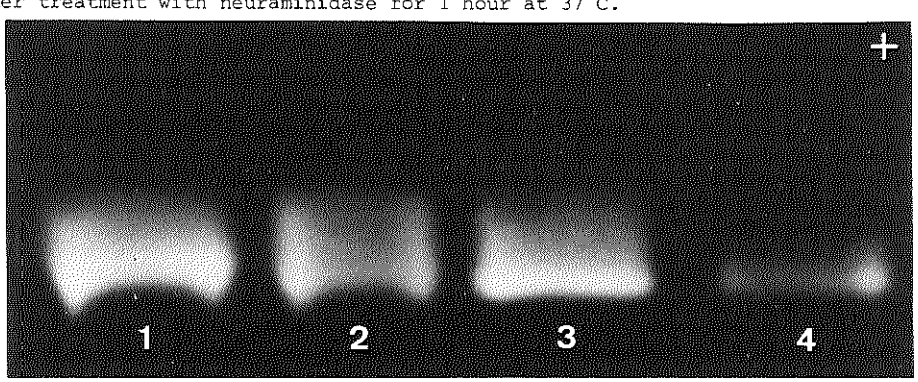

Figure 4. Electrophoresis of βGAL from infantile type 1 G_{M1}-gangliosidosis x Chinese hamster hybrids. lane 1: 3+22- hybrid; lane 2: 3-22+ hybrid; lane 3: 3+22+ hybrid; lane 4: Chinese hamster E36. Only freshly prepared cell homogenates were used.

the hybrids was characterized with an antiserum directed against purified human β-galactosidase from normal liver. The antiserum is able to precipitate the residual βGAL activity in fibroblasts from the adult type 4 patient (figure 2, lanes 7 and 8). In the hybrids, all the βGAL activity other than that of the Chinese hamster is removed by the antiserum (figure 2, lanes 2,4,6). The antiserum does not affect the βGAL activity of the Chinese hamster (figure 6, lanes 1 and 2).

The effect of neuraminidase treatment on type 4 fibroblasts and on the different types of hybrid cell lines is illustrated in figure 3. After incubation for 1 hour at 37°C in buffer, the βGAL of type 4 fibroblasts is present as a broad band of activity (figure 3, lane 7). This activity shifts to a more cathodal position after neuraminidase treatment (figure 3, lanes 7 and 8). Quantitative analysis showed that the type 4 βGAL is not affected by the treatment. The markerband in 3+22- hybrids is not removed, whereas the markerband in 3-22+ hybrids disappears after neuraminidase treatment (figure 3, lanes 1,2,3 and 4). The additional band of activity on the human βGAL_B position in 3+22+ hybrids remains present after treatment, but the activity between this band and the Chinese hamster band disappears.

The data of the chromosome and enzyme analysis of the hybrid clones are summerized in Table I. In one clone, in which the βGAL markerband is present before and after neuraminidase treatment, no chromo-

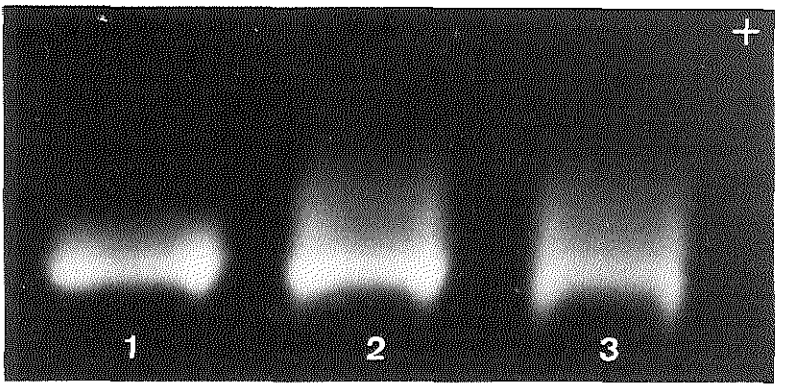

Figure 5. Electrophoresis of βGAL from type 1 x Chinese hamster hybrids after freezing to -70°C and thawing of the homogenates. Lane 1: 3+22- hybrid; lane 2: 3-22+ hybrid; lane 3: 3+22+ hybrid.

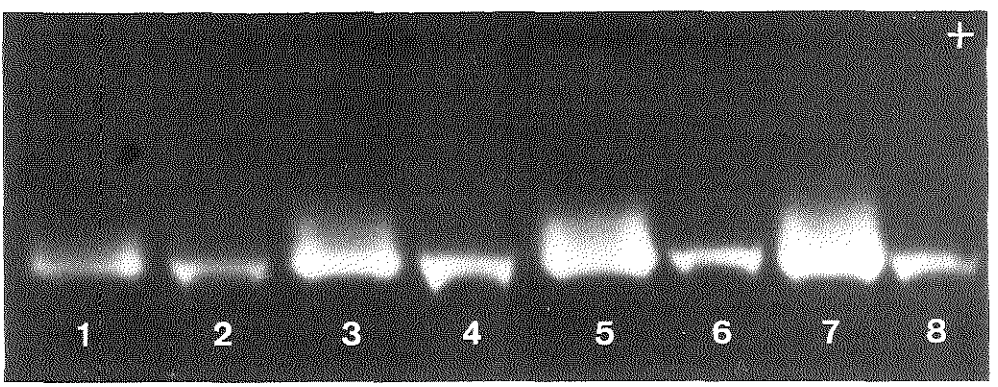

Figure 6. The effect of anti-human βGAL antiserum on the electrophoretic pattern of βGAL from type 1 x Chinese hamster hybrids. Lanes 1 and 2: Chinese hamster E36; lanes 3 and 4: 3+22- hybrid; lanes 5 and 6: 3-22+ hybrid; lanes 7 and 8: 3+22+ hybrid. The cell homogenates of lanes 2,4,6 and 8 were treated with the antiserum.

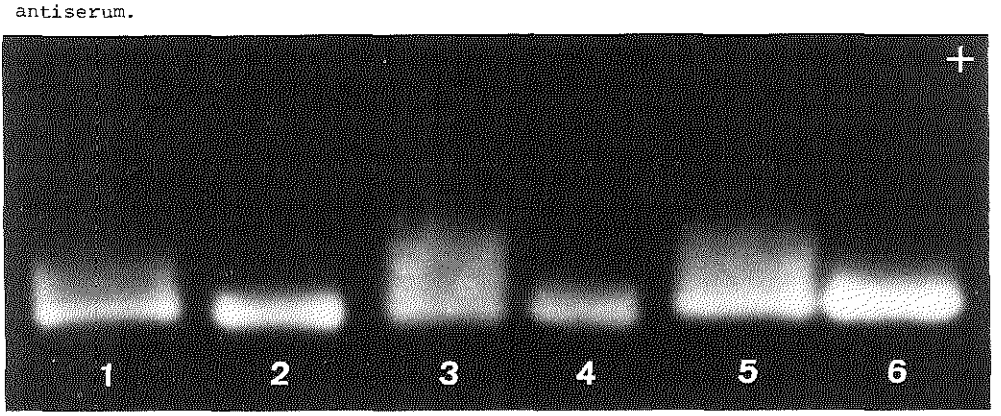

Figure 7. The effect of neuraminidase treatment on the electrophoretic pattern of βGAL from type 1 x Chinese hamster hybrids. Lanes 1 and 2: 3+22- hybrid; lanes 3 and 4: 3-22+ hybrid; lanes 5 and 6: 3+22+ hybrid. Lanes 1,3 and 5 show the βGAL patterns after incubation of the cell homogenates with buffer for 1 hour at 37°C. Lanes 2,4 and 6 show the βGAL patterns after treatment with neuraminidase for 1 hour at 37°C.

some 3 and GPX could be detected. This can be due to breakage of chromosome 3.

II. Hybrids between fibroblasts from a patient with infantile type 1 G_{M1}- gangliosidosis and Chinese hamster cells.

Eighteen primary and eighteen secondary clones were isolated following fusion of fibroblasts from the patient with infantile type 1 G_{M1}-gangliosidosis and Chinese hamster cells. Most of these clones retained the human chromosomes 3 and 22. Starting with a mixed cell population, another six hybrid clones were selected in the presence of 10 δ/ml 6-thioguanine in the culture medium (Table II).

Figure 4 shows that the βGAL electrophoretic patterns of three different types of hybrid cell lines (3+22-; 3-22+; 3+22+) are very similar. Each of them consists of activity at the position of the hamster-band (see figure 4, lane 4) and the activity anodal but not separated from this band which is called the markerband. The βGAL electrophoretic pattern shown in figure 4 was only observed in all types of hybrids if freshly prepared homogenates were used for electrophoresis. The residual βGAL activity in fibroblasts from the type 1 G_{M1}-gangliosidosis patient is less than 1% and is not detectable in our electrophoretic system.

When freshly prepared homogenates of the three types of hybrids were stored overnight at -70°C and

TABLE I ANALYSIS OF HUMAN ADULT TYPE β-GAL⁻ X CHINESE HAMSTER HYBRIDS

Number of clones	% chromosomes present		enzymes				
	3	22	GPX	$ACON_m$	β-GAL markerband -neur	β-GAL markerband +neur	β-GAL 'B'-band
71	25-100	35-90	+	+	+	-	+
19	-	20-60	-	+	+	-	-
1	-	-	-	-	+	+	-
1	60	-	+	-	+	+	-
4	-	-	-	-	-	-	-

TABLE II ANALYSIS OF HUMAN G_{M1}-GANGLIOSIDOSIS TYPE 1 X CHINESE HAMSTER HYBRIDS

Number of clones	% chromosomes present		enzymes			
	3	22	GPX	$ACON_m$	β-GAL markerband -neur	β-GAL markerband +neur
26	20-80	30-90	+	+	+	-
10	-	10-60	-	+	+	-
	<u>after selection in 6-thioguanine:</u>					
3	n.t.	n.t.	+	+	+	-
2	60	-	+	-	+	-
1	n.t.	n.t.	-	+	+	-

n.t.: not tested.

thawed shortly prior to electrophoresis, the markerband in 3+22- hybrids was no longer detectable (figure 5, lane 1). The βGAL isoenzyme pattern in 3-22+ hybrids was not affected by this treatment (figure 5, lanes 2 and 3).

Characterization of these βGAL electrophoretic patterns with an anti-human βGAL antiserum is illustrated in figure 6. The antiserum does not affect the βGAL activity of the Chinese hamster whereas the activity at the position of the makerbands was removed in all types of hybrids.

Neuraminidase treatment of type 1 x Chinese hamster hybrids resulted in a disappearance of the activities at the position of the markerband (figure 7). Incubation for 1 hour in buffer, which served as a control, reduced the activity of the markerband in 3+22- hybrids (figure 7, lane 1).

The results of the analysis of all 42 hybrid clones are summarized in Table II.

DISCUSSION

Gene localization studies revealed the presence of two structural β-galactosidase (βGAL) loci, on the human chromosomes 3 and 22 (de Wit et al., in press). The function of these two genes and their relation with different autosomal recessive diseases associated with β-galactosidase deficiency is as yet unknown. In the present study, hybrid cell lines were isolated after fusion of fibroblasts from two different types of β-galactosidase deficiency and Chinese hamster cells.

In the first fusion, fibroblasts from a patient with the adult type βGAL deficiency and Chinese hamster cells have been used. The hybrid cell lines isolated show βGAL electrophoretic patterns similar to the patterns from hybrids between control human fibroblasts and Chinese hamster cells ("control hybrids").

With the anti-human βGAL antiserum it could be shown that the markerbands as well as the additional band of activity on the human βGAL_B position in 3+22+ hybrids contain human determinants. Neuraminidase treatment of type 4 x Chinese hamster hybrids gave similar results compared to neuraminidase treatment of control hybrids.

In every respect type 4 x Chinese hamster hybrids behave as hybrids between control fibroblasts and Chinese hamster cells; no evidence for a structural βGAL mutation in the adult type 4 is obtained.

Wenger et al. (1978) found a neuraminidase deficiency in one of his adult patients with a βGAL de-

ficiency. In our laboratory we also found a neuraminidase deficiency in our adult type 4 βGAL deficiency (Hoogeveen et al., to be published). The absence of a structural βGAL mutation in the type 4 patient is in agreement with a primary defect of neuraminidase in this patient. How βGAL deficiency can occur due to neuraminidase deficiency is as yet unknown.

Another series of hybrid cell lines has been isolated following fusion of fibroblasts from a patient with the infantile type 1 G_{M1}-gangliosidosis and Chinese hamster cells. βGAL electrophoresis of these hybrid lines showed the presence of a markerband in 3+22-, 3-22+ and 3+22+ hybrids. Immunological studies revealed the presence of human determinants in the isoenzymes of these markerbands. Because of the low residual βGAL activity in the type 1 fibroblasts, it appears that the markerbands consist of heteropolymeric βGAL molecules formed by human and Chinese hamster subunits. In general, hybrids, isolated following fusion of fibroblasts with an enzyme deficiency and rodent cells, only show heteropolymeric molecules for the deficient enzyme if cross reacting material is present (Chern, 1977; Hamers, 1978).

The markerband in 3+22- type 1 x Chinese hamster hybrids is labile compared to the markerbands in 3-22+ and 3+22+ hybrids. This is in contrast with control hybrids, in which the markerband in 3+22- hybrids is stabile compared to the markerband in 3-22+ hybrids (de Wit et al., in press). Also the different behaviour of 3+22- type 1 x Chinese ham-

ster hybrids towards neuraminidase treatment demonstrates the abnormality of the markerband in 3+22- hybrids. The additional band at the human βGAL_B position in 3+22+ control hybrids, which is most distinct after neuraminidase treatment (de Wit et al., in press), is lacking in type 1 x Chinese hamster hybrids which contain both chromosomes 3 and 22. These data indicate the presence of a mutation in the structural βGAL gene on chromosome 3 in the type 1 patient. Because G_{M1}-β GAL is deficient in type 1 G_{M1}-gangliosidosis patients (Okada and O'Brien, 1968) and these patients' parents show heterozygous βGAL values (Wolfe et al., 1970), the β-GAL gene on chromosome 3 is apparently a structural gene for G_{M1}-β-galactosidase.

Both series of hybrid cell lines confirm the localization of two structural βGAL genes on the human chromosomes 3 and 22. As the result of the interac - tion of the gene products coded for by these βGAL loci an additional band of activity at the human βGAL_B position can be formed. Because the gene on chromosome 3 is a structural G_{M1}-βGAL gene, this suggests that also the gene on chromosome 22 is a structural G_{M1}-βGAL gene.

Biochemical studies revealed the presence of two G_{M1}-β-galactosidase isoenzymes (Norden et al., 1974). βGAL_A (Molecular Weight 70 000) is thought to be a monomer and βGAL_B (M.W. 700 000) is thought to be a multimeric aggregate of the monomeric protein. Whether this multimeric form also contains additional subunits is unknown (Frost et al., 1978). The

relationship between the structural G_{M1}-β-galactosidase genes on chromosomes 3 and 22 and the βGAL_A and βGAL_B isoenzymes has to be investigated.

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