ALPHA-N-ACETYLGALACTOSAMINIDASE: ENZYME PURIFICATION AND ISOLATION OF PUTATIVE cDNA CLONES

BY

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ABSTRACT

ALPHA-N-ACETYLGALACTOSAMINIDASE (ALPHA-GALACTOSIDASE B):
ENZYME PURIFICATION AND ISOLATION OF PUTATIVE cDNA CLONES

by

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Alpha-N-acetylgalactosaminidase (alpha-galactosidase B, alpha-gal B, E.C. 3.2.1.49) was purified to homogeneity, then used to provide information and to produce material necessary for the selection of putative cDNA clones from human cDNA libraries by several methods. The purified enzyme had a specific activity of 5.7 umoles of 4-methylumbelliferyl alpha-D-galactopyranoside hydrolyzed per minute per milligram protein which was completely inhibited by N-acetylgalactoside. The yield of this procedure (55%) was higher than previously reported. This result, in conjunction with decreased handling time and the higher specific activity obtained, makes this procedure more beneficial to continuing studies than previous procedures.

The molecular weight of the pure enzyme was 94,000 as judged by

gel filtration chromatography, with a subunit Mr = 45,000, determined by denaturing SDS-polyacrylamide gel electrophoresis. The deglycosylated, denatured subunit Mr was 38,000. Polyclonal, monospecific rabbit antibodies were raised against the pure enzyme and used in cloning attempts.

The carbohydrate moieties on alpha-gal A and B were not necessary for antigenicity of either enzyme, and antibodies recognized both the native and denatured forms of the enzymes. Antibodies against the human liver enzyme were specific for human enzyme, although there was some cross reactivity of coffee bean and watermelon alpha-galactosidase with the rabbit anti-alpha-gal B IgG when visualized by double antibody-enzyme conjugated detection systems.

Three methods were used to in attempts to isolate mRNA or cDNA coding for alpha-galactosidase B: Polysome immunoprecipitation with anti-alpha-galactosidase A IgG, screening a human fetal liver cDNA library with oligonucleotide probes homologous to alpha-gal B peptides, and screening a human cDNA lambda gt11 expression library with polyclonal, monospecific anti-alpha-gal B IgG.

Polysomal mRNA from human Chang liver cells was immunoprecipitated by anti-alpha-gal A IgGs, and translated in a wheat germ <u>in vitro</u> translation system. Isolated cDNA clones were characterized by restriction enzyme mapping, Maxam-Gilbert and dideoxy DNA sequence analysis, and antigenicity of expressed fusion proteins with

anti-alpha gal B IgGs in dot-blot and Western blot assays. One cloned phage remains promising, producing a fusion protein of approximately Mr=122,000 -- 6,000 to 8,000 mass units larger than the beta-galactosidase protein expressed by the control bacterial lysogen.

To Michael & Caitlin & ...

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INTRODUCTION

THE PHYSIOLOGICAL IMPORTANCE OF LYSOSOMAL ENZYMES

Since the lysosome was identified as a distinct organelle in 1955 by deDuve, (31, 32,) its complement of hydrolytic enzymes has been slowly identified and characterized, often in conjunction with a deficiency of one or more lysosomal enzymes. Although clinical descriptions of what are now referred to as lysosomal storage diseases date back to the 1880's, the biochemical nature of these disorders has been gradually established only in the past two to three decades (for a complete review of lysosomal storage disorders, see ref 176). This far-reaching scrutiny of the physiological importance of these enzymes has not only led to the elucidation of their biosynthetic and processing stages, but has also shed light on many cellular and subcellular functions, and the regulation of mechanisms involved in directing subcellular protein traffic.

Lysosomal enzymes degrade substances delivered to the lysosome by receptor-mediated endocytosis (28) or other mechanisms. Complex molecules are digested to simple sugars, lipids, amino acids and nucleotides. These products are then removed from the cell, or are recycled into other

molecules. Deficient enzyme activity results in the accumulation of complex molecules within the lysosome and a general disruption of cellular functioning; hence, the term lysosomal storage disease.

On an individual basis, lysosomal storage disorders are rare, but as a group they are much more common. Together, lysosomal storage disorders comprise about 1% of the total Mendelian disorders, but 10% of the total inherited conditions for which the primary cause has been identified (126). To date, all have proven to be recessive traits. Only two are X-linked: Fabry's and Hunter's disease.

Some lysosomal disorders, rare in the general population, are prevalent in certain races or cultures. Tay-Sach's disease, a deficiency of a hexosaminidase resulting in the storage of mucopolysaccharides, affects approximately 0.003% of the general population, but in communities of Ashkenazi Jews, occurs in 2-4% of the population (176).

One reason storage disorders are so well studied is that they are more easily identified than many other metabolic or genetic disorders by chemical identification of storage products in urine, plasma, or serum, and by morphological identification of abnormal lysosomal accumulation in various cell types. In addition, the identification of enzyme activity (or deficiency) in accessible cells such as

fibroblasts, leukocytes, and amniotic cells, and in body fluids is possible in most cases using artificial or natural substrates (23, 53).

Histological patterns of lysosomal accumulation are also used to identify a storage disorder by identifying the affected organ(s), or, more specifically, the cell type(s) accumulating substrate. Theoretically, all cells produce the same array of lysosomal enzymes, and should have the same deficient activity. However, since substrate accumulation causes the cellular damage, the organ(s) most actively metabolizing a specific substrate is the most affected. For example, Tay-Sach's disease is caused by a deficient hexosaminidase which normally degrades gangliosides, a major component common in developing brain. Accumulation of specific gangliosides results in extensive central nervous system (CNS) damage in the fetus and infant, resulting in death by approximately two years of age (176).

In contrast, Fabry's disease, a deficiency of alpha-galactosidase A, is marked by extensive accumulation of glycosphingolipids with terminal alpha-galactose residues at the non-reducing end of the carbohydrate chain in the visceral endothelia (94). Since the natural substrates are common components of endothelial cell membranes, but not neuronal cell membranes, the storage of undegraded substrates is limited primarily to visceral organs, with

some involvement of cells associated with the peripheral nervous system. The accumulation of substrates is gradual with clinical symptoms usually appearing around puberty, and more severe impairment of organ function in the second or third decade. Even with medical intervention designed to alleviate distress, or, in some cases, to replace damaged organs, death usually occurs in the fourth or fifth decade.

Lysosomal enzymes are substrate-specific and act in a cascade or series of reactions. Chemical identification of the accumulated material serves to identify the normal activity of the enzyme by defining the natural substrates. However, in some cases, the accumulating material may inhibit the activity of subsequent enzymes, causing the storage of secondary products. A deficiency of alpha-L-fucosidase, which cleaves fucose units from complex carbohydrate chains, results in the accumulation of glycoconjugates with other terminal residues such as galactose or mannose residues at the non-reducing ends. This indicates that not only does fucose removal precede the removal of these other residues, but that the presence of fucose on the carbohydrate chain sterically impairs the action of subsequent enzymes (214).

Most of the deficient enzymes are glycosidases, lipid-hydrolases, and sulfatases. However, not all lysosomal enzymes have been associated with a specific lysosomal

storage disorder in man. Considering the number of lysosomal enzymes known, only approximately 50% of the potential storage disorders have been identified (187). Of the thirty or so disorders identified, deficiencies of nucleases or proteinases have not been described thus far.

To account for this, two immediate hypotheses can be presented: 1) the accumulating substrate is not a major metabolite in any vital organ and, thus, does not cause serious distress, or, conversely, 2) the enzyme is so vital in the degratory cascade that any deficiency results in death *in utero*, or so immediately post-partum that detection by common diagnostic methods is impossible or at least unlikely.

In caprine beta-mannosidosis (84, 85) a fatal beta-mannosidase deficiency was discovered after inadvertent inbreeding of goats. Only after diagnosis and intervention by a team of veterinary and human clinical and research scientists was the specific enzyme deficiency identified. The family of goats was maintained as an animal model under extraordinary circumstances.

Another example of a lysosomal enzyme not associated with a storage disease may be a deficiency of alpha-N-acetyl-galactosaminidase (alpha-galactosidase B, alpha-gal B). Medical scientists linked the death of an infant diagnosed

with malignant histocytosis to a deficiency (2%-30% of normal levels, depending on the artificial substrate used for the assay) of alpha-gal B (46). Although gross histological data supported the idea of a lysosomal storage disease, results were disputed and a firm correlation could not be made.

Within a specific disorder, there is often an array of clinical presentations differing in the type, severity, and onset of symptoms. The biochemical basis for this heterogeneity is not well understood, but can be attributed to causes ranging from allelic mutations resulting in defective, unstable, or absent enzyme, to faulty cellular mechanisms affecting the biosynthesis and processing of the enzyme itself. While the biosynthesis of several lysosomal enzymes has been studied extensively, and cDNA clones of several lysosomal enzymes have been isolated (22, 45, 49, 77, 97, 98, 129, 136, 174), the exact molecular defect(s) resulting in deficient activity has yet to be determined in most cases.

No effective treatment for lysosomal storage disorders has been developed thus far. Some pain and discomfort have been alleviated through drug therapy (176), but depletion of accumulated substrates has not been successful. In some cases (33, 35, 141) affected organs were replaced with

normal human tissues. For example, kidney transplantations were performed on several patients with Fabry's disease. In the two cases where the kidney was not rejected, the kidney functioned normally and there was no detectable accumulation of substrate glycosphingolipids in the new kidney. However, the accumulation continued in other organs and the patients eventually died from complications caused by the deficiency. Presumably, this supports other data which show that even though some cells are capable of accepting and utilizing endogenously supplied lysosomal enzymes, cells rely on their own production of enzyme in order to function normally.

Early experiments involving enzyme replacement have been closely coupled to the elucidation of both enzyme-receptor mechanisms and biosynthetic pathways. Hickman, Neufeld and others showed that cultured fibroblasts with lysosomal accumulations of mucopolysaccharides due to a deficiency of alpha-iduronidase (Hurler's syndrome), iduronidate sulfatase (Hunter's syndrome) or beta-glucuronidase (mucopolysaccharidosis VII, MPS VII) could capture exogeneously supplied enzyme and transport it to the lysosome where it degraded the accumulating substrate, corrected the metabolic error, and returned the fibroblasts to a "normal state" (75, 76).

Attention focused on this "high uptake" form of alpha-iduronidase which could be selectively taken up by human

fibroblasts (128, 169).Others found similar forms of beta-glucuronidase, alpha-glucosidase and beta-hexosaminidase (76) which were secreted and recaptured by fibroblasts via the mannose 6-phosphate (M6P) residue on the enzymes' carbohydrate chains (73, 135) and the M6P receptor on the membrane of the fibroblast cell (90). This "high uptake" form is secreted from normal fibroblasts, taken up by the deficient fibroblasts and processed normally (194) as long as the defect does not lie in the processing mechanisms of the cell.

The observation that some cells, especially fibroblasts, can endocytose, process, and use endogenous enzyme has led to the elucidation and characterization of several types of recognition and receptor-mediated mechanisms which lead to proper sorting and transport of lysosomal enzymes. Studies of I-cell patient- and Hurler patient-derived fibroblasts, and other cell types indicate there must be alternatives to the M6P system. I-cell patients are unable to phosphorylate mannose residues on lysosomal enzymes, leading to the secretion of a large number of lysosomal enzymes (78, 148). However, not all lysosomal enzymes are secreted from these fibroblasts. In addition, parenchymal, Kuppfer, brain, and macrophage cell lines derived from these patients process lysosomal enzymes properly and do not accumulate substrate.

Several carbohydrate-mediated receptor systems clearing

circulating glycoproteins by the recognition of specific sugar residues of the carbohydrate chains and functioning in the transport of newly synthesized lysosomal enzymes have been well documented. These systems include recognition of galactose by a receptor on hepatocyte cells-- the Ashwell receptor (4, 82, 88, 89, 189, 195), a second, distinct M6P receptor (148, 189), receptors for glucose, fucose and sialic acid (1, 88, 189) and a mannose-N-acetylglucosamine receptor found in reticuloendothelial cells (1).

The elucidation and characterization of these carbohydrate recognition and receptor-mediated mechanisms involved in the sorting and transport of lysosomal enzymes have fueled attempts to treat affected patients by enzyme replacement. Pilot trials of alpha-galactosidase A replacement for the treatment of Fabry's disease showed that infusing patients with alpha-gal A, isolated from the plasma, spleen and placenta of normal individuals, cleared accumulating substrates from the patients' plasma. The disappearance was transient, returning to normal levels as the enzyme was cleared from the patients' plasma and after administration was ended (18, 119).

The different forms of alpha-gal A were cleared from the circulation at different rates: the highly sialylated plasma form remained in circulation ($t_{1/2}$=70 min.) longer than the less sialylated splenic form which was rapidly

cleared ($t_{1/2}$=10 min.) (34). These results are consistent with the Ashwell receptor model (4, 195) confirming the prolonged retention of sialylated glycoproteins in the circulation and the rapid clearance of desialylated glycoproteins. In addition, the activity of the administered enzyme could be detected in biopsies of the liver one hour after administration (12).

These studies, along with others, give hope that enzyme replacement therapy might prove to be a worthwhile endeavor in some cases. In those lysosomal storage disorders involving the visceral organs, infusion of endogenous enzyme might cause the clearance of accumulated substrate. Clearly, more research is needed to determine ways to target the enzyme to the specific organs metabolizing the substrate as well as the most effective and stable forms of the replacement enzymes. Not only do researchers need to define the primary lesions involved in many disorders, but the effects of long term enzyme replacement need to be be determined.

Besides identifying the exact defect or cause(s) of deficient activity, and possible immunological complications, there are other drawbacks to enzyme replacement therapy. Those disorders which affect the central nervous system (CNS) such as Hurler's syndrome, GM_1 gangliosidosis, infantile Gaucher's disease, and

Krabbe's disease among others, will not respond to the infused enzyme unless the blood-brain barrier can be altered, or the enzyme can be complexed to a carrier that can traverse the blood-brain barrier. While the effects of the accumulating substrate in the skeletal system and visceral organs may be overcome, the severe mental retardation and brain damage due to the accumulation will prove to be the most difficult consequence to correct.

In addition, the logistics of enzyme replacement will be difficult to overcome. Obtaining sufficient enzyme for biochemical analysis has proven difficult: purification is usually tedious and results in miniscule amounts of the enzyme. For example, alpha-fucosidase represents approximately 0.02% of the total cellular protein (49) and alpha-galactoidase A perhaps 0.01 to 0.001% (22). In most cases the enzymes are obtained from human cadaver organs (liver, spleen, kidney, lung) or other tissues such as plasma or placenta. Obtaining normal human tissue in many cases is quite difficult and limited.

Recently, cDNA clones or partial clones of several lysosomal enzymes have been isolated including: alpha-fucosidase (49), alpha-galactosidase A (22), alpha-glucosidase (19), cathepsin B (45), the alpha chain of human beta-hexosaminidase (98, 129), the pre-beta-polypeptide of hexosaminidase (136), human glucocerebrosidase (174), alpha-mannosidase,

beta-galactosidase, acid lipase (204), and beta-glucuronidase (136). Investigators have hopes of isolating entire genes, then using the DNA sequence information and the structural genes themselves to shed more light on the biosynthesis and function of these enzymes, and in future engineering and production experiments.

The possibility of engineering a cell line to overexpress and secrete enzyme which could later be used in replacement therapy exists. Cloning the cDNA into an expression vector (i.e., a retrovirus vector), then infecting an appropriate cell line that could synthesize and process the enzyme properly is a viable alternative to purification of the enzyme from human sources.

A last resort-- possibly the only hope for those disorders affecting the CNS-- is gene replacement. Recent progress in cloning genes into defective retrovirus vectors, infecting bone marrow cells and transplanting these cells into animals causes researchers to consider this action seriously (3). Of course, the type of vector to use (perhaps a DNA virus) as well as structural features of the DNA sequences controlling insertion, stability, and expression must still be determined as well as the appropriate target cell.

THE BIOSYNTHESIS OF LYSOSOMAL ENZYMES

Like secretory proteins, lysosomal enzymes are synthesized as precursors of higher molecular weight (154), and are transported by specific cellular mechanisms which recognize both protein and carbohydrate structures. Processing of both protein and carbohydrate structures is substantial, requiring specific mechanisms at various subcellular locations. A number of lysosomal enzyme deficiencies can be traced back to disturbances in the processing and/or transport of precursor enzymes.

The biosynthesis of lysosomal enzymes begins with the translation of the mRNA on polysomes bound to the endoplasmic reticulum (ER) and translocation of the nascent polypeptide into the ER lumen where it undergoes co-translational proteolytic processing, as well as the addition and processing of carbohydrate chains.Subsequently, the precursor enzyme moves to the Golgi apparatus where it undergoes more processing, sorting, and transport to other subcellular or extracellular locations. Finally, lysosomal enzymes are delivered to mature lysosomes where some undergo a final round of processing before they are capable of degrading specific substrates.

Initial synthesis on the rough endoplasmic reticulum is coupled to both protein and carbohydrate processing. The

biosynthesis and processing of lysosomal hydrolases resembles that of many other glycoconjugates, and the initial synthesis and transitory stages follow the pattern of many secretory proteins (40).

Initially, the 5'end of the mRNA codes for a transient peptide segment at the amino terminus of the nascent peptide. This stretch of about twenty, mostly hydrophobic amino acids, called the signal sequence (41), is important for the movement of the protein through the ER membrane.

The signal sequence is recognized by an 11S ribonucleoprotein composed of six polypeptide chains, the signal recognition particle or SRP (212). The SRP and signal sequence form a complex which, in turn, binds to a receptor or docking protein on the ER membrane. Then, the nascent polypeptide is translocated into the lumen of the ER and translation of the remainder of the mRNA continues.

Proteolytic processing of the nascent peptide begins immediately after translocation of the protein through the ER membrane and before translation is completely finished. The term "prepropeptide" or "preproenzyme" commonly refers to the precursor form of the enzyme containing the amino-terminal signal sequence and the immature, higher molecular weight polypeptide. In most cases, the signal sequence is removed by a signal peptidase within the ER, leaving the

proenzyme form (41).

Erickson and others demonstrated the existence of protein precursors of cathepsin D and their role in the biosynthetic phenomena of lysosomal enzymes in general (40, 41). When porcine spleen mRNA was translated in a cell-free wheat germ translation system, a non-glycosylated 43,000 M_r prepro-cathepsin D was synthesized. The addition of dog pancreas microsomes to the in vitro translation system resulted in the synthesis of a glycosylated (endoglycosidase H sensitive) 46,000 M_r protein. N-terminal sequencing of the two polypeptides revealed two distinct sequences, providing direct proof for a transient amino-terminal sequence.

Others have indirectly shown that several other lysosomal enzymes are synthesized with transient amino-terminal sequences, including mouse cathepsin D, rat beta-glucuronidase (150), and the alpha and beta-chains of hexosaminidase (143). By culturing cells in the presence of tunicamycin, an inhibitor of glycosylation, non-glycosylated precursors were synthesized that had a lower apparent molecular weight than the prepro forms synyhesized in vitro from mRNA isolated from the same cell lines. The non-glycosylated proenzymes had different sensitivities to endoproteases than the in vitro prepro enzymes. This Lack of sensitivity was correlated with the lack of an N-

terminus peptide segment in the proenzyme.

Secretory, transmembrane, and lysosomal proteins all require a signal recognition particle/receptor mechanism for translocation into the ER and for subsequent processing (2, 43, 213). When microsomal membranes are salt-extracted to remove the SRP and are added to an *in vitro* translation system, only the prepro form of the protein is synthesized. The addition of SRP restores translocation into the membranes and proteolytic processing events. When purified SRP is present in the *in vitro* system without membranes, the elongation of the nascent peptide is arrested until the salt-extracted membranes are also added.

These events have been repeated with both secretory and lysosomal proteins (43) and show that these glycoproteins share a common receptor-mediated system for translocation of the nascent peptides across the ER membrane. Whether the SRP is a universal recognition protein particle, or whether subtypes of the SRP recognize the slight amino acid variations in the signal sequences of various proteins is not known.However, secretory and lysosomal enzymes are co-segregated in the lumen of the ER, and apparently separated in the Golgi apparatus.

Translocation into the ER lumen is accompanied by the addition of an endoglycosidase sensitive high mannose

oligosaccharide (41, 150, 212). Initial biosynthetic studies (41) showed that precursor enzymes synthesized in vitro have a net increase in molecular weight despite the observed proteolytic events. Microsomal membranes added at the start of in vitro synthesis are responsible for the glycosylation activity. They also protect the precursor from both proteolytic and glycosidic enzymes unless the integrity of the membranes is compromised by the addition of detergents.

The oligosaccharides are important for the stability of both the precursor during biosynthesis and the mature enzyme in the extra- and intra-cellular millieu, for recognition phenomena such as receptor-mediated endocytosis, and for transport from the ER to the Golgi, cell surface and other organelles. Cell cultures treated with inhibitors of glycosylation synthesize the proenzyme peptide (150, 212) and the increase in mass is not seen. The non-glycosylated propeptide is neither proteolytically processed, nor transported intracellularly to any significant extent. Secretion of the nonglycosylated propeptide from the cell is greatly decreased compared to secretion rates of the normal enzyme. Subcellular fractionation of treated cells reveals the accumulation of non-glycosylated lysosomal precursors in the lumen of the ER (150).

The carbohydrate chains of glycoproteins are classified

according to the molecular linkage between a sugar and an amino acid on the peptide chain (100). The most common linkage in mammalian glycoproteins is an N-glycosidic linkage between an asparagine residue (Asn) and an N-acetyl-glucosamine (GlcNAc) residue in the oligosaccharide. Three other linkages, containing an O-glycosidic bond, occur between N-acetyl-galactosamine (GalNAc) and a serine or threonine, galactose and hydroxylysine, and xylose and serine (162). The carbohydrate chains described on lysosomal enzymes thus far are all N-glycosyl GlcNAc-Asn bonds.

Lennarz and co-workers found that the peptide sequence -Asn-X-(Ser/Thr)- is required for glycosylation, although not all such sequences are glycosylated (179). Assessment of the three dimensional structure of the peptide sequence around possible glycosylation sites showed that those sites in beta-turns tend to be glycosylated while those in alpha helices are not (180). Interestingly, the predicted amino acid sequences from several cDNA clones of lysosomal enzymes (13, 45, 98, 174) all have four potential glycosylation sites, although nearby amino acids (i.e., prolines which may produce unusual kinks in the secondary structure) may decrease the probability of all four being glycosylated (60).

The first step in the synthesis of an asparagine-linked

glycoprotein is the assembly of an oligosaccharide containing three terminal glucose residues and nine mannose residues on an N-acetyl-glucosamine core (Glc_3Man_9-$(GlcNAc)_2$) by the stepwise addition of each sugar carried by a nucleotide donor or dolichol-phosphate donor (80, 112). The entire activated oligosaccharide complex is assembled on both the both the cytoplasmic and lumenal sides of the ER membrane by different membrane-bound glycosyltransferases (139). The oligosaccharide complex is transferred from the lipid-linked carrier dolichol pyrophosphate to an asparagine on the nascent polypeptide chain (9, 66, 80, 81, 93, 113). This final transfer takes place in the ER lumen, usually before the protein backbone is completely folded (Figure 1).

Three types of carbohydrate structures have been identified on lysosomal enzymes: high mannose, complex and hybrid chains (Figure 2). High mannose chains result from the removal of few, if any, of the alpha mannosyl residues present on the precursor complex. Complex chains have two or more structures attached to the $Man_3(GlcNAc)_2$ core. Sialylated, complete complex chains (NANA-Gal-GlcNAc) and incomplete (Gal-GlcNAc or GlcNAc) have been identified (57, 108, 177). The complex chains may be bisected by a GlcNAc residue at the core beta-linked Man. Hybrid-type carbohydrate structures contain a high mannose antenna on the alphal-6 core Man, and one or two complete or incomplete complex-type chains on the alphal-3 core Man. The hybrid

FIGURE 1. Assembly and Processing of N-linked Oligosaccharides.

The activated oligosaccharide complex (A) is transferred to (B) the Asn residue of the nascent polypeptide. The three terminal glucose residues (G) are removed by alpha-glucosidase I and II. The resulting structure (D) can be further processed to high mannose chains, or can be processed (F) by alpha3-6 mannosidase II to complex chains or by GlcNAc transferase III to hybrid chains.

G: glucose residues; M: mannose residues; Gn: N-acetylglucosamine residues; P-P-Dol represents the dolichol phosphate lipid carrier; R: the oligosaccharide core (C)

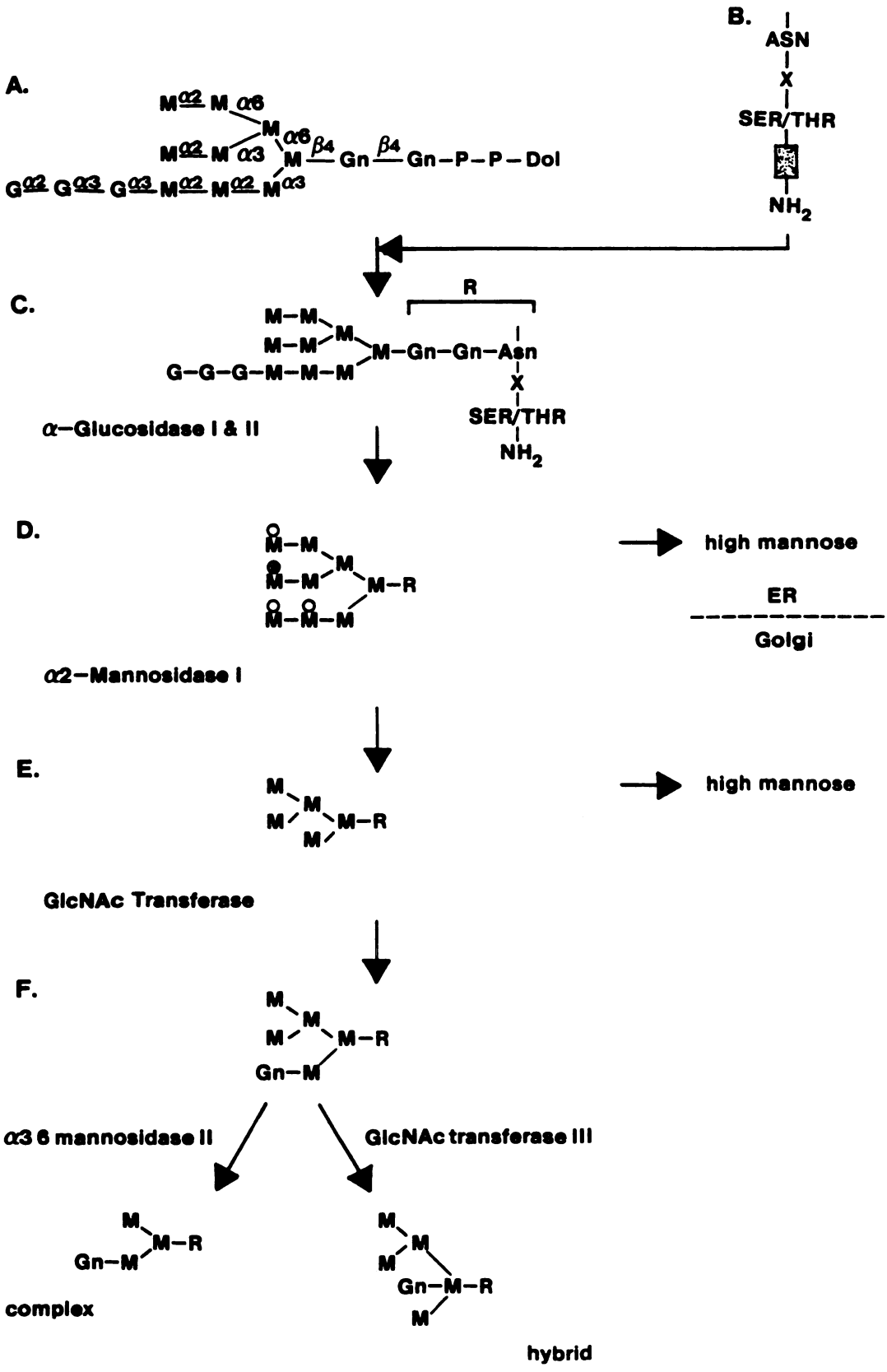
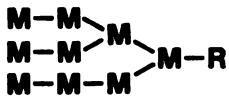

A.
B.
ASN
X
SER/THR
NH2
C.
R
M-Gn-Gn-Asn
G-G-G-M-M-M
α-Glucosidase I & II
D.
high mannose
ER
Golgi
α2-Mannosidase I
E.
high mannose
GlcNAc Transferase
F.
Gn-M
α3 6 mannosidase II
GlcNAc transferase III
complex
hybrid

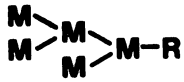
FIGURE 2. Examples of N-linked Carbohydrate Structures Found on Lysosomal Enzymes.

G: glucose residues; Gn: N-acetlyglucosamine residues; M: mannose residues; NANA: sialic acid residues; R: core oligosaccharide/protein.

High Mannose

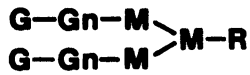


(M_9)

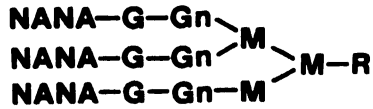


(M_5)

Complex

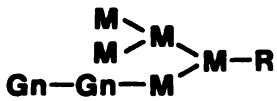


biantennary

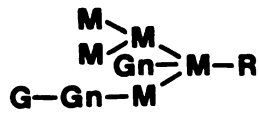


trisialylated
triantennary

Hybrid



non-bisected
biantennary



bisected
biantennary

chains may be bisected or non-bisected and may contain sialic acid (NANA) (71) and phosphate (P) (200).

Processing or "trimming" of the oligosaccharide begins immediately after its en bloc transfer to the nascent polypeptide chain, sometimes even before the polypeptide is completely synthesized (5). The terminal glucose residue is quickly removed by alpha-glucosidase I, accompanied by the slower removal of the following two glucose units by alpha-glucosidase II, leaving the $Man_9(GlcNAc)_2$ structure (80, 132). Final trimming in the ER involves the removal of a mannose residue by alpha-mannosidase I to yield $Man_8(GlcNAc)_2$ (185). The removal of the terminal alphal-2 Man from the middle branch (*, Figure 1) seems to enhance the possibility of processing to the complex and hybrid structures, rather than the high mannose structure. Further oligosaccharide processing is associated with transfer from the ER to the cis-face of the Golgi apparatus and subsequent passage through the Golgi stacks.

Transport from the ER to the Golgi complex is mediated by the formation of vesicles from the transitional elements of the ER. Inhibition of the ER alpha-glucosidases by 1-deoxyniojirimycin (159) causes inhibition of the transport of fibroblast lysosomal enzymes from the ER (110). Although some mature, properly processed lysosomal enzymes may eventually make it to the lysosome, the majority are not

transported to the Golgi and subsequently, are not processed. This may be due to the inability of the unprocessed ER precursors to interact efficiently with a transport receptor in the ER elements as has been proposed for secretory enzymes (115, 116). In addition, processing of any precursor enzyme which may move to the Golgi by another mechanism would be limited due to the rigorous substrate specificity of the Golgi-associated glycosyl transferases.

The Golgi complex consists of three ill-defined regions, the cis-face, mid-region and the trans-face (7, 152). These regions are associated with progressive changes in membrane components (55, 151) and different carbohydrate modifications made by specific glycosyl transferases (206).

Proteins move through the Golgi stacks in a unidirectional or vectoral flow. As seen by the carbohydrate structures identified on lysosomal enzymes, the enzyme precursors can move through the entire Golgi complex.However, it has not been determined if complete passage through the entire Golgi complex is obligatory for all lysosomal enzymes. Small secretory vesicles found throughout the Golgi stacks may be intermediate transport vesicles for some lysosomal enzymes and may thus be involved in an early sorting mechanism (20, 55).

Many glycoproteins entering the cis-Golgi from the ER undergo trimming by alpha2-mannosidase I (190) to $Man_5(GlcNAc_2)$, the common starting point for all complex and hybrid chains. This structure may be left as a high mannose chain, or a GlcNAc residue may be added, most likely in the mid-cisternae, by N-acetylglucosaminyl transferase I (GlcNAc transferase I) (69, 137).

The $GlcNAcMan_5(GlcNAc)_2$ product is a branching point for the formation of complex or hybrid structures. Golgi mannosidase II (GlcNAc-transferase I dependent alpha3-6 mannosidase) removes the two terminal mannose residues (105) leading to the formation of complex chains. The action of at least four glycosyl transferases, localized throughout the mid- and trans- cisternae, results in the synthesis of non-bisected and bisected structures with as many as four antennae. Galactose (gal), fucose (fuc), sialic acid (NANA), and other sugars are also added by specific transferases in the trans-cisternae (100).

If alpha-mannosidase II does not remove the two terminal mannose residues, hybrid structures result. Again, a number of transferases may act on the $GlcNAcMan_5(GlcNAc)_2$ to add GlcNAc, Gal, NANA, and phosphate (71, 200).

The exact mechanisms involved in the regulation of specific glycosylation of glycoproteins is unknown.The carbohydrate

moieties themselves play an essential role in sorting and transport mechanisms as ligands, receptors and recognition signals (206). In addition, the carbohydrate chains are specific substrates for sequential glycosyl transferases (161).

The primary protein sequences also influence the type of oligosaccharides added. Proper glycosylation may be accomplished when a nascent polypeptide assumes a conformation that allows a transferase access to some Asn-X-Ser/Thr sites, but not others (50). The folding of the polypeptide and the three dimensional structure of added oligosaccharides can influence the further addition of oligosaccharides by limiting the access of carbohydrate chains to further modification by transferases. Proposals of this sort have been made to explain the processing of RNAse B (219) and immunoglobulins (160). Along this line, the inhibition of alpha-mannosidase II by the rapid addition of a galactose or other residues to GlcNAc-$Man_5GlcNac_2$-Asn has been described (191).

Cell type also plays an important role in the determination of carbohydrate composition and structure. Since glycosyl-transferases are usually substrate specific, the presence or absence, relative activity and relative abundance of specific transferases-- which varies with cell type in many cases (79, 171)-- all play a role in oligosaccharide

processing.

With this array of factors influencing the carbohydrate structures present on an enzyme, the carbohydrate itself seems to play a foremost role in the transport to various subcellular locations, sequential processing and, therefore, proper activity.

The general movement of lysosomal enzymes from the Golgi to mature lysosomes can be summarized, regardless of specific recognition factors (Figure 3). Precursor enzymes bud from the Golgi apparatus in small, secretory-like vesicles. The pathway may diverge, some enzymes remain in the cytosol while some receptor bound enzymes are brought to the plasma membrane. Those receptor-bound enzymes transported to the plasma membrane may be expelled from the cell with the other contents of the pre-lysosomal or secretory vesicle. This enzyme pool may be recaptured by the same or neighboring cells and are internalized into coated vesicles. From there they move to the endosomes and pre-lysosomal vesicles. Again, a pH drop causes the dissociation of the receptor and ligand, followed by progression to mature lysosomes.

Some receptor-bound enzymes brought to the plasma membrane may not be expelled, but are cycled to and fuse with the coated vesicles or endosomes, then are transported with exogeneous enzymes to the lysosomes.

FIGURE 3. Transport of Lysosomal Enzymes

The enzyme is transported from the endoplasmic reticulum (ER) to the cis-Golgi where further processing of the carbohydrate (▲) takes place. Some enzymes bind to a receptor (ie., M6P-R;) and bud from the Golgi apparatus (G) in secretory vessicles, perhaps as soon as the late cis- or mid-regions. Other enzymes pass completely through the Golgi apparatus and are transported in secretory vesicles to the plasma membrane. The secretory vesicles may remain in the cytosol and fuse with coated vesicles (CV) which fuse with the endosomes or pre-lysosomal vesicles, then with CURL (compartment for uncoupling of receptor and ligand) elements.

Some enzyme brought to the plasma membrane in the Golgi-derived secretory vesicles may be expelled from the cell. This enzyme may be recaptured by the same or another cell by a specific receptor (▬) which moves to the coated vesicles becomes internalized and is transported to the CURL elements. Other Golgi-derived secretory vesicles may fuse directly with coated vesicles or endosomes, them move to the CURL.

In the CURL compartments, receptors and ligands dissociate, and the enzymes are processed in the early lysosomes and fuse with mature lysosomes (L).

Those enzymes in secretory vesicles remaining in the cytosol move to vesicles similar to endosomes or receptosomes which associate and develop into a membraneous system often referred to as CURL (compartment for uncoupling receptor and ligand). Here, or in similar vesicles, there is a drop in pH which causes the dissociation of the pro-lysosomal enzyme from the receptor. The contents of the vesicle (often referred to as early lysosomes or light lysosomes) fuse with other subcellular vesicles, and the enzymes are delivered to the late, mature, or dense lysosomes.

The mannose-6 phosphate system is a well characterized receptor/transport mechanism for lysosomal enzymes. This system functions primarily, or most efficiently, in fibroblasts, but has also been described in bovine liver, human hepatocytes and Chinese hamster ovary cells (153, 178).

Briefly, a GlcNAc-phosphotransferase in the Golgi complex (209) transfers a GlcNAc-phosphate from UDP-GlcNAc to carbon six of one or two of any five residues on the high mannose chains (88) after the three terminal glucose residues have been removed (Figure 1). The covering GlcNAc residue is subsequently removed by N-acetyl-glucosamine 1-phospho-diester alpha-N-acetylglucosaminidase.

The GlcNAc-phosphotransferase is highly specific for lysosomal enzymes (197, 199, 207), recognizung a unique structural element common to all lysosomal enzymes which is not found in other glycoproteins. This entity, yet to be identified, is denaturable (144), and separate from the acceptor oligosaccharide (105). Non-lysosomal proteins containing high mannose structures are poor substrates for the GlcNAc-phosphotransferase (144, 208) as are heat denatured and deglycosylated enzymes (105, 144).

A receptor protein, the mannose 6-phosphate receptor, M6P-R, binds to the phosphorylated mannose residue(s) and directs the enzyme to the lysosome. The receptor functions intracellularly (44), moving the precursors from the Golgi (19) to pre-lysosomal vesicles (25, 137) to the mature lysosome, as well as extracellularly (138), binding to extracellular enzyme, endocytosing the enzyme (156) and delivering the enzymes to the lysosomes.

Intracellularly, the M6P receptor binds to at least one uncovered M6P residue probably in the mid-Golgi region, although the M6P receptor has been localized throughout the Golgi (19, 56) of different cell types. In some cases, with the addition of galactose and sialic acids by terminal glycosyltransferases in the trans-Golgi, binding is likely to occur in the trans-cisternae after processing is complete.

Segregation of the lysosomal enzymes from other proteins takes place from anywhere in the Golgi complex to the plasma membrane. Co-segregation of newly synthesized, non-lysosomal and lysosomal enzymes has been demonstrated at the coated vesicle buds of the trans-Golgi reticulum and plasma membrane (56, 202).Factors other than M6P binding must play a role in the segregation of glycoproteins as seen by the varying processing times and routes for different lysosomes with hybrid (M6P and complex antenna) chains (126).

The M6P-R is recycled and participates in a number of transport cycles (62, 149). It is not found in the mature lysosomes as acidification of the CURL-associated pre-lysosomal vesicles causes dissociation of the receptor-ligand complex before reaching the mature lysosomes. The M6P receptors are then recycled via membrane mechanisms which are unknown.

Tietz et al. confirmed two functionally distinct intracellular pools of M6P receptors on the basis of the effect of weak bases on the recycling of the M6P receptor and the release of the ligand (189). Although the receptor is found on the cell surface, the majority of the receptor is intracellular and functions in intracellular transport.

The two M6P receptors have been well characterized (51, 52, 189). The more extensively studied receptor described here is a membrane bound glycoprotein of 215,000 M_r. The majority of this receptor is found intracellularly, although some does cycle to the plasma membrane where it binds extracellular enzyme. This receptor is independent of divalent cations, unlike the second M6P receptor.

The second M6P receptor has been described in cells such as macrophages, hepatocytes, and others where the cation-independent receptor is in relatively low abundance (51). The general transport pathway for both M6P receptors is similar, although a second recognition signal in the lysosomal enzyme may play a role in receptor binding.

Other transport-receptor recognition systems must exist along with the M6P system and in other cell types where the M6P receptor is at lower levels than in fibroblasts. GlcNAc-phosphotransferase is absent in I-cell disease fibroblasts (Mucolipidosis II, ML II) (74) and low in pseudo-Hurler dystrophy (Mucolipidosis III, ML III) (145). As a result, mannose residues are not phosphorylated and are not recognized by the M6P receptor. In these two storage diseases, an unusual number of lysosomal enzymes from cultured fibroblasts are secreted into the culture medium. However, there are normal levels of active acid phosphatase

and beta-glucosidase (74) and alpha-glucosidase and cathepsin D are 20% - 50% of normal levels, allowing normal

functioning of the cell with respect to these enzymes (75, 76). There are also normal levels of the membrane bound enzymes beta-glucocerebrosidase and acetyl CoA:alpha-glucosaminidase N-acetyltransferase (72). Yet, there is a complete lack of other lysosomal enzymes in the endosomes and early and mature lysosomes which results in the symptoms associated with these disorders. As previously mentioned, other cell types from I-cell patients such as hepatocytes, bone marrow cells and neurons from I-cell patients have normal levels of mature, properly processed lysosomal enzymes (138).

Other receptor mechanisms may play an important role in the transport of lysosomal enzymes in these cell types. A galactosyl receptor on hepatocytes recognizing galactosyl-terminal asialoglycoproteins has been described (4, 89, 195), purified and characterized (82, 88). This galactose recognition receptor is a glycoprotein specifically localized in hepatocytes, along with receptors for fucose, N-acetylglucosamine (GlcNAc), mannose and M6P.

Anchord et al. (1) described the uptake of human beta-glucuronidase by liver cells, confirming existence of a mannose-N-acetylglucosamine clearance system localized in reticuloendothelial system organs such as the liver, spleen, and lungs, and, possibly, that the major organ involved in such clearance is the liver. The "low uptake" form of beta-

glucuronidase, so called because it lacks the phospho-mannosyl recognition marker (M6P residue) and thus is not efficiently captured by fibroblasts, was cleared from the plasma preferentially by non-parenchymal cells lining the liver sinuses, and could be taken up by alveolar macrophages.

Final proteolytic processing of several lysosomal enzymes takes place after transport to the lysosome. Several investigators have described the cleavage of a single polypeptide into subunits which are then assembled to form active, mature enzyme. Oude Elferink et al. have shown that proteolytic processing of an inactive, precursor form of alpha-glucosidase to the mature, active form took place during final transport to the mature lysosome (146). In addition, C-terminal processing of several enzymes has been demonstrated including cathepsin D and beta-glucuronidase (42, 91). This may be a common biosynthetic step for many lysosomal enzymes, coupled to the final transport from the pre-lysosomal vesicles to mature lysosomes.

In many lysosomal disorders there is an array of defects causing the overall deficient activity (for reviews, see 146, 204). These may include disturbed or defective processing, decreased stability of the enzyme, or the absence of an "activator" or "protector" protein. Investigators have pursued the discovery of the common

denominators which function in the proper synthesis, transport, processing and activity of lysosomal enzymes in hopes of understanding the specific defects and, therefore, the most effective means of overcoming the deficiency. Understanding the biosynthesis of lysosomal enzymes may lead to a greater understanding of the proper function and the vast array of defects seen in lysosomal enzyme deficiencies overall. Purification of the enzymes, in vivo studies in multiple cell types, molecular studies involving isolation of mRNA and subsequent in vitro translation as well as the isolation of cDNA clones have all contributed significantly to future solutions.

ALPHA-GALACTOSIDASE A AND ALPHA-N-ACETYLGALACTOSAMINIDASE: FUNCTION, BIOLOGICAL SIGNIFICANCE, BIOSYNTHESIS AND PURIFICATION

Fabry's disease, a fatal, X-linked, recessive lysosomal storage disease, is due to a deficiency of alpha--galactosidase A (alpha-gal A). Glycosphingolipids with a terminal alpha-linked galactose accumulate in a variety of tissues in Fabry males and are responsible for a variety of ailments which include skin lesions, renal impairment, cardiovascular disease, corneal opacities, and lesions in the gastrointestinal tract. Sweeley and Klionsky (183) identified the major accumulating lipid as ceramide trihexoside, $GbOse_3Cer$ and digalactosylceramide as a minor component. Wherrett and Hakomori (218) found blood group glycolipids accumulating in Fabry patients with type B blood. Several other glycoconjugates are likely substrates of this enzyme (Figure 4) and the disruption of a degradative pathway may cause the accumulation of secondary products.

Death is the result of the slow accumulation of the lipids in the lysosomes of the cells. As previously mentioned, therapeutic methods are limited to relief of pain. Heterozygous carrier females with 50% of normal enzyme

FIGURE 4. Typical Glycoconjugates Containing Terminal alpha-Galactoside Units.

(1) Digalactosyl Ceramide

(2) Ceramide Trihexoside

(3) Blood Group B II glycolipid

(4) Pancreatic Glycolipids from blood type B Fabry patient

(5) P_1 Glycolipid

(1) Gal(α1–4)Gal(β1–1′)Cer

(2) Gal(α1–4)Gal(β1–4)Glc(β1–1′)Cer

(3) Gal(α1–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–1′)Cer
|
Fuc(α1–2)

(4) Gal(α1–3)Gal(β1–3)/GlcNAc(β1–3)Gal(β1–4)Glc(β1–1′)Cer
|
Fuc(α1–2)

(5) Gal(α1–4)Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc(β1–1′)Cer

activity are affected to a smaller extent: lipid accumulation is seen, but does not interfere with normal organ function.

Brady et al. (16) correlated the lipid accumulation with the deficiency of an enzyme they called ceramide trihexosidase. Kint (94) determined that the enzyme was an alpha-galactosidase using synthetic substrates. Later, Beutler and Kuhl (10) and Kint et al. (95) found that there were two different alpha-galactosidase activities in human tissues, the A and B forms. The A activity is deficient in Fabry's disease while the B activity is normal or, in some cases, elevated.

Several notable attempts to separate and purify the alpha-galactosidase activities have been made and have met with varying degrees of success. Bishop and Desnick (33) made the greatest improvements in the purification of alpha-gal A in terms of yield, purity and number of steps. Their success was primarily due to the use of an alpha-galactose affinity ligand previously used by Harpez and Flowers (219) for the purification of coffee bean alpha-galactosidase.

Kusiak et al. (103) partially purified alpha-gal B from human placenta. In their preparation, contained several protein bands visualized by SDS-PAGE, the major component, which they identified as alpha-gal B, had a subunit

M_r=57,700, higher than the subsequent reports of M_r=45,000-49,000. Dean and Sweeley (30) were able to separate A activity from B activity in human liver and were able to determine some kinetic parameters and substrate specificities for the enzymes. Although there was some alpha-galactosidase A activity in their preparation of B, their studies showed that alpha-gal B was actually an alpha-N-acetylgalactosaminidase with activity toward some important substrate classes such as globopentaglycosylceramide (Forssman antigen; $GbOse_5Cer$), a minor component in human intestine and an important antigen in some human tumors (87) as well as blood group A-active glycolipids and glycoproteins containing terminal alpha-N-acetylgalactosamine residues (30, Figure 5).

Wilkinson (219) was able to purify alpha-gal A completely with alpha-gal B as a by-product using an alpha-galactose affinity ligand. N-terminal sequencing of the A and B enzymes obtained from this procedure revealed significant homology in the first nineteen amino acids (219, Figure 6). Further sequencing of alpha-gal B tryptic peptides resulted in the identification of approximately 18% of the total protein sequence (Figure 7).

The carbohydrate moieties of lysosomal enzymes are significant in both the biosynthetic pathway and in biological function. Desnick *et al.* injected human

FIGURE 5. Typical Glycoconjugates Containing Terminal alpha-N-Acetylgalactosaminyl Units.

(1) Globopentaglycosylceramide (Forssman antigen)

(2- 4) Typical Blood group A active glycolipids

(1) GalNAc(α-3)Gal(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1')Cer

(2) GalNAc(α1-3)Gal(β1-4)GlcNAc(β1-3)$(Gal)_n$(β1-4)Glc(β1-1')Cer
Fuc (α1-2)

(3) GalNAc(α1-3)Gal(β1-4)GalNAc(β1-3)Gal(β1-4)GalNAc(β1-3)$(Gal)_n$(β1-4)Glc(β1-1')Cer
Fuc (α1-2)

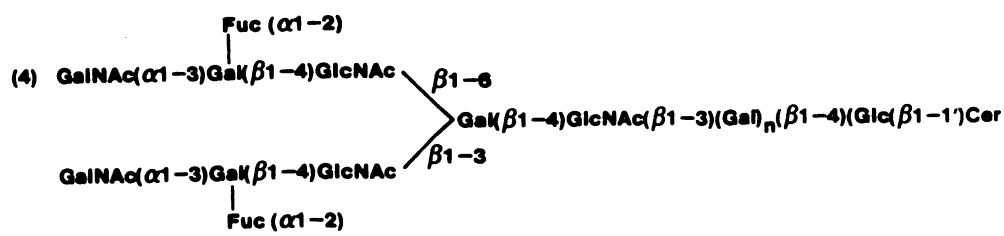
(4)


FIGURE 6. N-terminal Sequences of alpha-Galactosidase A and alpha-N-Acetylgalactosaminidase from Human Liver.

X^a represents an undetermined amino acid residue.

α-Gal A: Leu-Asp-Asn-Gly-Leu-Ala-Arg- X^8-Pro-Thr/Tyr-Met-Gly- X^8- X^8-Leu

α-Gal B: Leu-Asp-Asn-Gly-Leu-Leu-Gln-Thr-Pro- Pro -Met-Gly-Trp-Leu-Ala-Trp-Glu-Arg-Phe

FIGURE 7. Amino Acid Sequences of alpha-N-Acetylgalactosaminidase Tryptic Peptides.

(1) Ser-Ala-Asp-Gln-Val

(2) Leu-Leu-Ile

(3) Val-Gln-Tyr

(4) Ala-Gln-Met-Ala

(5) Thr-Asp-Met-Pro-Tyr-Arg

(6) Met-Ala-Gln-Asp-Gly

(7) Asp-Met-Gly-Tyr-Thr-Tyr-Leu-Asn/Gly

(8) Val-Val-Gln-Asp-Ala-Gln/Glu-Thr-Phe-Ala-Gln/Glu-X^a-Lys

splenic and plasma alpha-gal A separately into two brothers affected with Fabry's disease (34). Although the splenic form had a higher specific activity than the more highly sialylated plasma form, the plasma form persisted longer in circulation and cleared more of the circulating $GbOse_3Cer$ than the splenic form. The disappearance of circulating alpha-gal A is consistent with the clearance of desialylated glycoproteins from the serum by the Ashwell receptor (82, 141, 195).

LeDonne (108) and Sweeley (184) studied alpha-gal A in Chang liver cells and alpha-gal B in FS4 human foreskin fibroblasts. The largest glycosylated subunit precursor of alpha-gal A identified was M_r=58,000, and was subsequently processed to M_r=49,000. In the fibroblasts, a glycosylated alpha-gal B precursor of M_r=65,000 was processed to M_r=49,000.

One-half of the carbohydrate chains of alpha-gal A were processed to complex tri- or tetraantennary structures while the remainder were high mannose chains with eight or nine mannoses. In contrast, alpha-gal B had only high mannose chains with eight or nine mannose residues in the fibroblast cell line. A phosphorylated form of alpha-gal B was found in the fibroblasts and was exported to the medium. While there was no phosphorylation of alpha-gal A seen in Chang liver cells, others have reported that 50% of the enzyme

purified from human placenta had one or more phosphorylated chains (35).

STATEMENT OF THE PROBLEM

A number of serious metabolic disorders are due to the deficiency of one or more lysosomal enzymes. Studies of enzymes in this group, such as cathepsin D, beta--glucuronidase, alpha-galactosidase A and alpha-N-acetyl-galactosaminidase have rendered important information about their proper biological function and role in metabolic disorders.

However, our understanding of these enzymes is incomplete. While progress has been made in defining lysosomal storage disorders, little is actually known about the specific lesions in many cases. In fact, few of the enzymes involved have been defined as far as primary sequence, molecular mechanisms of catalysis, interaction of carbohydrate chains and biosynthesis and processing in different cell or tissue types.

This project was undertaken to expand our knowledge of the lysosomal hydrolases alpha-galactosidase A and alpha N-acetylgalactosaminidase. The ultimate goal was to isolate a cDNA clone homologous to the gene for alpha-N-acetyl-galactosaminidase (commonly referred to as alpha-gal B).

Specific objectives toward this end included:

1.) To improve the purification scheme for alpha-gal B in order to improve the yield while simplifying the overall procedure and reducing the handling time, thus eliminating the proteolysis and loss of activity seen with other methods.

2.) To generate monospecific, polyclonal antibodies suitable for both biosynthetic cellular studies as well as for screening expression libraries.

3.) To determine properties of non-glycosylated enzyme in several cultured human cell lines.

4.) To isolate mRNA or cDNA clones of alpha-gal B by one of several possible methods including polysome immuno-precipitation, screening a cDNA library with synthetic oligonucleotides, and immunological screening of a cDNA expression library.

These studies have resulted in a better understanding of the structure and biosynthesis of alpha-gal B and have provided at least the ground work in the isolation of the gene for alpha-gal B. This should give rise to other studies involving the expression and synthesis of alpha-gal B and similar enzymes.

STUDIES OF ALPHA-N-ACETYLGALACTOSAMINIDASE (ALPHA-GALACTOSIDASE B)

Substrates of alpha-N-acetylgalactosaminidase (alpha-galactosidase B, alpha-gal B, E.C. 3.2.1.49) are a widely occurring, structurally diverse group of glycoproteins and glycolipids. Although this suggests that alpha-gal B has an important physiological function, there is no known disorder of glycoconjugate metabolism involving this enzyme.

Alpha-gal B catabolizes terminal, alpha-linked N-acetylgalactose (GalNAc) residues. This activity has been demonstrated in a variety of organisms including bacteria (70, 125, 215), mollusks (47, 192, 193), the earthworm (21, 163), and a number of mammals including sheep and man (217). Preparations from rat, porcine, bovine, and human livers have been obtained (24, 216, 217). Dean (29, 30) and Wilkinson (219) purified both A and B enzymes which were suitable for substrate, kinetic and protein studies.

Intense interest in alpha-gal B has arisen in conjunction with studies on alpha-galactosidase A, the enzyme deficient in Fabry's disease. Comparisons of the protein sequence, oligosaccharides, kinetics, substrate specificity and biosynthesis of the two enzymes are greatly desirable. Several investigators have postulated that the two enzymes may have a product-precursor relationship (166), or are

interconvertible (164). While these theories now seem unlikely, the N-terminal homology and similarities toward some substrates are intriguing.

Several problems still plague investigators. Despite improvements, significant amounts of activity are lost during purification and cannot be recovered. Enzyme integrity and activity are also lost due to proteolysis during prolonged purification schemes. These drawbacks make it difficult to study the human enzymes since there is approximately 1 mg or less of each enzyme per kilogram of human liver, and most purification schemes have yields between 20-30% (219).

In this report, a more rapid, and higher yielding procedure for the isolation of pure alpha-galactosidase B is described. Several experiments characterizing the physical properties of the enzyme are also presented, along with information from several cellular studies.

I. THE ISOLATION OF ALPHA-N-ACETYLGALACTOSAMINIDASE

MATERIALS

Reagents and artificial substrates for the enzyme assays were from Sigma Chemical Co. Concanavalin A Sepharose was from Sigma. Sephadex G-150 was from Pharmacia Fine Chemicals. N-glycanase was from Genzyme, Inc. Nitrocellulose was from Schleicher and Schuell. The affinity ligand, N-6-Aminohexanoyl-alpha-D-galactosyl-pyranosylamine-sepharose 4B was synthesized by F. E. Wilkinson. All other reagents were from general chemical suppliers.

METHODS

ENZYME ASSAYS WITH ARTIFICIAL SUBSTRATES

Assays with 4-Methylumbelliferyl-alpha Galactoside. Total alpha-galactosidase activity (A + B) was measured by a modification of the method of Desnick et al. (124) with 4.3 mM 4MU-alpha-Gal in NaCit, pH 4.6. An aliquot of the enzyme source to be measured (25-50 ul) was placed in a 10 x 75 mm glass test tube, adjusted to 50 ul with 0.1 M citrate, pH 4.6, and 150 ul of a stock solution of 5.0 mM 4-MU-alpha-Gal

in 0.1 M citrate buffer, pH 4.6 was added to the tube. The reaction mixture was incubated at 37°C for 1-120 min. The reaction was terminated with 2.35 ml of 0.1 M ethylene-diamine, pH 11.0.

Alpha-gal A activity was measured by incubating the enzyme solution with 5.0 mM 4MU-alpha-gal/150 mM GalNAc in citrate buffer.Alpha-gal B activity was the difference between the total alpha-gal activity and alpha-gal A only activity since alpha-gal B is completely inhibited by 50 mM GalNAC (219).

Fluorescence of the 4-MU assay was read in an Aminco Fluoro-Colorimeter previously calibrated with standard solutions of 4-methylumbelliferone prepared in water and diluted to a final volume of 2.55 ml with 0.1 M ethylenediamine immediately before use.The 4-MU assay was linear from 0 to 500 nmol 4-methylumbelliferone, and most reliable when run from 5-15 min at 37°C.

Alpha-Gal B Activity Measured with p-Nitrophenol-N-acetyl-galactosaminide. This assay is a modification of the method of Sung and Sweeley (181). An aliquot of the enzyme solution to be assayed (up to 50 ul) was diluted to 100 ul with 0.1 M citrate buffer, pH 4.3, and 100 ul of 10 mM p-NP-GalNAc in 0.1 M citrate buffer, pH 4.3 was added. The reaction mixture was incubated at 37°C for 30 min. and terminated by the addition of 3.0 ml of saturated sodium

borate solution, pH 9.7. The optical density of the resulting yellow solution was read at 410 nm with a standard curve constructed with p-Nitrophenol (p-NP) dissolved in water (molar extinction coefficient at 410 nm is 1.77 x 10^4 l/mol x cm) from 0 to 200 nmol. Blanks were prepared by omitting the enzyme solution from the reaction mixture until after the addition of the saturated borate solution. Activity is expressed as nmoles of p-NP-GalNAc hydrolyzed/min x ml of enzyme solution. The assay is linear up to 30 min with up to 200 nmol substrate hydrolyzed for 30 min.

Protein assays Two protein assays were used which compare favorably with the Lowry method values using bovine serum albumin as a standard. The Markwell method (121) was used for samples of 40-100 ug. The Peterson assay (140) was used for samples of 10-50 ug.

PURIFICATION: All steps were performed at 4°C unless noted.

Homogenization of tissue. Frozen human cadaver liver (approximately 500g) was cut into small pieces while still frozen, and homogenized in two volumes of 0.025 M sodium cacodylate buffer, pH 6.5 (NaCac) until the homogenate (approx. 33% liver) was smooth. The homogenate was strained through three layers of cheese cloth to remove connective tissue, then centrifuged in a Sorvall GS-4 rotor at 16,000 x

g for 20 minutes. The supernatant was stored at 4°C. The pellet was rehomogenized in one volume of NaCac, centrifuged, and the supernatant was added to the first supernatant. Approximately one volume of buffer was added to the pellet, then the suspension was vigorously vortexed and an aliquot (25ul) of the pellet suspension and the collective supernatant was tested for 4-MU-alpha-gal activity with and without GalNac added.

After aspirating floating lipid material from the top of the tube, the supernatant (approx 1l) was centrifuged for 30 minutes at 100,000 x g in a Beckman Ti70 rotor. Solid material was again aspirated from the surface before collecting the supernatant.

Concanavalin A - Sepharose 4B Chromatography. Conconavalin A-Sepharose 4B chromatography beads (100 ml settled volume) were suspended in 0.05 M sodium citrate (NaCit), pH 6.0 and allowed to settle. The buffer was exchanged in this manner four to five times, then a loose cake was formed by gently suctioning the buffer from the beads.

The 100,000 x g supernatant (approximately 1l) was combined with the beads and gently shaken on an orbital shaker (Lab-line orbit, 50-100 rpm) at room temperature for 1-2 hr. Occasionally, the beads were completely resuspended in the solution by swirling by hand.

The suspension was then filtered through a medium glass-fritted funnel. The filtrate was passed over the beads two more times, then gentle suction was applied to remove the liquid. The Con A bead cake was resuspended in two volumes of NaCit buffer with 1M NaCl, swirled briefly, and again filtered. The washing procedure was repeated until the A_{280} of the filtrate was zero. Again, gentle suction was applied to remove liquid.

Bound substances were eluted with alpha-methlymannoside (methyl-alpha-D-mannopyranoside). The cake was resuspended in two volumes of elution buffer (0.05M NaCit, pH 6.5/1M NaCl/0.4M methyl-alpha-D-mannopyranoside) and shaken gently as above for 15 minutes at room temperature. The suspension was filtered through the glass-fritted funnel and the filtrate was collected in a cooled vessel. The elution was repeated at least three more times until an assay of the beads with 4-MU-alpha-gal resulted in less than 10% of the total enzyme activity originally applied remained on the beads. (Usually, 5-10% of the original activity could not be removed from the beads unless the pH was lowered to 3.5, or raised to 8.0, resulting in the denaturation of the desired enzymes and co-elution of undesirable proteins.)

The alpha-methlymannoside eluted enzyme was concentrated in an Amicon Ultrafiltration Manifold with a PM10 membrane to

200-300 ml, finally exchanging the buffer with approximately five volumes 0.025 M NaCac, pH 6.5, until the ionic strength of the enzyme solution was equal to 0.025 M NaCac, pH 6.5. The enzyme solution was then centrifuged in a Beckman Ti70 rotor at 100,000 x g for 1 hr.

Affinity Chromatography on N-6-Aminohexanoyl-alpha-galacto-pyranosylamine Sepharose. The affinity ligand was synthesized by F.E. Wilkinson of the laboratory and is described in ref. 219.

The alpha-gal A and B activity from the Con A fraction was concentrated to 250 ml (4-MU-alpha-gal activity = 0.11 umols/min x ml), acidified with 1% citric acid to pH 4.8, then added to a 15 ml bed volume of affinity ligand beads equilibrated with NaCit, pH 5.0 in a 500 ml flask. The bead/protein solution was allowed to incubate at $4^{o}C$ for 2hr with occasional gentle swirling. The matrix was then loaded into a 1.1 x 18.5 cm column and allowed to settle with elution of the solution. The eluate was collected, assayed for activity and concentrated. The column was then washed with NaCit, pH 4.8/0.1M NaCl/0.05M N-acetyl-alpha-D-glucosamine (GlcNAc) until the A_{280} was essentially zero. The column was then washed with 0.05M NaCit, pH 6.5/0.1M NaCl/0.75M N-acetyl-alpha-D-galactosamine (GalNAc). The eluate was monitored with A_{280} and 4-MU +/- GalNAc. One ml fractions containing alpha-gal B activity were collected

until the activity was negligible and the A_{280} was nearly zero, then pooled and concentrated in an Amicon concentrator with a PM10 membrane to approximately 1U/ml exchanging the buffer with approximately 10 volumes of 0.025 M NaCac, pH 6.5.

The column was then washed with 10 volumes of 0.05M NaCit, pH 5.0/0.1M NaCl. Alpha-gal A activity was eluted with 0.05M NaCit, pH 5.0/0.1M NaCl/0.8M galactose (Gal). Fractions (1ml) were monitored for A_{280} and 4-MU activity, pooled, and concentrated as above to approximately 4U/ml, again exchangeing the buffer with 0.025 M NaCac, pH 6.5.

The alpha-gal B and A fractions were re-chromatographed on the affinity column after the column was washed with 10 volumes each of 0.1M Tris-Cl, pH 8.0/1M NaCl, then 0.05M NaCit, pH 3.5, and re-equilibrated with 0.05M NaCit, pH 4.8. Each fraction was separately applied and eluted exactly as described above, separating and pooling all activity initially eluting from the column and subsequently eluting with the specific inhibitor (GalNAc- or Gal-containing buffer). If the initial flow-through protein contained significant activity, it was acidified to pH 4.8 and rechromatographed exactly as the crude Con A fraction was processed.

Three pools containing 4-MU-alpha-gal activity were finally made: the non-binding or weakly-bound alpha-gal A fraction (collected when the A_{280} of the initial wash was less than 0.05), the activity eluted with GalNac and completely inhibited by GalNAc (alpha-gal B), and the activity eluted with Gal and not inhibited by GalNAc (alpha-gal A).

Gel-filtration on Sephadex G-150. Standard proteins, phosphorylase B (MW=96,000), bovine serum albumin (MW=66,000), and cytochrome C (MW=12,000) (supplied suitable for gel filtration from Boehringer Mannheim) were run through the column first in order to characterize the separation and provide an estimation of the molecular weights. The exclusion volume was estimated with blue dextran and the inclusion volume was determined with phenol red. Elution was monitored by A_{280}.

Affinity purified enzyme (0.5-1.0 mg in 0.5 ml NaCac, pH 6.5/0.1M NaCl/0.001% SDS (w/v)) was applied to 1 cm x 120 cm Sephadex G-150 column, and eluted with a flow rate of 2.5 ml/hr (approximately 2.0 ml/cm^2/hr). Fractions of approximately 0.35 ml were collected and assayed by A_{280}, 4-MU +/- GalNAc, and PNP-GalNAc. Enzyme pools were concentrated to 1.0 U/ml in 0.025M NaCac, ph 6.5.

Ion-exchange chromatography. The enzyme solution was applied to a 250 ml Zeta-Prep cartridge (DEAE cellulose, LKB

Sciences) which was pre-equilibrated with 0.01M NaCac, pH 6.5 at a flow rate of 40 ml/min. After the enzyme solution was applied, the column was washed with 500ml of the loading buffer, and a 2.5 l linear gradient of 0-0.3M NaCl in 0.01 M NaCac was started. Fractions of 25ml were collected, monitored by A_{280} and assayed for activity with 4-MU +/- GalNAc. The column was finally washed with 500 ml 1M NaCl in NaCac, pH 6.5. Fractions 15 to 50 were collected, pooled and concentrated. A second pool of fractions 70 to 78 were collected separately.

Each pool was concentrated and buffer was exchanged as described until the ionic strength of the enzyme solutions were equal to 0.025 M NaCac, pH 6.5. Each pool was rechromatographed as above and fractions corresponding to activity peaks were collected, pooled and concentrated.

Digestion of Alpha-Gal B with N-Glycanase. N-linked carbohydrates were removed by digestion with N-glycanase (GenZyme). Approximately 50 ug of purified alpha-gal B was diluted in 20 ul 0.5% SDS/0.1M 2-mercaptoethanol and boiled for five minutes. The denatured enzyme was lyophilyzed, then resuspended in 30 ul 50 mM sodium phosphate, pH 8.6/5 mM EDTA, and 2 ul 10% NP-40 was added. N-glycanase was added (5 units) and the digestion mixture was incubated at $37^{o}C$ for 12 hr.

Digestion of Alpha-Gal B with Mercuric Cyanide/KOH/Mercuric oxide. The following procedure was developed and performed by Peter B. Weber, Ph.D., Department of Biochemistry, Albany Medical College, Albany, NY.

One-half milligram of alpha-gal B was lyophilyzed in a 1.5 ml Eppendorf tube, then mixed with 200 ul 20% (w/v) mercuric cyanide in 2 M KOH saturated with mercuric oxide and 3.0 mg mercuric oxide was added. The mixture was sonicated in a bath sonicator for 15 minutes, then shaken on a vibrator for 6 hr at 25°C. Potassium borohydride (3.5 mg) was added and allowed to react for 50 min., essentially stopping the reaction. Acetic acid (50 mg) was then added and the reaction tube was immediately centrifuged to collapse rising foam, remixed and frozen. The sample was later thawed and diluted with 1 ml deionized water and recentrifuged for five minuted at 10,000 rpm (Eppendorf microfuge). The supernatant and second aqueous wash were combined and lyophylized. The mercury containing sediment was extracted with SDS and anhydrous trifluoro acetic acid to extract any insoluble peptides. The supernatant from the extraction was added to the dried, soluble peptides.

Separation of Mercuric-peptides by HPLC. Peptide separation was performed on a U6K Waters modified-Beckman model 342 binary HPLC with a 4.6 x 250 mm Brownlee Aquapore RP-300 (C18, 300 An pore) column. The dried peptides were

dissolved in 200 ul 0.1% trifluoro acetic acid (TFA), and separated in a linear gradient of 0.1% TFA (A) in water versus 0.1% TFA in MeCN (B). The flow rate was 1 ml/min and the gradient was increased from 5% (A) to 50% (B) at 2%/min. The elution was monitored with a Beckman 165 detector at 215 nm/1.0 AVFS and 275 nm/ 0.1 AVFS and peaks were collected by hand after a 2 sec delay in acid washed 10 x 75 mm borosilicate tubes.

Each collected peak was rerun in a uBondapak Phenyl column (3.9 x 300 mm) in the 0.1% TFA gradient used in the primary eparation. The flow rate was 1 ml/min and the gradient was developed from 1% (A) to 50% (B) at 1%/min. Peaks were monitored and collected as before, dried and frozen until subjected to amino acid sequencing.

SDS-Polysacrylamide Gel Electrophoresis. SDS-Page was performed essentially by the Procedure of Laemmli (104). The stacking gel was normally 3% acrylamide (30:0.8::acrlyamide:bis). pH 6.8 and the separating gels were 10 % or 12%, pH 8.8.

Silver Staining. The silver staining procedure of Wray et al. was the stain of choice in the purification procedure because of its sensitivity (222). The gel was soaked for at least 8 hr with two changes of reagent grade 50% methanol before staining.

Western Transfer. Proteins separated by electrophoresis were transferred to nitrocellulose (0.45u Schleicher and Schuell) using the Bio-Rad Trans-Blot cell as directed by the manufacturer. The gel and nitrocellulose were pre-equilibrated in transfer buffer (25 mM Tris base/192 mM glycine/-20% reagent grade methanol, approx. pH 8.3) for at least 30 min, but no longer than 1 hr. The transfer was run at 30V for at least 6 hr, followed by 70V for 2 hr. Filters were used immediately.

"Dot-blotting". Proteins (and DNA) were applied to nitrocellulose through a multi-well vacuum manifold from Schleicher and Schuell according to the manufacturer's directions.

Native gel electrophoresis. Native polyacrlyamide gel electrophoresis was carried out as described by Gabriel (224), using a cooled 7% gel and tris-glycine buffer, pH 6.5.

Activity Staining Native Gels. The methods of Sung and Sweeley (181) and Krisman (102) was modified to detect 4-MU-alpha-gal activity (A or A+B activity), 4-MU-alpha-gal activity in the presence of GalNAc (A activity only), and p-NP-GalNAc activity (B activity only). Gels were equilibrated in 0.1M citrate buffer with 150 mM GalNAC (for

A activity) or without GalNAc (for B activity measured with 4-MU-alpha-gal or with p-NP-GalNAc) by shaking at room temperature 15 min. Gels were then incubated in the respective substrate solution. Gels stained for A activity were incubated in 0.1 mM 4-MU-alpha-gal/150 mM GalNAC/0.1 N citrate, pH 4.3 for 15 min. and active bands were visualized under UV light after terminating the reaction with 0.1 M ethylenediamine, pH 11.0. Gels stained for B activity were incubated with 0.1 mM 4-MU-alpha Gal or 0.17 mM p-NP-GalNAc in 0.1 mM citrate, pH 4.3 and the reactions were terminated with 0.1 M ethylenediamine or saturated sodium borate, pH 9.7 respectively. Gels stained for 4-MU activity were visualized with long and short wave UV light, and p-NP-GalNAc activity was visualized under fluorescent light or scanned with a Gilford 2400 spectrophotometer at 410 nm.

RESULTS

Isolation of Alpha-N-Acetylgalactosaminidase. This purification scheme was devised to maximize the yield of alpha-gal B rather than alpha-gal A. Our interest in obtaining cDNA clones of alpha-gal B and in continuing biosynthetic and processing studies demanded pure and intact enzyme be available for amino acid sequencing, use in the production of antibodies and for information leading to the synthesis of oligonucleotide probes.

The combination of conventional techniques and the application of the use of an affinity ligand described here resulted in a 30,820-fold purification of alpha-gal B and a 55% overall yield of pure enzyme. The final specific activity was 5.7 umol 4-methylumbelliferyl-alpha-galactoside hydrolyzed per min per mg protein which was completely inhibited in the presence of 50 mM GalNAc.

The results, averaged from four complete purifications are given in Table 1. Approximately 0.627 mg of enzyme was isolated from an initial 500g human liver, or 1.25 mg/kg.

The purification scheme did not maximize the yield or purity of alpha-gal A as Wilkinson's procedure did (219). Rather, a 29% yield resulted in the purification of 0.4 mg A/kg liver with a specific activity of 36.8 U.

TABLE 1. The Isolation of alpha-N-Acetylgalactosaminidase From Human Liver.

Step	Total Activity μmol. min^{-1}	Specific Activity μmol. min^{-1}. mg^{-1}	% Yield	Purification fold
Crude Homogenate (37%)	A. 26.00	0.000740	100	1
	B. 6.50	0.000185	100	1
Post Nuclear Supernatant	A. 26.08	0.000960	103	1.29
	B. 10.72	0.000390	109	2.10
Con A Sepharose	A. 27.50	0.020000	105	27.0
	B. 12.50	0.009100	192	49.0
Affinity (2x's)	A. 19.50	3.672	75	4962
	B. 4.87	1.900	75	10270
Sephadex G-150	A. 7.54	36.800	29	49730
	B. 3.575	5.701	55	30816

Units of activity are μmol 4-methylumbelliferyl α-D-galactopyranoside hydrolyzed per min at 37°C in the presence of 150 mM N-acetylgalacteside.

Dean's purification scheme (29) was designed primarily to separate alpha-gal A from alpha-gal B activity. Although a number of substrate and kinetic studies showed some separation, there was still a considerable amount of cross-contaminating activity in each preparation.

Wilkinson (219) used an affinity ligand to separate the A and B enzymes completely. However, that procedure maximized the yield of alpha-gal A while procuring alpha-gal B as a side product. The B product obtained was not always intact, subject to the effects of proteolysis and degradation while A, the enzyme of interest in that project was purified completely.

Several steps seemed to make a difference in the purification scheme as performed by others. Homogenization of the tissue in 0.25 M sodium cacodylate buffer seemed to afford the most stability and maximum specific activity. When the use of this buffer was compared to the same steps using NaP or NaCit buffer or water, there was an overall increase of 20% in the total yield of B. The activity of alpha-gal A was similarly affected.

Ion exchange chromatography was beneficial to other purification schemes as reported by the investigators (29, 181, 219) for separating the A and B activities, although

this step rarely increased the fold purification. This step was unreliable in our hands, often leading to disastrous effects on the yield while not increasing the purification. After several resins were tried (DE-52 from Whatman, and LKB's ZetaPrep cartridge as described, among others), this step was dropped in favor of repeated affinity column applications. However, this also forced us to use smaller amounts of liver to start with, since both enzymes are unstable in an impure state.

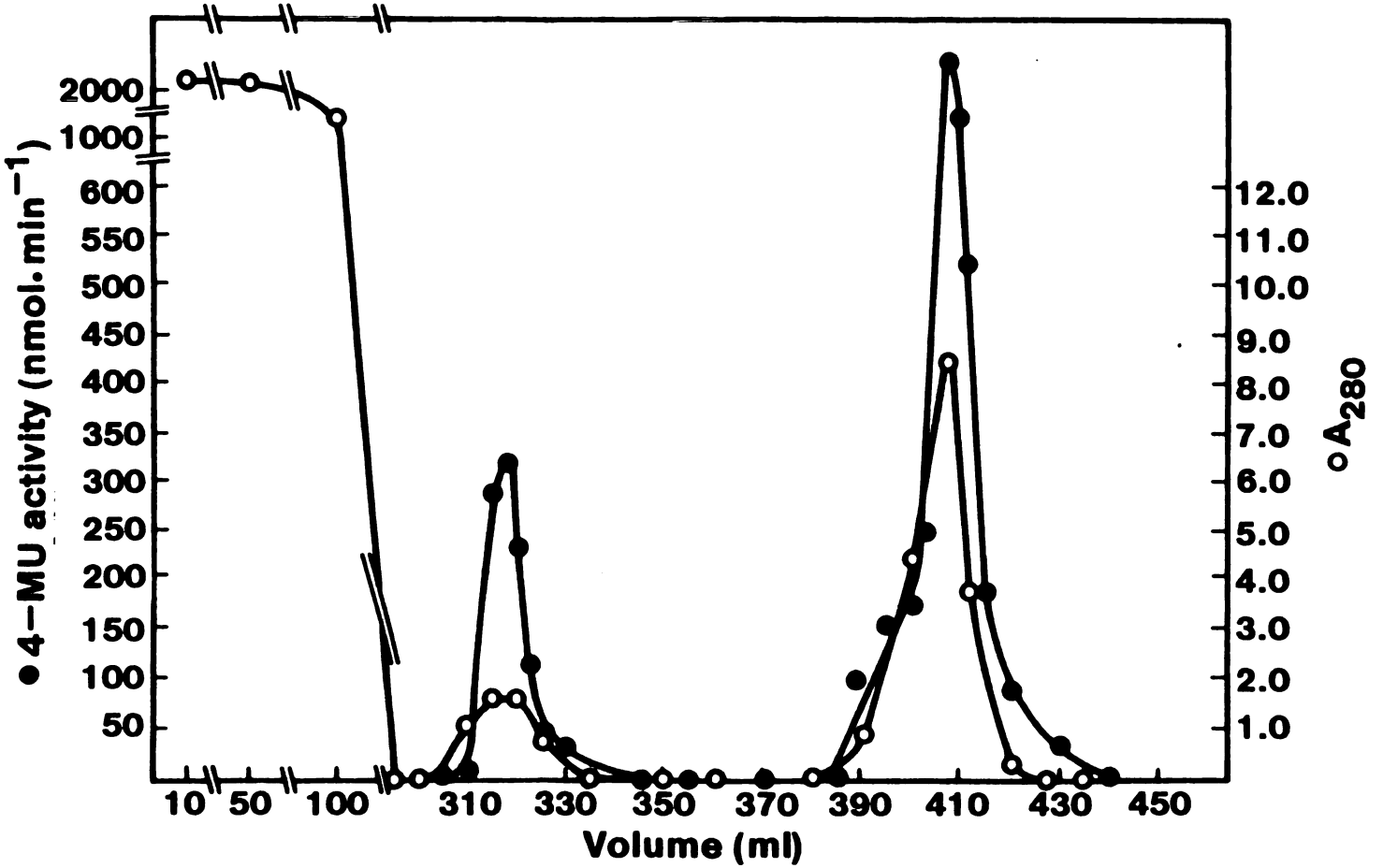
The affinity chromatography step was repeated twice, then the first eluate (or non-binding fraction) was rechromatographed in order to increase the yield. All enzyme was pooled into "pure A", "pure B", and an impure fraction that had slight activity, but would not stick to the ligand (Figure 9). The addition of N-acetylglucos-amine (GlcNAC) to the binding and elution buffers decreased the amount of non-specific (non-4-MU-alpha-gal active) protein that interacted with the column, which otherwise caused excessive washing times and impurities in the eluted product (mostly observed in the B fraction). N-acetyl-glucosamine does not affect alpha-gal A or B activity as measured by either the 4-MU assay or the p-NP assay (unpublished observations).

By skipping the ion-exchange steps and the gel-filtration step used by others, we were able to substantially decrease

FIGURE 9. Affinity Chromatography of alpha-Galactosidases A and B.

The enzyme mixture, obtained after Con A Sepharose chromatography, was batch attached to the affinity ligand which was then poured into a 1.0 x 18.5 cm column. Non-binding protein was eluted with 300 ml 0.05 M sodium citrate buffer, pH 4.8/0.1 M NaCl/0.05 M N-acetyl-glucosamine. Alpha-gal B activity was eluted with 0.05 M sodium citrate, pH 4.8/0.1 M NaCl/0.75 M N-acetyl-galactosamine. Alpha-gal A activity was eluted with 0.05 M sodium citrate, pH 5.0/0.1 M NaCl/0.8 M galactose, after washing the column with at least 30 ml 0.5 M sodium citrate, pH 5.0/0.1 M NaCl. Activity was monitored with 4-MU-alpha-Gal. The non-binding pool, GalNAc-eluted pool (B) and the Gal-eluted pool (A) were concentrated and rechromatographed to insure yield and separation of the two enzyme acivities.

○ A_{280}
● 4-methylumbelliferyl-alpha-D-galactoside activity (4-MU-alpha-gal)


● 4–MU activity (nmol. min^{-1})
2000
1000
600
550
500
450
400
350
300
250
200
150
100
50
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
○ A_{280}
10
50
100
310
330
350
370
390
410
430
450
Volume (ml)

the handling time of the enzyme. This is an important consideration in the purification of the two enzymes since their activity is substantially decreased as purification time increases. Proteolytic degradation was observed (as judged by a decrease in molecular weight of alpha-gal B on SDS-PAGE) when enzyme was stored in an impure or dilute state (sp.act. = 0.009 U) from the con A step or from the DEAE step before being affinity-purified.

The Sephadex G-150 purification was performed as the last step in the purification. The placement at this point was more beneficial to the purification and stability of the enzyme. One major concern and problem has been the separation of the alpha-gal A and B activities. Others have shown that the native wolecular weight of alpha-gal A was approximately 104,000 as determined by gel filtration, and the molecular weight of B was approximately 90,000 (29). This difference was not enough to separate the glycoproteins efficiently by gel filtration, although it was valuable as far as removing other impurities. One problem with the gel filtration step with these glycoproteins is that the enzymes typically "smeared" on the column, often eluting from the column in a volume that increased or decreased the molecular weight by nearly 10,000 as judged by the elution of standard proteins. This phenomenon is not atypical of the hands-on experiences of other investigators working with glycoproteins (personal communication J.Brauker, Y.

Moutsatsos and J.Laing, Dept. of Biochemistry, MSU). Consequently this step did very little to separate A and B activities.

In order to circumvent this problem since this step was necessary to remove other impurities, the A and B activities were separated by affinity chromatography first, then separately applied to the Sephadex column. In addition, 0.001% SDS was added to the running buffer and to the enzyme solutions. While this concentration did not affect the activity of alpha-gal B over four days as measured by p-NP-GalNAc activity, and only slightly reduced the 4-MU-alpha-gal activity (the presence of GalNAc still completely inhibited the activity), the activity of alpha-gal A as measured by 4-MU-alpha-gal was decreased and continued to decrease over the four day period. Whether this was merely an artifact of the assay with the artificial substrate, or whether the integrity of the enzyme was affected is unknown. SDS-PAGE resulted in the expected bands even when activity was decreased by the presence of SDS in the elution buffer.

Using the standard proteins as parameters, alpha-gal A had a M_r=105,000, and alpha-gal B had an M_r of 94,000, values comparable to others' data (Figures 10 and 11). The proteins eluted with less smearing than previously observed with the gel filtration step. However, with the elution of alpha-gal B, a second peak of 4-MU activity eluted at

FIGURE 10. Sephadex G-150 Chromatography of alpha-Galactosidase A

Approximately 0.5 mg protein from the second affinity purified A pool of enzyme was applied to a 1 x 120 cm column at a flow rate of 2.5 ml/hr (2.0 ml/cm^2/hr) in 0.05 M sodium citrate/0.1 M NaCl/0.001% SDS. Fractions (0.35 ml) were collected and assayed by 4-MU-alpha-Gal (●), p-NP-alpha-GalNAc (○), and A_{280} (■). The column was previously calibrated with standard proteins beta-galactosidase from E. coli, phosphorylase B, BSA, and cytochrome C.

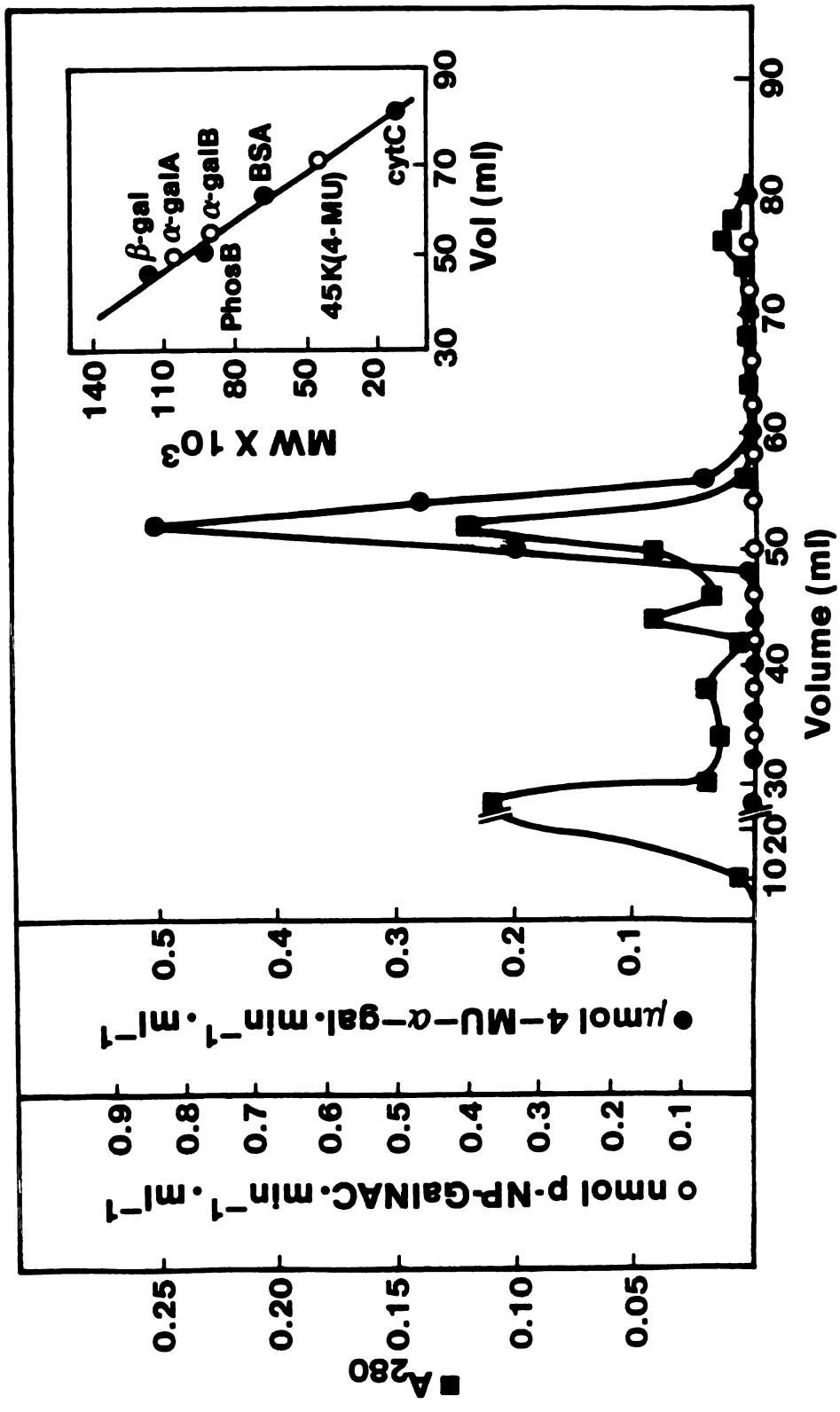
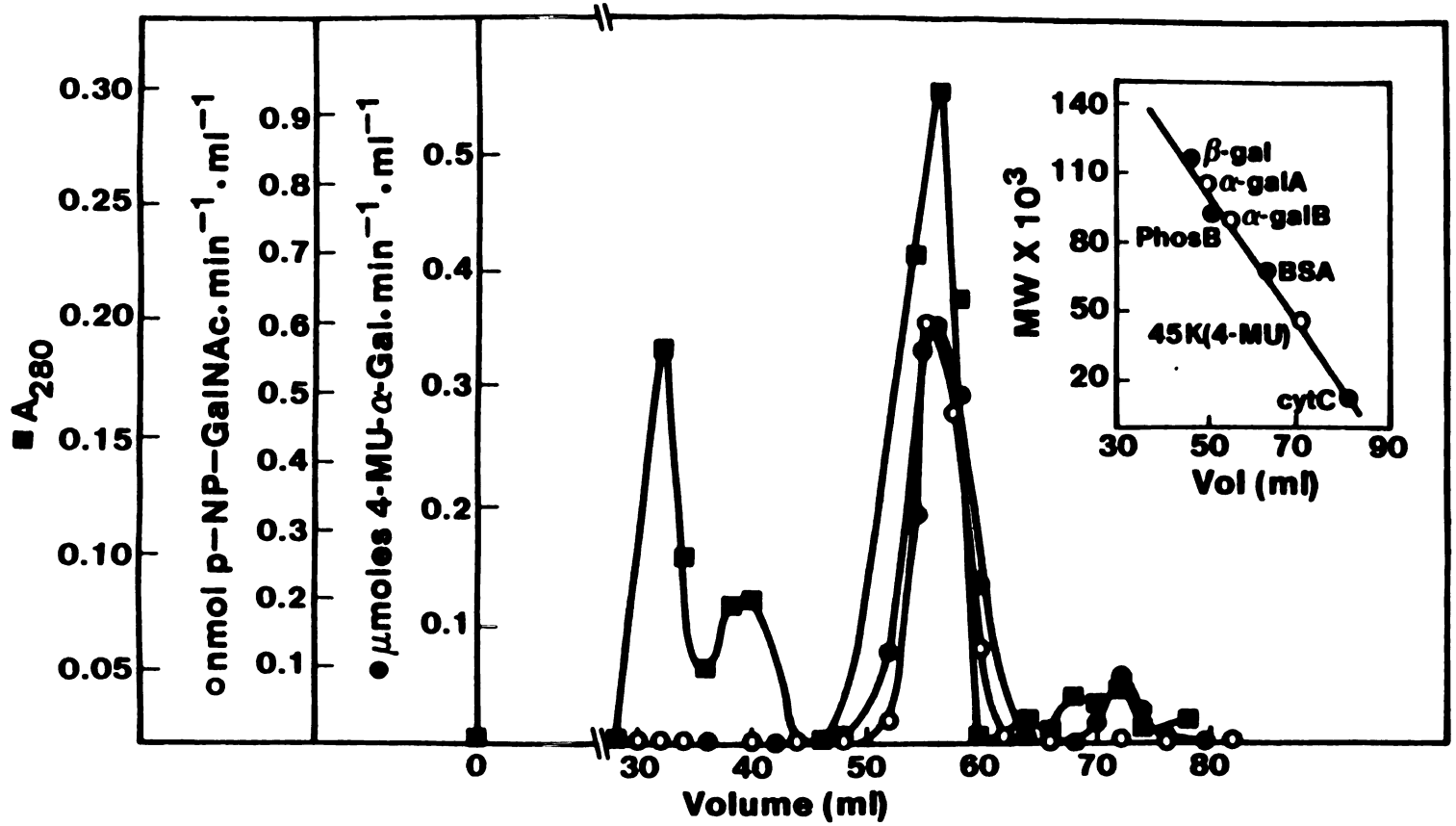

■ A_{280}
0.25
0.20
0.15
0.10
0.05
○ nmol p-NP-GalNAC. min^{-1}. ml^{-1}
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
● μmol 4-MU-α-gal. min^{-1}. ml^{-1}
0.5
0.4
0.3
0.2
0.1
10 20 30 40 50 60 70 80 90
Volume (ml)
MW X 10^3
140
110
80
50
20
30 50 70 90
Vol (ml)
β-gal
α-galA
α-galB
PhosB
BSA
45K(4-MU)
cytC

FIGURE 11. Sephadex G-150 Chromatography of alpha-N-Acetyl-galactosaminidase (alpha-Galactosidase B; alpha-Gal B).

Approximately 0.5-1.0 mg protein from the second affinity purified B pool of enzyme (eluted with GalNAc) was applied to a 1 x 120 cm column at a flow rate of 2.5 ml/hr (2.0 $ml/cm^2/hr$) in 0.05 M sodium citrate ph 6.5/0.1 M NaCl/0.001% SDS. Fractions (0.35 ml) were collected and assayed by 4-MU-alpha-Gal (●), p-NP-alpha-GalNAc (○), and A_{280} (■). The column was previously calibrated with standard proteins beta-galactosidase from E. coli, phosphorylase B, BSA, and cytochrome C.


■ A_{280}
0.30
0.25
0.20
0.15
0.10
0.05
○ nmol p-NP-GalNAc.min^{-1}.ml^{-1}
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
● μmoles 4-MU-α-Gal.min^{-1}.ml^{-1}
0.5
0.4
0.3
0.2
0.1
0
30
40
50
60
70
80
Volume (ml)
MW X 10^3
140
110
80
50
20
β-gal
α-galA
α-galB
PhosB
BSA
45K(4-MU)
cytC
30
50
70
90
Vol (ml)

approximately 43,000-46,000. This activity had no p-NP-GalNAc activity and GalNAc did not inhibit the 4-MU-alpha-gal activity. On SDS-PAGE this protein migrated to an M_r=45,000, and resembled the silver stained protein bands of pure alpha-gal A and B. On Western blots, antibodies to alpha-gal B detected the protein band, but anti-alpha-gal A antibodies did not.

Native gels were run on the affinity purified enzyme, before gel filtration through Sephadex G-150. While the results were visually less-than-spectacular, the alpha-gal A preparation showed three bands of approximately 400,000 M_r, 300,000 M_r and 120,000 M_r. The alpha-gal B preparation showed two bands: 100,000 M_r and 45,000 M_r (Figure 12).

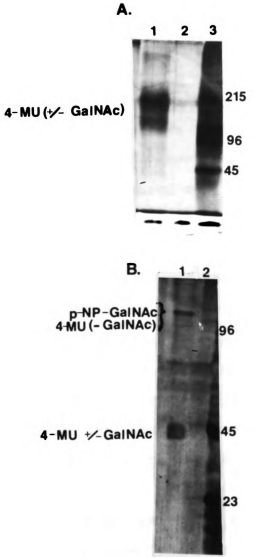
When the native gels were stained for activity, the alpha-gal A lane had no p-NP-GalNAc activity, but the 4-MU-alpha-gal activity was a smear which encompassed all three bands and was not diminished by the presence of GalNAc. The alpha-gal B preparation was run in three lanes each stained separately for 4-MU-alpha-gal activity, 4-MU-alpha-gal with GalNAc and for p-NP-GalNAc activity. The p-NP-GalNAc activity was seen as a diffuse yellow band around the area of the 100,000 MW band. The upper band also had 4-MU activity which was inhibited (no fluorescence was seen under UV) when GalNAc was added. The lower band around 45,000 had 4-MU-alpha-gal activity, was not inhibited with GalNAc, and

FIGURE 12. Native Gel Electrophoresis of Partially Purified alpha-Gal A and alpha-Gal B.

Enzyme collected after the second affinity chromatography step was electrophoresed through a native polyacrylamide gel. Lanes were incubated with substrate as described in Methods, or were silver stained. Molecular weight standards are indicated.

(A) lane 1: alpha-gal A
lane 2: buffer
lane 3: molecular weight standards
The bands floresced with 4-MU-alpha-gal with and without GalNAc, and no activity was seen with p-NP-GalNAc.

(B) lane 1: alpha-gal B
lane 2: buffer
The high molecular weight band (100,000) had activity with p-NP-alpha-GalNAc and 4-MU-alpha-gal activity which was inhibited with GalNAc.
The lower molecular weight band had 4-MU-activity with and without
GalNAc present, but no p-NP-alpha-GalNAc activity.


A.
1
2
3
4-MU (+/- GalNAc)
215
96
45
B.
1
2
p-NP-GalNAc
4-MU (- GalNAc)
96
4-MU +/- GalNAc
45
23

had no detectable p-NP-GalNAc activity either visually or when scanned (Figure 12).

On denaturing SDS-PAGE gels (Figure 13), alpha-gal A had was a single band with a subunit M_r=46,000.Alpha-gal B (Figure 14) was also a single band with a subunit M_r=45,000. Alpha-gal B treated with N-glycanase which cleaves N-linked oligosaccharides had a subunit M_r=38,000 and was again a single band.

Glycoproteins typically are diffuse bands on SDS-PAGE, as seen consistantly with A and B. When the oligosaccharides were removed, alpha-gal B appeared as a much more compact band (Figure 14).

Western blots of the two enzymes and N-glycanase treated B showed that alpha-gal A did not react with the anti-alpha-gal B antibodies while alpha-gal B and N-glycanase treated B showed the expected bands. The 45,000 MW peak that eluted from the Sephadex column also reacted with the B antibodies (appearing as a diffuse band about 43,000 M_r on SDS-PAGE). Only alpha-gal A reacted with the anti-A IgGs.

Approximately 0.5 mg of alpha-gal B was sent to Dr. Peter Weber to be digested by a novel chemistry using a mercuric cyanide resulting in cleavage of the protein at glycine residues. This would mean that alpha-gal B would be cleaved

FIGURE 13. SDS-PAGE and Western Blot of alpha-Gal A and N-glycanase digested alpha-Gal A.

Protein was electrophoresed through a 10% SDS-PAGE. One-half the gel was silver stained (lanes 1-5) and the other half was Western blotted (lanes 6-9) as described in Methods, then incubated with anti-alpha-gal A IgGs. The bands were visualized with the GAR-HRP enzyme conjugate-double antibody method as described.

SDS-PAGE
Lane 1: B-enzyme from the affinity step (partially purified)
Lane 2: A-enzyme from the Gel Filtration step (pure)
Lane 3: N-glycanase digested alpha-gal A
Lane 4: Buffer and N-glycanase enzyme.
Lane 5: Molecular weight standards (beta-galactosidase, BSA, OVA, alpha-lactalbumin)
Western Blot on Nitrocellulose
Lane 6: Partially purified alpha-gal B
Lane 7: Alpha-gal A
Lane 8: N-glycanase digested alpha-gal A
Lane 9: Buffer and N-Glycanase enzyme

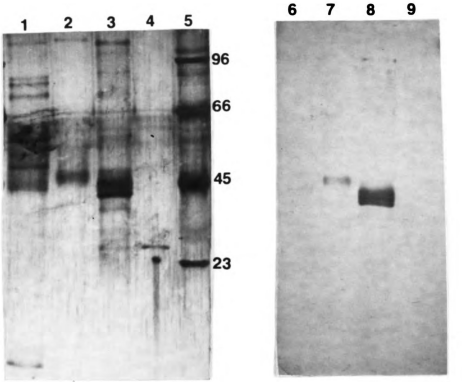
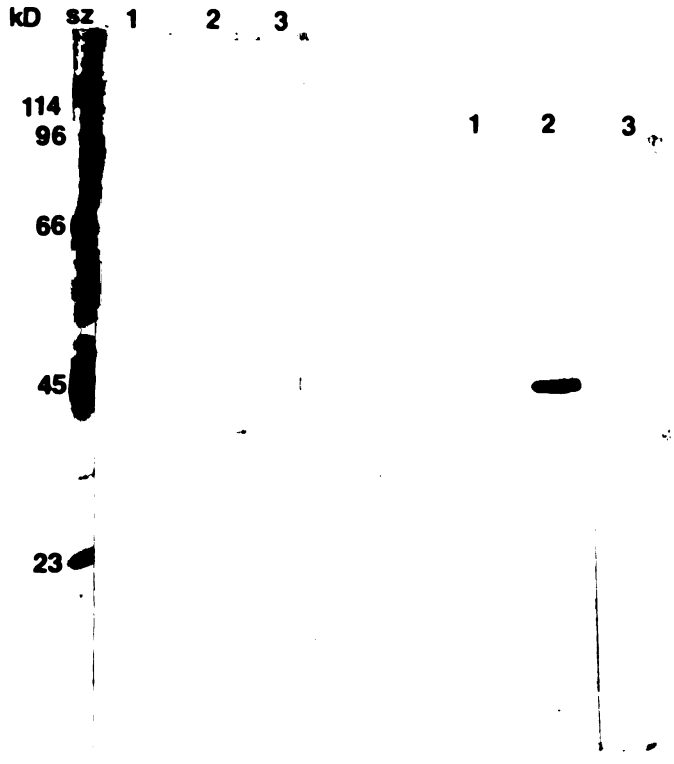

1
2
3
4
5
96
66
45
23
6
7
8
9

FIGURE 14. SDS-PAGE and Western Blot of alpha-N-acetyl-galactosaminidase (alpha-Gal B)

Pure enzyme was electrophoresed through a 10% SDS-PAGE. One-half was silverstained (A: lanes 1-3) and the other half was Western blotted (B: lanes 1-3) and reacted with mono-specific anti-alpha-gal B IgGs, and the bands were visualized with GAR-HRP double antibody method as described.

(A) Lane 1: Alpha-gal A
Lane 2: N-glycanase treated alpha-gal B
Lane 3: Alpha-gal B

(B) Lane 1: Alpha-gal A
Lane 2: N-glycanase treated alpha-gal B
Lane 3: Alpha-gal B


kD
sz
1
2
3
114
96
66
45
23
1
2
3

into a number of small peptides (10 - 20 amino acids) which could then be purified by HPLC and sequenced by conventional Edmond degradation and by mass spectrometry on a JEOL apparatus in the MSU Mass Spectrometry Facility. Mass spectrometry could also be used to determine peptides with carbohydrate residues.

The digested peptides were returned and, with the assistance and equipment at the University of Michigan Sequencing Facility, eighteen potential peptides were separated (Figure 15A). After repurifying the collected peptides (Figure 15B), three were selected for sequencing, based on the estimated quantity of peptide. The other fifteen, although pure, were relatively small amounts (approximately <1 ug per peak).

After these procedures were performed, it was determined that the chemistry used to cleave the peptides also resulted in blocked N-termini on the peptides, thus rendering them unusable for routine sequencing. When sequencing by mass spectrometry was attempted, the amount of material was too little to give any sequence information.

FIGURE 15a. HPLC Profiles of Isolated alpha-Gal B Hg/HCN Peptides.

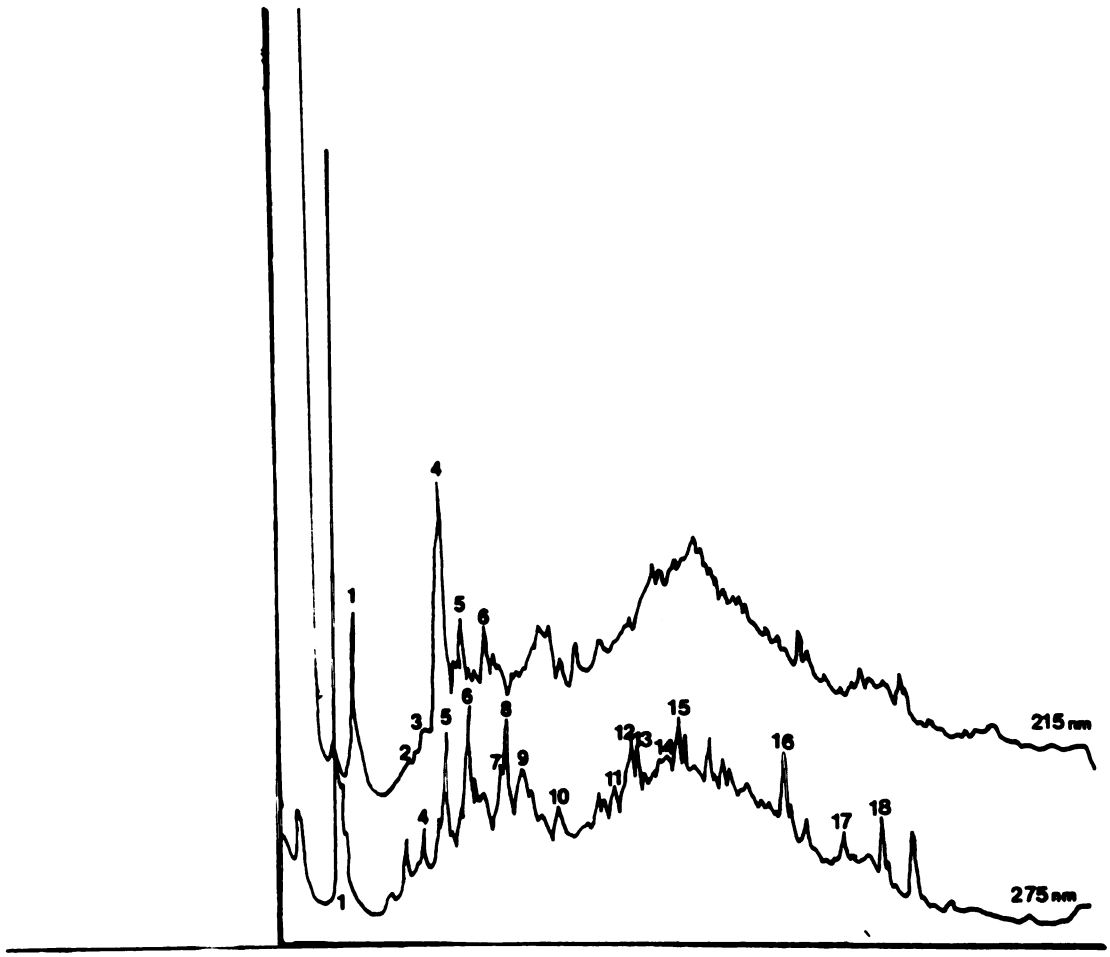
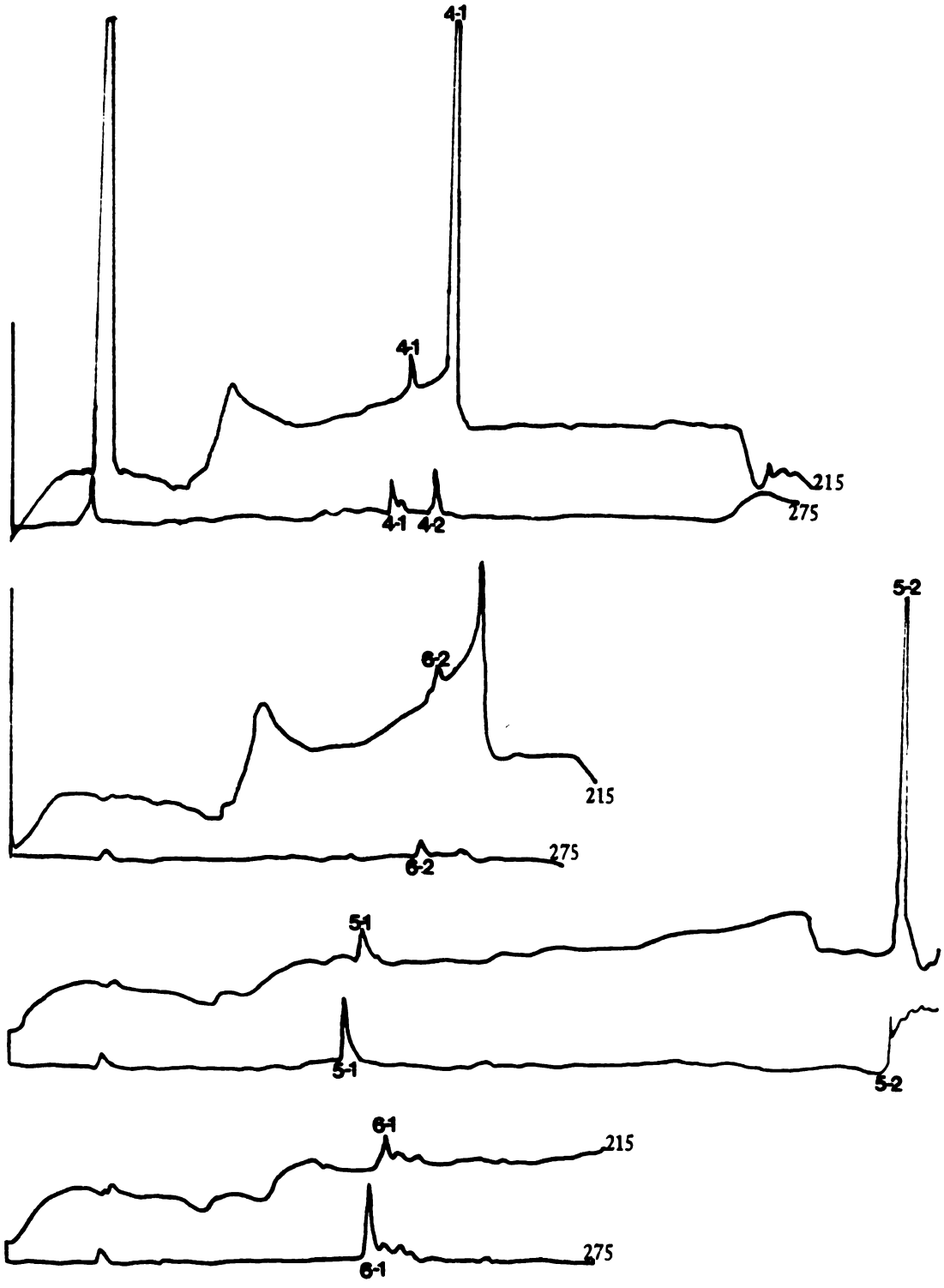

1
2
3
4
5
6
215 nm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
275 nm

Figure 15b. HPLC Profiles of Rechromatographed alpha-Gal B Peptides Selected For Sequencing.


4-1
4-1
215
275
4-1
4-2
6-2
215
275
6-2
5-2
5-1
5-1
5-2
6-1
215
275
6-1

II. THE ISOLATION OF THE GENES FOR ALPHA N-ACETYLGALACTOSAMINIDASE (ALPHA-GAL B) AND ALPHA-GALACTOSIDASE A

The need to define the exact lesion in Fabry's disease and to compare the biosynthetic pathways, processing mechanisms and functions of the protein and carbohydrate structures associated with the lysosomal enzymes alpha--galactosidase A and alpha-N-acetylgalactosaminidase has led beyond the physical purification of the enzymes from human sources. While studying the enzymes in cultured cells has resulted in valuable information, these means of investigating the properties of the enzymes are limited by the low abundance of alpha-gal A and B, and the ability to identify and manipulate such minute quantities.

Studying the enzymes on the molecular level-- on the RNA and DNA level rather than the cellular level-- would provide a unique means of identifying areas where the two enzymes are similar or different, what structures, coded for by the DNA, influence the transport, subsequent processing and activity of the enzymes, and where lesions or mutations have the greatest effect on the function of the enzyme and, therefore, on an organism.

The major problem in undertaking the isolation and characterization of alpha-gal A and B coding sequences has been the lack of a specific probe for these genes. Although the proteins have been purified, the small quantities of protein have limited the information obtained from protein sequencing. Secondly, the low abundance of protein in a cell has limited the isolation of specific mRNA, as well as the ability to immunoprecipitate gene products.

This report details the attempts to isolate mRNA coding for alpha-gal A, and cDNA clones for alpha-gal B. Several *in vivo* cellular studies are described which help determine the efficacy of several methods as well as provide more information about the biosynthesis and processing of alpha-gal A and B in human cells.

A. POLYSOME IMMUNOPRECIPITATION WITH ANTI-ALPHA-GAL A IgG

Without protein sequence amenable to translation into oligonucleotides useful for screening cDNA libraries, or a semi-homologous cDNA from another source, our initial cloning strategy for alpha-gal A rested upon our ability to enrich alpha-gal A mRNA by immunoselecting polysomes from a human cell line using antibodies specific for human liver alpha-gal A. Several investigators had demonstrated the ability to immunoprecipitate the nascent protein chains, thus precipitating specific mRNA. Although most of these cloned genes coded for products which represented a significantly higher proportion of the total translated products than alpha-gal A, (0.01% of total cellular protein vs. 0.001%; 225), several investigators (129) reported success in precipitating polysomes assembling minor products from tissue cultured cells.

The limits of sensitivity of this procedure necessitated finding a tissue source high in the enzyme of interest, but low in RNase. Human liver has the highest abundance of alpha-gal A, but is difficult to obtain, and is notoriously rich in RNase. The use of other tissues was not only limited by the same concerns, but was also

limited by the ability to immunoprecipitate enzyme using antibodies raised against the liver enzyme.

To this end, a number of human cell lines were surveyed for activity of alpha-gal A and the immunoprecipitability of the enzyme. Cells were also grown under conditions which prevented the glycosylation of the enzyme, presumably mimicing the nascent polypeptide being translated and available to the antibodies. Results are presented on the immunoprecipitatability of the lysosomal enzymes, the isolation of specific mRNA and the translation of the products.

MATERIALS

Ultrapure chemicals were purchased specifically for RNA work. Cycloheximide, heparin, Tris-Cl, Trizma Base, protein A-Sepharose 4B, ultrapure sucrose and diethylpyrocarbonate (DEPC) were purchased from Sigma Chemical Company (St. Louis, MO). Bentonite was purchased from Fischer Scientific (Rochester, NY). RNase inhibitor (RNasin) from human placenta was purchased either from Sigma Chemical Company as a lyphilized power containing 30,000 units/mg solid or from Biotec (Madison, WI) in storage buffer (40,000 units/ml). Beckman Ultraclear ultracentrifuge tubes were used on the polysome isolation (Beckman Instruments, Palo Alto, CA).

X-ray film for fluorography/autoradiography was Kodak X-OMAT. Cell culture media, serum and other additives were from Gibco, Inc., and disposable, plastic culture flasks, dishes, etc. were from Corning.

METHODS

RNase Free Technique. To minimize the possibility of RNase contamination, all glassware was washed in 1 M KOH/50% ethanol and baked for at least 4 hours at 280°C. Sterile plastic test tubes and pipettes were used when possible. All chemicals were kept separate from general laboratory use. Dialysis tubing (6000 MW cut-off) was boiled in 10 mM KOH, then in 1 mM EDTA/0.5% DEPC and finally rinsed in glass distilled, DEPC-treated (0.5% DEP), autoclaved H_2O. All buffers were autoclaved before use.

Enzyme Assay for alpha-Galactosidase A with Artificial Substrates. The enzyme assays are described in Part I.

Protein Assays. Protein was determined by modifications of Lowry et al. The Peterson assay (140) was used for low concentrations of proteins, 5 ug to 50 ug and the Markwell procedure (121) was used for greater concentrations, 50 ug to 100 ug. Both assays give results comparable to the standard Lowry for the assay of

BSA standards, but are subject to less interference from additives (i.e. sucrose, salts, detergents, etc.).

Preparation of alpha-gal A antisera. Alpha-gal A was the generous gift of F.E. Wilkinson of this lab. Whole antisera was prepared by injecting 30 ug of homogeneous alpha-gal A suspended in Freund's complete adjuvant at multiple sites on the back of a young New Zealand White rabbit. Booster injections were of 15 ug of alpha-gal A in Freunds incomplete adjuvant were given on days 14 and 28. Rabbits were bled on day 42.

Preparation of IgG. Whole antiserum was allowed to clot at room temperature and the serum was collected, acidified to pH 8.2 with 0.1 M Na_2HPO_4, and applied to a 1.0 x4.0 cm column of protein A Sepharose previously equilibrated with Na_2HPO_4, pH8.0. The IgG fraction was eluted with a step gradient of 20 ml each 0.1 M NaCit, pH6.0, pH 4.5, pH 3.0. Fractions (1.0 ml) were collected, immediately titrated to pH 7.4 and assayed for anti-alpha-gal A activity. Active fractions were pooled and concentrated in an Amicon concentrator with an XM50 membrane to 2.0 mg/ml, and 0.5 ml aliquots were frozen at $-20^{o}C$.

Cell lines. Four human cell lines were tested for activity of alpha-gal A and B. FS4 human foreskin

fibroblasts (Massachusetts Institute of Technology Cell Culture Center) were grown in Minimal Essential Media supplemented with 10% fetal calf serum. Human Chang liver cells (American Type Culture Collection [ATCC] CCL13) were grown in MEM and 10% calf serum. Human hepatoma cells Hep 3B and Hep G2 (ATCC HB 8064 and ATCC HB 8065) were grown in OptiMem supplemented with BME and 5% FCS. Cultures were incubated in a humidified chamber at 37°C with 5% CO_2.

Cell Culture for Immunoprecipitation of Enzyme from Media and Cell Lysates. Cells were plated at a density of 1 x 10^4 cells per plate (100mm dishes) in OptiMem supplemented with 0.4% BME and 5% FCS. Four hours after seeding, the media was removed and replaced with OptiMem/0.4% BME without serum. The cells were serum starved for 24 hr, then the media was replaced with OptiMem/0.4% BME/5% FCS. Culturing continued for approximately 120 hours when the cells were nearly confluent (approximately 25-30 x 10^4 cells/cm^2). Media was drawn off and concentrated 10-fold in an Amicon concentrator with a PM10 membrane, exchanging the media with PBS. Cells were harvested by trypsinization (0.02% trypsin in Hank's buffered salts; Gibco), washed from the plates with 5 ml PBS, and pelleted by centrifugation at 1,500 x g. Pellets and media were stored frozen at -80°C until used for immunoprecipitation.

Immunoprecipitation of Alpha-Gal A. Pure alpha-gal A and cell lysates were used as the enzyme sources. The cell pellet from one 100 mm plate was frozen-thawed, then disrupted in 0.5 ml of PBS containing 0.5% Triton X-100. The homogenate was allowed to stand at 0°C for 15 min, then cell debris was pelleted by centrifugation at 11,000 x g for 15 min. Approximately 100 units of alpha-gal A activity (by the 4-MU-alphal-gal assay as described in Section I) was added to 5 ul of a Staph A preparation previously incubated for 16 hr with preimmune serum or antiserum.The cell homogenate/Staph A immune complex was incubated at 37°C for 15 min, then at 0°C for 30 min. Immunocomplexed alpha-gal A was then removed by centrifugation for 1 min at 11,000 x g, and the supernatant was assayed for alpha-gal A activity.

Culturing Cells in Tunicamycin. FS4, Chang liver, and HepG2 cells were selected for study based on the specific activity of alpha-gal A and B per cell. Cells were plated as described except that the media was supplemented with 0.05 ug, 0.01ug or 0.15 ug/ml tunicamycin homologue B2 (Boehringer Mannheim) added directly to each plate. Three series of cell growth experiments were run: A) control cells were seeded at low density and media was replaced every 48 hr with no other supplements; B) cells were refed every six hours:one set received no tunicamycin, one set received

0.15ug/ml tunicamycin; ^{3}H-mannose (100 uCi/ml) was added to one plate in each set 6 hr before it was harvested (radiolabelled cells were fed with the same media lacking mannose); C) cells were refed every 24 hours: one set received no tunicamycin, one set received 0.15 ug/ml tunicamycin; ^{3}H-mannose in labelling media was added to one plate in each set 24 hrs before harvesting.

Incorporation of ^{3}H-Mannose into Chang Liver Cells. ^{3}H-mannose was added to cultures in order to assess the extent to which glycosylation was prevented and whether potentially unglycosylated enzyme could be immunoprecipitated by anti-alpha-gal A IgGs. Cells were harvested, lysed and immunoprecipitated as described.

Both the immunoprecipitate pellet and supernatant were prepared as follows using the whole immune-complex pellet (immunoprecipitated alpha-gal B) and 1/10 the supernatant.Aliquots of the sonicate from unimmunoprecipitated cells (1/10 th of the total cell pellet, and of the media) were assayed for ^{3}H-incorporation into proteins. Bovine serum albumin (50 ug) was added to each sample as carrier protein and 200 ul of cold 20% (w/v) trichloroacetic acid (TCA) was added.Solutions were incubated at room temperature for 10 min, then centrifuged at 11,000 xg for 10 min. The supernatant was removed from each tube. Pellets were dissolved in 200 ul 1.0 M NaOH. One-half of the

preparation was counted by liquid scintillation spectrophotometry and one half was saved for electrophoresis (7.5% SDS-PAGE) and fluorography.

Preparation of Cell Lysates for Polysomes from Chang Liver Cells. Chang liver cells were grown to a density of approximately 1.8 x 10^5 cells/cm^2. The nearly confluent cells were refed with DMEM-10% CS with 200 ug/ml of cycloheximide, 1 U/ml RNasin for 30 min. The cells were then washed with cold PBS and harvested by scraping the cells from the plate with cold PBS containing cycloheximide (200 ug/ml), heparin (200 ug/ml) and RNasin (100 U/ml). Cells were pelleted at 800 x g and RNasin (100 U) was added to the pellet. If the cells were not to be used immediately, they were quick-frozen in a dry ice-ethanol bath and stored at -80 C.

Preparation of Polysomes. Newly harvested or frozen cell pellets were homogenized in a hand-held glass Dounce homogenizer in 5 volumes of Polysome Buffer A (50 mM Tris, pH 7.5/23 mM NaCl/5 mM $MgCl_2$/0.25 M sucrose) containing bentonite (1 mg/ml), heparin 200 ug/ml), cycloheximide (200 ug/ml) and RNasin (1000 U/ml) with 5 strokes of a tight-fitting pestle. Two volumes of Polysome Buffer A were added and the mixture was homogenized with 5 strokes of a loose-fitting pestle. The homogenate was transferred to silanized Corex

centrifuge tubes and centrifuged in a Sorvall SS34 rotor for 10 min at 17,000 xg. The supernatant was transferred to sterile plastic culture tubes, diluted with 0.1 vol of 10% (w/v) sodium deoxycholate/10% (w/v) Triton X-100, put on ice for 5 min, and layered over a discontinuous sucrose gradient containing 2.0 ml of 2.5 M sucrose, 3.0 ml of 1.0 M sucrose and 1.0 ml of 0.5 M sucrose in 25 mM Tris, pH 7.5/150 mM NaCl/5 mM $MgCl_2$ containing cycloheximide, heparin, and RNasin as in Polysome Buffer A. The gradients were centrifuged for 4 hr in a Beckman SW-41 rotor at 38,000 rpm and fractions (0.5 ml) were collected from the top of the tube until about 0.5 ml was left above the 2.5 M-1.0 M interface. The tube was punctured with an 18 gauge needle approximately 0.5 ml below the interface and the entire opalescent band at the interface was collected. The polysome-containing sucrose was diluted 1:1 with Polysome Buffer B (0.25 mM Tris, pH 7.5/150 mM NaCl/5 mM $MgCl_2$/0.1% Nonidet P-40 containing cycloheximide, heparin, and RNasin as in Polysome Buffer A) and the solution was dialyzed against dialysis buffer for 16 hr (Polysome Buffer B plus 1 mg/ml bentonite. Polysomes were quick-frozen in an ethanol-dry ice bath and stored at -80°C or were used for immunoprecipitation after additon of 100 U RNasin.

Polysome Immunoprecipitation. Polysomes were immunoprecipitated and immobilized using the method

described by Krause and Rosenberg (225). The polysome preparation was thawed, then centrifuged for 10 min at 15,000 x to remove aggregates. The polysome concentration was adjusted to 15 A_{260} units/ml with Polysome Buffer B. Purified IgG was incubated at room termperature for 30 min with 1000 U RNasin/mg IgG, then reacted with polysomes at a ratio of A_{260} units of polysomes per mg IgG for 1 hour at 4°C.

The protein A-Sepharose 4-B column used for IgG purification was washed with 3 vol 1 M acetic acid and equilibrated with Polysome Buffer B until the pH of the eluate was 7.5.The polysome-antibody mixture was applied to the column at a flow rate of 5 ml/hr. The breakthrough eluate of free polysomes were collected, quick-frozen and saved for translation. The resin was removed after the polysome/Ab mixture was applied, resuspended in 2 volumes Polysome Buffer B and repacked in the column.The column was washed with 100 ml Polysome Buffer B (5 ml/hr) at 4°C, then eluted with 25 mM Tris/20 mM EDTA, pH 7.5, containing heparin (200 ug/ml)and RNasin (10 U/ml).

<u>Purification and Precipitation of RNA.</u> Proteinase K (200 ug/ml) was added to the polysome suspensions and the solutions were incubated at 37°C for 30 min. The proteins were removed by extracting with

phenol/chloroform, and RNA was precipitated with 0.1 vol 3M NaAc, pH 5.0, 2.5 vol 100% ethanol.

In Vitro Translation. A wheat germ translation system 54) was used to translate the purified RNA (courtesy of D. Lorimer, Department of Biochemistry, Michigan State University). Translations were made with 0.4 uCi ^{14}C-leucine (New England Nuclear) and 6 ug polysomal RNA (from breakthrough eluate of immunoprecipitation) or approximately 0.005 ug immunoprecipitated polysomal RNA (estimated by assuming alpha-gal A is 0.02% total Chang liver cell protein and, therefore, the alpha-gal A mRNA is 0.02% of the total mRNA; then, basing calculations on recovered RNA in the breakthrough eluate). The translation mixtures were incubated for 60 min at 37^{o}C along with a control containing no RNA. An aliquot (20 ul) of each was removed, spotted on a 3 mm Whatman fiberglass filter disc, placed in a Buchler funnel and washed with cold 5% (w/v) TCA, 95% ethanol, then ether. The filters were air dried and ^{14}C-leucine content was determined by liquid scintillation spectrometry. An aliquot (45 ul) of the breakthrough RNA translation mixture was immunoprecipitated as described. Electrophoresis (6% SDS-PAGE) of immunoprecipitated breakthrough translation mixture (pellet and supernatant), total breakthrough translation mixture (45 ul), immunoprecipitated polysome RNA translation mixture,

and the control translation was performed and the gels were prepared directly for fluorography by soaking in 500 ml 1 M sodium salycilate for 1 h and then fluorographed as previously described (26).

RESULTS

In previous experiments using Chang liver cells, we observed that the specific activity of alpha-gal A increased as the cells became confluent and contact inhibited. We wanted to determine in vitro cell culture conditions which would maximize the specific activity of alpha-gal A and, assuming a 1:1 correlation between mRNA and protein, the maximum rate of synthesis and amount of alpha-gal A-specific mRNA present.

Figure 16 shows the cell growth curve generated as cells plated at a low density (1.2×10^4 cells/cm^2) reach density dependent inhibition of growth (2.95×10^5 cells/cm^2). Specific activity of alpha-gal A was assayed with 4-MU-alpha-Gal and the amount of alpha-gal A as a percent of total cellular protein (%TCP) was calculated using Wilkinson's value of specific activity for pure alpha-gal A (219). Alpha-gal A increased from 0.002% to 0.008% TCP when cells were in log phase (Table 2). This percentage increased further when cells were harvested for the polysome immunoprecipitation procedure. After a 30 min treatment

FIGURE 16. Effect of Tunicamycin B2 Homologue on Chang Liver Cell Growth

Cells were treated every 48 hr in the following manner: ● control: DMEM/10% CS, no other supplements; ▲ 0.05 ug/ml tunicamycin; ○ 0. 1 ug/ml tunicamycin B2; □ 0.15 ug/ml tunicamycin B2.

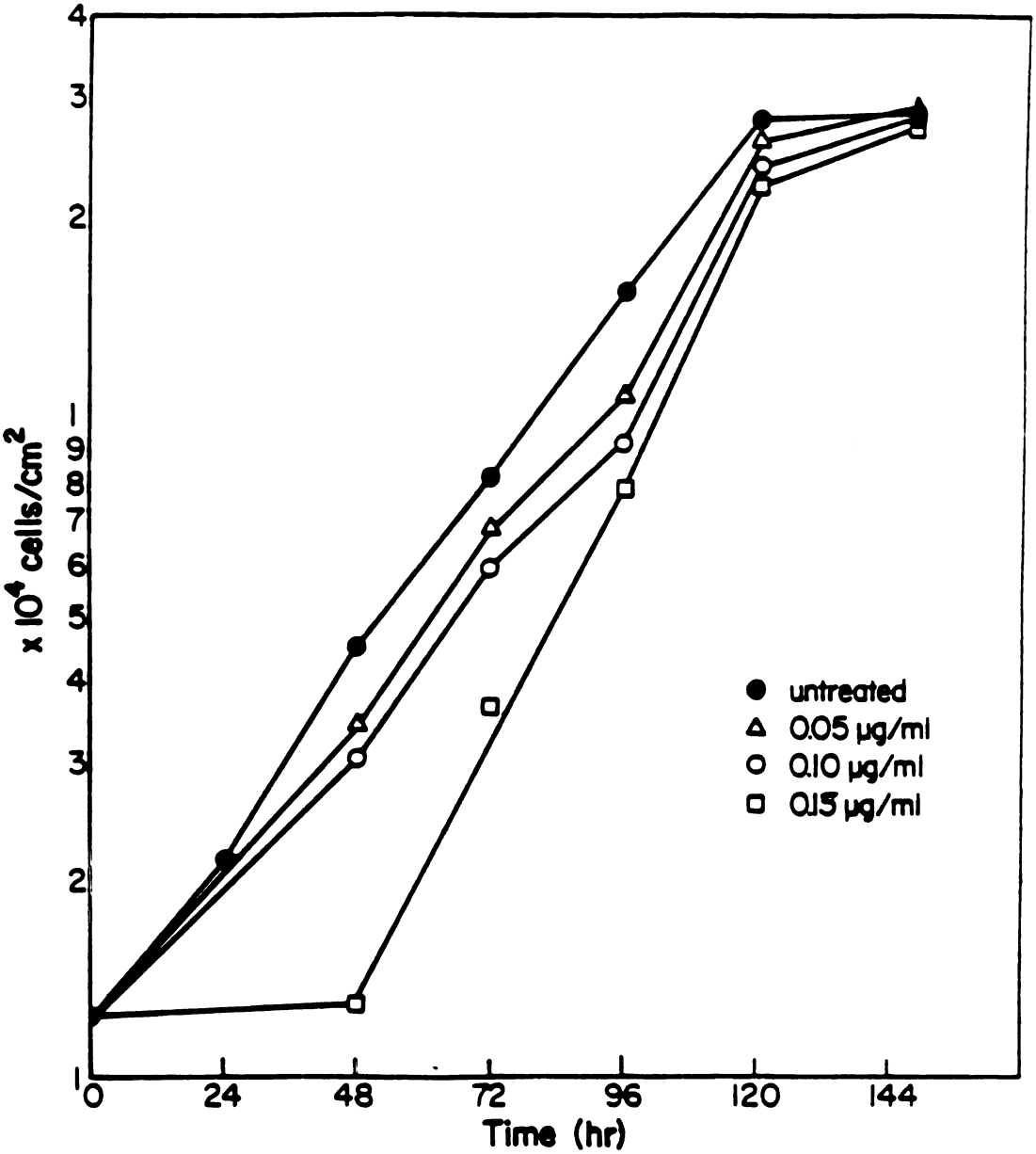

x 10⁴ cells/cm²
4
3
2
1
9
8
7
6
5
4
3
2
1
0
24
48
72
96
120
144
Time (hr)
untreated
0.05 µg/ml
0.10 µg/ml
0.15 µg/ml

TABLE 2. Effect of Cell Density on alpha-Gal A Activity in Chang Liver Cells.

Cells were plated in DMEM/10% CS, refed every 48 hr, and harvested as described. (*) indicates plate treated and harvested as for the polysome preparation.

Time (hr)	Cell Density (cells . $10^4/cm^2$)	Specific Activity (nmole . hr^{-1} . mg^{-1})	Percent Total Cellular Protein (% TCP)
0	1.2	37.6	0.002
48	4.5	94.9	0.005
96	15.5	122.1	0.0065
100	17.9	136.9	0.007
120	28.0	144.9	0.008
148	29.5	105.0	0.006
120.3*	28.0	226.0	0.012

with DMEM-10% CS containing cycloheximide and RNasin, alpha-gal A rose to 0.012% TCP. In practice, this scheme has increased alpha-gal A to as much as 0.02% TCP.

At the same time, we wanted to determine if glycosylation of alpha-gal A was necessary for its enzymatic activity and its reactivity with anti-alpha-gal A Ig. Cells were treated with tunicamycin B2 to prevent glycosylation (39). This homologue has been shown to prevent the glycosylation of proteins without inhibiting protein synthesis. As seen in Figure 16, tunicamycin did not affect cell growth to any extent. The highest concentration (0.15 ug/ml) did cause an extended lag period, but the cells seemed to recover after 48 hours despite a second treatment at this time. Specific activity of alpha-gal A was not affected (Table 3). Ninety-three percent of the alpha-gal A activity was immunoprecipitated with anti-alpha-gal A antiserum which compares with cells not treated with tunicamycin (Table 4).

The control and tunicamycin-treated cells were labelled with 1 uCi ^{3}H-mannose/60 mm plate for 6 hours before harvest to assess the extent of N-linked glycosylation by measuring the incorporation of mannose into glycosylated proteins (alpha-gal A and B both contain mannose units). Table 5 shows that the tunicamycin treated cells incorporated less than 5% of the mannose incorporated into control cells.Alpha-gal A activity was unaffected,

TABLE 3. Effect of Tunicamycin B2 on alpha-Gal A Activity in Chang Liver Cells.

Chang liver cells were treated with 0.05 ug/ml, 0.10 ug/ml, and 0.15 ug/ml tunicamycin in DMEM/10% CS every 48 hr and harvested in PBS by scraping the plates.

Time	Tunicamycin (μg/ml)	Cell Density (cells x $10^4/cm^2$)	Specific Activity nmole$\cdot hr^{-1} \cdot mg^{-1}$	Percent Total Cellular Protein (% TCP)
48	0.05	4.2	83.8	0.004
96		11.8	123.8	0.0065
148		29.0	90.5	0.005
48	0.10	3.1	92.8	0.004
96		9.4	112.5	0.006
148		28.0	84.6	0.005
48	0.15	1.5	63.2	0.003
96		7.8	103.7	0.006
148		27.0	110.3	0.006

TABLE 4. Immunoprecipitation of alpha-Gal A Activity from Different Cell Lines and the Effect of Tunicamycin Treatment.

Cells were grown and harvested as described. Approximately 100 U (nmol 4-MU liberated /hr x mg) of activity were reacted with the Staph A/anti-alpha-gal A IgGs as described. The supernatants from the immunoprecipitation reaction were assayed for activity remaining. Two cell lines, Chang liver and HepG2 were selected for tunicamycin treatment. Cells were grown in 15 ug/ml tunicamycin, then the immunoprecitation was repeated as described above.

Table 4. IMMUNOPRECIPITATION OF ∝-GAL A ACTIVITY FROM DIFFERENT TREATED AND UNTREATED CELL LINES.

Cell line	spec. act. nmol x hr^{-1} x mg^{-1}	%activity remaining	% activity precipitated	
			preimmune	immune
Chang Liver	144.0	99	8	92
HepG2	138.2	98	19	81
Hep3B	36.5	100	21	81
FS-4	236.3	103	77	23
Human liver enzyme (control)	683.3	105	5	95
<u>Tunicamycin B2 treated cells</u>				
Chang liver	144.5	98	7	93
HepG2	137.0	99	20	80

TABLE 5. Incorporation of (2-^{3}H)-Mannose into Chang Liver Cells

Chang liver cells were grown with and without tunicamycin in the media. Six hours before harvest, media containing (2^{3}H)-mannose was added. ^{3}H incorporation was determined in the soluable fraction of the cell lysate, and in the immune complex pellet from the anti-alpha-gal A precipitated cell lysate supernatant. Alpha-gal A activity, determined in the cell lysate supernatant and immunoprecipitated supernatant, was as in Table 4.

Table 5. INCORPORATION OF ^{3}H-MANNOSE INTO CHANG LIVER CELLS

	^{3}H-man incorporated per 104 cells (cpm)	cpm precipitated preimmune	cpm precipitated immune	% Activity Precipitated
Untreated Cells	21,500	512	21,251	92%
	20,030	500	19,800	
Tunicamycin B2 treated cells (15 ug/ml)	775	52	750	91.5%
	798	38	778	

and 100% of the immunoprecipitable activity compared to the control cells could be precipitated by the anti-alpha gal A IgGs. Fluorography of the precipitated products did not give conclusive results: the autoradiogram of the control cells and media showed a large smear of radioactivity while the treated cell pellets showed a smaller smear from approximately 40,000 MW to 70,000 and a small band at approximately 30,000. The fluorography was not repeated.

The procedure used for the rapid isolation of pure polysomes is illustrated in Figures 17 and 18. For optimal recovery of polysomes, ribonuclease (RNase) inhibition and the prevention of polysome disaggregation is important. Polysome disintegration results in the polysomes spreading throughout the sucrose gradient and not sedimenting onto the 2.5 M sucrose "cushion", lowering the yield. When cycloheximide (200 ug/ml), sodium heparin (200 ug/ml), and RNase inhibitor (RNasin) were included in all buffers, a high yield of polysomes was obtained.

Fractions were collected from the top of the centrifuge tube to a point 0.5 cm above the 2.5 M-1.0 M interface. The tube was then punctured approximately 0.5 cm below the opalescent band at the interface and the band was

FIGURE 17. Schematic Diagram for the Cytoplasmic Lysate for Polysome Isolation.

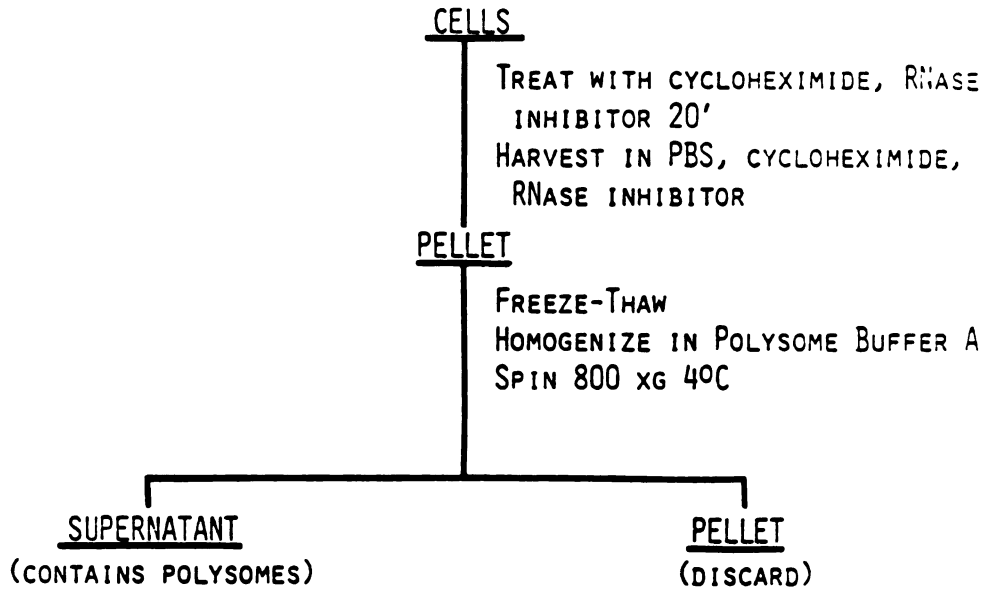
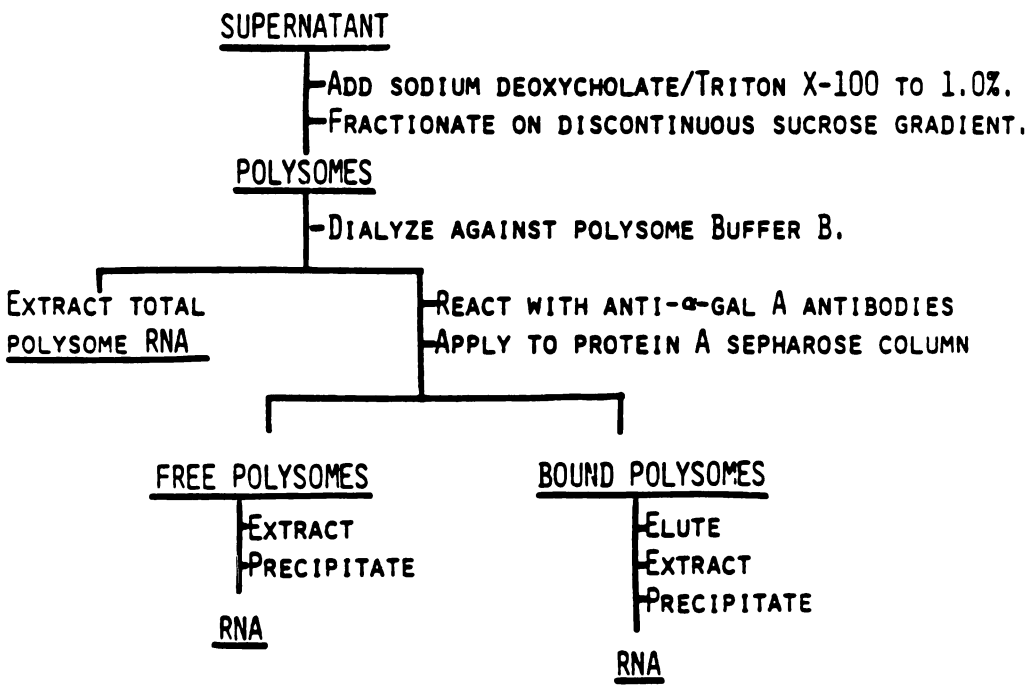

CELLS
TREAT WITH CYCLOHEXIMIDE, RNASE INHIBITOR 20'
HARVEST IN PBS, CYCLOHEXIMIDE, RNASE INHIBITOR
PELLET
FREEZE-THAW
HOMOGENIZE IN POLYSOME BUFFER A
SPIN 800 XG 4°C
SUPERNATANT
(CONTAINS POLYSOMES)
PELLET
(DISCARD)

FIGURE 18. Schematic Diagram for the Isolation of mRNA from Polysomes From Chang Liver Cells.


SUPERNATANT
ADD SODIUM DEOXYCHOLATE/TRITON X-100 TO 1.0%.
FRACTIONATE ON DISCONTINUOUS SUCROSE GRADIENT.
POLYSOMES
DIALYZE AGAINST POLYSOME BUFFER B.
EXTRACT TOTAL POLYSOME RNA
REACT WITH ANTI-α-GAL A ANTIBODIES
APPLY TO PROTEIN A SEPHAROSE COLUMN
FREE POLYSOMES
EXTRACT
PRECIPITATE
RNA
BOUND POLYSOMES
ELUTE
EXTRACT
PRECIPITATE
RNA

withdrawn with a syringe. This proved to be the best method to recovery polysomes and avoid mixing.

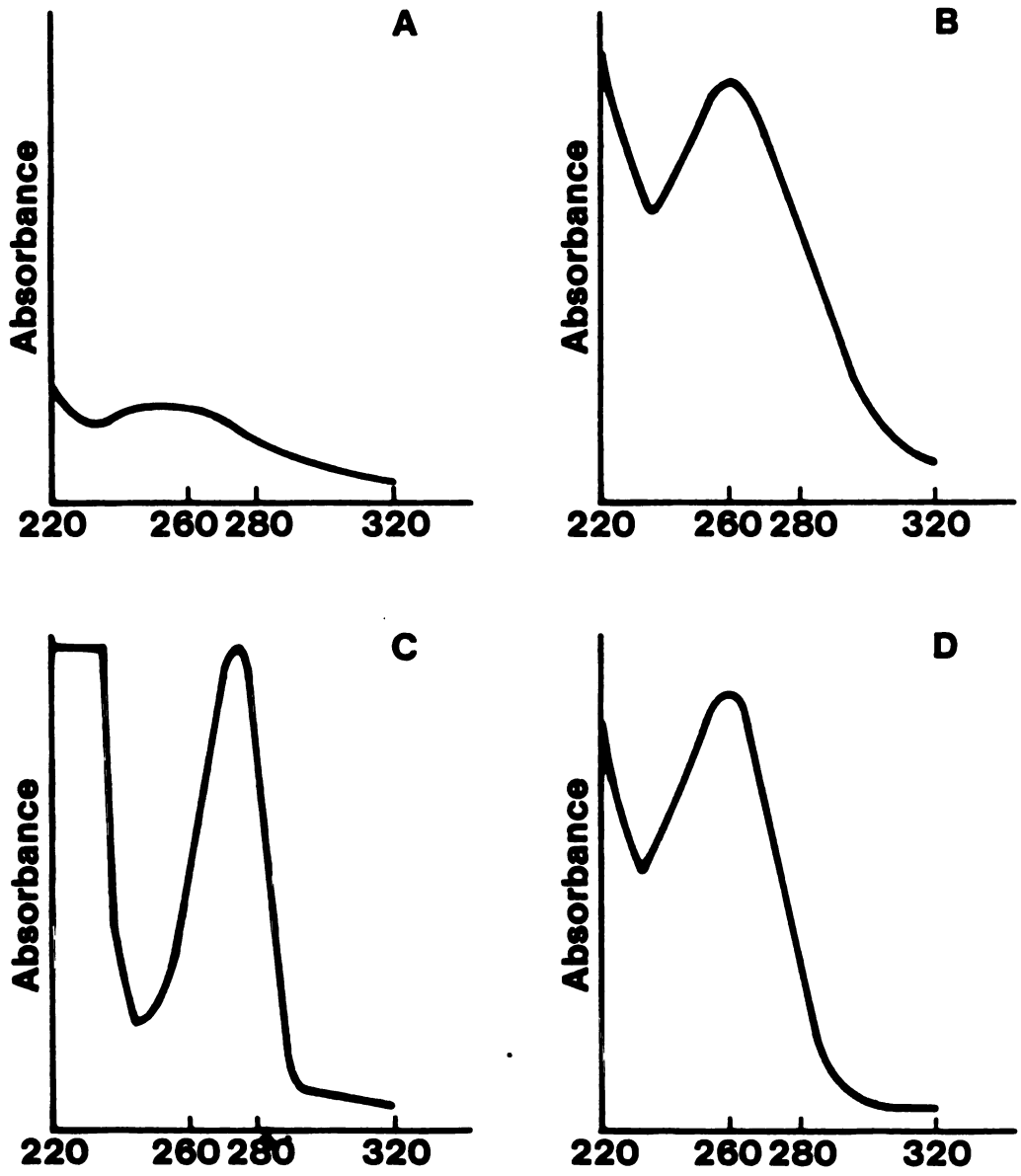
Sucrose fractions were analyzed by ultraviolet light spectroscopy. Typical scans from 220 to 320 nm are shown in Figure 19A-D. Polysomes have a characteristic A_{260}/A_{280} ratio of 1.9, whereas DNA has a ratio of 1.8 and RNA a ratio of 2.0. Fractions having an A_{260}/A_{280} of 1.9 were pooled, dialyzed and frozen as described. Quick-freezing and storing the polysomes changed neither the OD at 260 nm nor the A_{260}/A_{280} ratio. The typical yield of polysomes was 30-40 A_{260} units per gram of pelleted Chang liver cells.

RNA extracted from the polysomes had an A_{260}/A_{280} of 2.1. An OD of 1 at 260 nm is approximately 40 ug RNA/ml (22). On the average, 10-15 ug RNA was recovered from 1 gram of Chang liver cells by this method.

The immunoprecipitation of polysomes occurs through the specific immunological recognition of the nascent polypeptide chain synthesized on the ribosome. Previous work in this laboratory showed that anti-alpha-gal A IgG was specific for alpha-gal A by sodium dodecyl sulfate gel electrophoresis of immunoprecipitated, ^{3}H-mannose labeled Chang liver alpha-Gal A (26) (Figure 13). Polysomes from 3 grams of Chang liver cells (95 A_{260}

FIGURE 19. Spectral Scans of Sucrose Gradient Fractions and Extracted RNA.

A) 2.5 M sucrose; B) 2.5-1.0 M interface; C) Top 1.0 M fraction; D) Polysomal RNA extracted from the 2.5-1.0 M interface. Fractions were diluted 1/50 and scanned from 220 nm to 320 nm.


A
Absorbance
220
260
280
320
B
Absorbance
220
260
280
320
C
Absorbance
220
260
280
320
D
Absorbance
220
260
280
320

units) were reacted with 0.6 mg purified IgG for 1 hour at 4°C. A second aliquot of anti-alpha-gal A IgG was added and the reaction continued for another hour.

After application of the polysome-antibody mixture to the protein A-Sepharose column, the breakthrough fraction was collected.Approximately 95% of the polysome fraction was recovered as determined by OD_{260}. The OD_{260} of the bound polysomes after elution of the column could not be determined due to the presence of EDTA in the elution buffer. Assuming that alpha-gal A activity and mRNA are 1:1, and that alpha-gal A was at least 0.012% TCP and at best 0.02% TCP, the yield of alpha-gal A mRNA should be between 4-7 pg. This immunoprecipitation was repeated twice more with the same results. The breakthrough and immunoprecipitated polysomes were pooled separately and RNA was extracted. The yield from the breakthrough fraction was 32 ug RNA. This RNA had an A_{260}/A_{280} of 2.05 which is comparable to reported values for polysomal RNA (109).

Precipitated RNA was resuspended in RNase-free H_2O with 10 U RNasin and translated as described. Incorporation of ^{14}C-leucine into both the breakthrough fraction (free polysomes) and into the immunoprecipitated polysome was measured after TCA precipitation.Although background radioactivity was high (214) in a control translation

mixture (no RNA added), 840 total cpm were detected in the breakthrough fraction and 817 cpm were detected in the immunoprecipitated fraction. This indicated that translation of mRNA was successful in each case.

A second aliquot of the translated breakthrough fraction was immunoprecipitated with anti-alpha-gal A as was an aliquot of the control. Electrophoresis of the following translations was performed: control, immunoprecipitated control, breakthrough fraction, immunoprecipitated breakthrough fraction, and the immunoprecipitated polysomal fraction.

Non-specific trapping of radioactivity in all lanes except the immunoprecipitated control caused the film to be overexposed, obscuring any bands that might have shown up with a longer exposure. The gel was fluorographed for varying times from 6 hours to one week, overexposure by the trapped radioactivity could not be eliminated. The remaining RNA was translated in the same way, except the products were precipitated and washed with 5% TCA, acetone and ether. Product bands were seen by fluorography, but alpha-gal A specific bands could not be positively identified.

At this time partial amino acid sequences of the N-termini of alpha-gal A and B were determined. The

polysome precipitation project was set aside in favor of screening a cDNA library with an oligonucleotide probe synthesized to the N-terminus of alpha-gal B since protein sequencing was continuing on that protein.

B. THE ISOLATION OF cDNA CLONES WITH AN ALPHA-GAL B-DERIVED SYNTHETIC OLIGONUCLEOTIDE.

N-terminal sequencing of alpha-gal A and B revealed extensive homology which was not common among other lysosomal enzymes. This information, the first available the amino acid sequence of either alpha-gal A and B, raised the possibility that a specific probe could be synthesized and used for the isolation of the enzymes' coding sequences.

From the N-terminal amino acid sequence of alpha-gal B, a mixed octadecanucleotide (18-mer) was synthesized, homologous to alpha-gal B and semi-homologous to alpha-gal A. The total number of possible sequences of the 18-mer was 256, since a mixture of appropriate nucleotides were substituted at degenerative positions.

Synthetic, mixed-sequence oligonucleotides have been used successfully to identify DNA fragments carrying genes of interest from cDNA libraries. Wallace et al. (210, 227) were able to demonstrate that the hybridization of small oligonucleotides to large DNAs can be controlled by specifically regulating the temperature of the hybridization reaction in high salt buffers based on the

thermal stability of exactly matched sequences and single mis-matched duplexes. Although the number of sequences of the mixed probe was quite high, the fact that a very similar enzyme was only semi-homologous to alpha-gal B, and that no other sequenced lysosomal enzyme was homologous, made it seem possible that hybridization of the synthetic 18-mer to human DNA could be specific and that exact base pair hybridization could be accomplished. Hybridization conditions were determined, and the 18-mer was used to screen a human fetal liver cDNA library for clones homologous to alpha-gal B.

MATERIALS

Nitrocellulose filters were from Schleicher and Schuell. Bacterial culture supplies were from Difco (Difco laboratories, Detroit, MI). $\gamma[^{32}P]$-ATP (3000 Ci/mmol) was from New England Nuclear. T-4 polynucleotide kinase was from Bethesda Research Laboratories (Gaithersburg, MD). Restriction enzymes were from Bethesda Research Laboratories, or New England Biolabs and used as directed by the supplier. Human, chicken, E. coli and lambda DNA samples were supplied by P. Bates, M. Raines, and C. Gibbs (Departments of Biochemistry and Microbiology, Michigan State University). Cell and Bacterial culture

media and additives were purchased from Gibco, and cultureware was from Corning.

METHODS

Preparation of genomic DNA. High molecular weight genomic DNA was isolated from human tissue samples and human cell lines for use in stringency determinations and as hybridization controls by a modification of the method of Blin and Stafford (14). Confluent 75 mm flasks of human cells (Chang liver, HepG2 and FS4 fibroblasts as described previously) were washed with ice cold PBS and scraped from the plates, centrifuged and frozen at -80^{o}C until cells from 15 flasks each had been harvested. Cell pellets were thawed in ice cold TE, pH 7.4 (10 mm Tris-Cl, pH 7.4/1 mm EDTA, pH 8.0) at a concentration of 10^{8} cells/ml. Ten grams of mouse and human liver and human placenta were minced with a scalpel while semi-frozen, then blended in a Waring blender and liquid nitrogen until the tissue was ground to a fine powder. All samples were treated the same at this point. Ten volumes of lysing solution (10 mm Tris-Cl, pH 8.0/0.1 mm EDTA, pH 8.0/0.01 M NaCl/0.5% SDS/100 ug/ml proteinase K) were added to each sample and the suspensions were vigorously vortexed, then incubated at 50^{o}C for 3 hr with periodic inversion. The DNA was then extracted three times with Tris-buffered phenol, phenol/chloroform, and chloroform,

dialyzed against twenty volumes 50 mm Tris-Cl, pH 8.0/10 mm EDTA, pH 8.0/10 mm NaCl at 4°C, incubated with 100 ug/ml DNase-free RNase at 37°C for 3 hr, reextracted with phenol/chloroform, and dialyzed extensively against TE. The DNA was finally concentrated by ethanol precipitation and spooling onto glass rods.

Synthesis of a Mixed Oligonucleotide Probe. A mixed 18-nucleotide probe was synthesized by M.H. Caruthers (Univ. Colorado, Boulder) using an automated system based upon the solid-support phosphodiamidite method (122) and purified by reversed-phase high performance liquid chromatography (Applied Biosystems Model 380A Automated DNA Synthesizer, Applied Biosystems, Foster City CA). The probe sequences were synthesized based on the protein sequence of alpha-Gal B near the N-terminus at a site semi-homologous to alpha-gal A (amino acids 8-13 of alpha-gal B, Figure 21). The probe was synthesized as a mixture of 256 sequences, since all four nucleotides were added at the third position of four ambiguous codons predicted by the amino acid sequence.

Two other oligonucleotides were synthesized later in this project as the DNA sequencing of a plasmid, isolated by hybridization of the 18-mer to a cDNA library, was concluded. Several alpha-gal B tryptic-peptides were isolated and sequenced, then two oligonucleotides, a

FIGURE 20. The N-terminal Sequences of alpha-Gal A and B and the Octadacanucleotide Probe (18-mer).

α-Gal A:	Leu	Asp	Asn	Gly	Leu	Ala	Arg		Pro	Thr / Tyr	Met	Gly			Leu				
α-Gal B:	Leu	Asp	Asn	Gly	Leu	Leu	Gln	Thr	Pro	Pro	Met	Gly	Trp	Leu	Ala	Trp	Glu	Arg	Phe

		(coding)	5'	A	C	X	C	C	X	C	C	X	A	U	G	G	G	X	U	G	G	3'
18-mer		(non-coding)	3'	T	G	X	G	G	X	G	G	X	T	A	C	C	C	X	A	C	C	5'

mixed 17-mer (64 sequences) and a mixed 14-mer (16 sequences), corresponding to amino acids from two peptides were synthesized by Applied Biosystems and supplied as crude mixtures (Figure 21). These probes were purified as suggested by the company through 30% gels.

Kinase Labelling. This method, a modification of Watseth and Johnson (211), was used to reliably label the 5'end of the oligonucleotide probe as well as DNA fragments used for sequencing. The 18-mer (80 pmoles) or DNA fragment was mixed with ^{32}P-γ-ATP (sp.act. 3000 Ci/mmol; Amersham), 25 U T4 polynucleotide kinase, and 2.5 ul 10X kinase buffer (10X buffer = 0.5 M Tris-Cl, pH 7.6/0.1 M $MgCl_2$/50 mM dithiothreitol/1 mM spermidine/1 mM EDTA), and the volume was brought to 25 ul with H_2O. The mixture was incubated at 37°C for 30 min and the reaction was terminated by heating to 80°C for 3 min. The mixture was diluted with 100 ul 1X SSC (Standard Saline Citrate = 0.15 M NaCl/0.015 M sodium citrate, pH 7.4) and loaded onto a 0.5 ml DE-52 (Whatman) column. The column was washed with 1X SSC (2.5 ml), then with 20X SSC (2.5 ml). Fractions (0.5 ml) were collected and radioactivity was determined by liquid scintillation spectrometry (Cerenkov counts).

Determination of Stringent Conditions for Hybridization and Washing. The conditions for hybridizing the ^{32}P-

Figure 21. The DNA Sequences of the Synthetic 17-mer and 14-mer Mixed Oligonucleotides.

Synthetic Oligonucleotide Probes

N-terminal 18-mer: 3' C C A (G/C/A/T) C C C A T (G/C/A/T) G G (G/C/A/T) G G (G/C/A/T) G T 5' (256)

Internal peptides:

14-mer 3' T A C C G (G/C/A/T) G T (C/T) C T (A/G) C C 5' (16)

17-mer 3' C T (A/G) T A C C C (G/C/A/T) A T (A/G) T G (G/C/A/T) A T 5' (64)

labelled probe to DNA bound on nitrocelllulose filters and washing non-specific hybrids from the filter were determined using modifications of the procedures described by Wallace et al. (210).

Human placental DNA (100 ug) was digested with Eco RI endonuclease (200 units) for 3 hours. Digested DNA was divided into four 25 ul aliquots to which 65 ul LoTE (10 mM Tris/0.1 M EDTA, pH 7.4) and 10 ul tracking dye (0.25% bromophenol blue/0.25% xylene cylanol/30% glycerol) was added. Samples were loaded into individual wells of a horizontal 0.7% agarose gel and electrophoresed at 1 V/cm overnight. The gel was stained with ethidium bromide and photographed under UV light, then Southern blotted (75) onto nitrocellulose (Schleicher and Schuell BA85). The filter was washed in 6X SSC/0.5% SDS (1X SSC = Standard Saline Citrate 0.15 M NaCl/0.015 M sodium citrate, pH 7.4) for 5 min, then prehybridized at room temperature in 6X SSC/5X Denhard's/10 ul/ml single-stranded salmon sperm DNA for 4 h (1X Denhart's = 0.02% Ficoll/0.02% polyvinyl-pyrrolidone/0.02% BSA). The filters were hybridized with 0.24 ug <32P-labelled probe at 12°C for 24 h in 6X SSC/5X Denhardt's/10 ug/ml salmon sperm DNA, then were washed in 2 changes of 6X SSC at 12°C for 1 h and air dried. The 4 lanes were cut apart and each lane was washed in 6X SSC at a different temperature (12°C, 20°C, 37°C, or 42°C).

The filter strips were autoradiographed on X-OMAT x-ray film at -80°C.

Determination of Probe Specificity. Human placental DNA, chicken DNA, and E. coli DNA were individually digested with Eco RI and Bam HI in separate digestions. Aliquots containing 10 ug DNA were electrophoresed through a horizontal gel, transferred to a nitrocellulose filter, washed and prehybridized as described. The filter was hybridized in 6X SSC/5X Denhardts/10 ug/ml salmon sperm DNA with a total of 0.24 ug of the ^{32}P-labelled probe at 20°C for 24 h, then washed in 6X SSC successively at 20°C, 37°C and 42°C.

Screening a Human Fetal Liver cDNA Library. The cDNA library was obtained from S.H. Orkin (Children's Hospital Medical Center, Boston, MA) (127). Essentially, double-stranded cDNA greater than 400 base pairs (bp) in length was dC tailed and inserted into the dG tailed plasmid vector pKT218 at the Pst I site. The colonies produced after transformation of the E. coli strain MC 1061 are tetracycline-resistant. The library was obtained as a glycerol stock, titered and plated onto 150 mm LB-agarose (Lurea-Bertani medium: 1% Tryptone/1%NaCl/0.5% yeast extract (w/v) pH 7.5) plates with 15 ug/ml tetracycline at a density of 15,000 colonies/plate. A total of

200,000 colonies were screened as described by Hanahan and Meselman (65).

An aliquot of the glycerol stock library was diluted into LB. After a 20 min incubation at 37°C, 1.0 ml containing approximately 15,000 bacteria was plated onto perfectly dry, day-old 1.5% LB-agarose plates containing 15 ug/ml tetracycline. the plates were then incubated approximately 24 hr until colonies were approximately 0.4 mm in diameter. An 82 mm nitrocellulose filter was placed carefully on the plate, keyed and quickly pulled from the plate. Plates were incubated an additional 8 hr to regenerate bacteria, and a duplicate filter was pulled.

The colonies on the filters were denatured by laying the filter (colonies up) on puddles of 0.3 M NaOH (3 min), then neutralized and washed in 1M Tris, pH 7.4 (5 min), then in 1.5 M NaCl/0.5 M Tris, pH 7.4/5% SDS (3 min). Filters were baked *in vacuo* at 80°C. The filters were then hybridized as described, except that the hybridization of the probe and successive washes were done at 42°C.

After autoradiography, colonies positive on duplicate filters were picked and diluted into LB+tet medium. The filters were rehybridized at 37° in order to detect any additional, less stringently hybridizing clones.

Colonies were picked from plates, diluted in LB with 15 ug tetracycline/ml and stored at 4°C.

Autoradiography. Filters or gels were taped to a backing of Whatman 3MM paper, asymmetricly marked with radioactive ink or a fiber tipped-pen, wrapped in Saran Wrap, placed against X-ray film, sandwiched between two calcium-tungstate-phosphor screens (DuPont Cronex Lightening Plus Intensifying screens) and exposed at -80°.

Plasmid DNA mini-Preparation. The DNA was prepared from 4 ml overnight cultures of selected plasmids by a modification of the alkaline lysis procedure (96, 210). The pelleted bacteria were lysed with 200 ul GET buffer (50 mm glucose/10 mm EDTA/25 mm Tris, pH 8.0) for 5 min at room temperature, then 400 ul alkaline SDS (0.2 M NaOH/1% SDS) was added and the mixture was incubated for at least 5 min at 0°C. Pre-cooled 3 M sodium acetate was added and, after at least 5 min at 0°C, the mixture was centrifuged at 11,000 x g. The supernatant was recovered, phenol/chloroform extracted, and the DNA was precipitated with ethanol.

Large Scale Plasmid DNA isolation. A 1 litre, chloramphenicol-amplified overnight bacterial culture containing a selected plasmid was centrifuged at 5000 x

g, and the washed pellet was resuspended in 3.6 ml GET buffer at room temperature, vortexed, and 0.4 ml lysozyme (50 mg/ml in GET) was added. After the vortexed mixture incubated at room temperature for 10 min, 8.0 ml alkaline SDS was added and the mixture was incubated at 4°C for 10 min. Cold sodium acetate, pH 4.8, was added (6.0 ml), and the mixture was mixed by inversion, then centrifuged at 20,000 x g for 20 min. The supernatant was recovered, precipitated, resuspended in LoTE, pH 8.0, then centrifuged through a cesium chloride gradient containing ethidium bromide, as described (118). The ethidium bromide was extracted, and the DNA was dialyzed against LoTE, pH 8.0, then precipitated with ethanol.

Restriction Enzyme Digestions and Mapping. Restriction endonucleases were purchased from various manufacturers and used according to their suggestions, often including 1 ug/ml DNase-free RNase.

Agarose GelElectrophoresis. DNA was electrophoresed generally through 0.7% to 1.5% DNase free, ultrapure agarose as described (118) using TBE electrode buffer (0.89 M Tris-borate/0.89 M boric acid).

Isolation of DNA Fragments. DNA of interest was restriction enzyme-digested, kinase labelled, and electrophoresed through an appropriate concentration of

agarose gel containing ethidium bromide. A slot was cut either before or after the fragment of interest and the DNA was electrophoresed into a piece of dialysis tubing inserted into the slot. The DNA was then released by quickly reversing the current, and the buffer containing the DNA was aspirated, extracted with butanol, then phenol/chloroform, and DNA was precipitated with ethanol.

Blotting. DNA was transferred from agarose gels to nitrocellulose as described by Southern (175). "Dot-blots" were prepared by applying DNA to nitrocellulose through a vacuum manifold (Schleicher and Schuell).

DNA Sequence Analysis. Maxam-Gilbert Sequencing. cDNA was isolated, digested and labelled as described above. A plasmid, pI-33, was selected and the Pst I cDNA insert was isolated. The fragment (1.1 kb) was cut with Bam HI, kinase labelled, redigested with Eco RI, and the Eco RI-Bam HI fragment was isolated. Alternatively, the whole plasmid was cut with Bam HI, kinase labelled and redigested with Pvu II. The Bam HI-Pvu II fragment, containing the cDNA insert, was purified. Both fragments were sequenced by Maxam-Gilbert reactions (123).

Dideoxy Sequencing in M13. Fragments of pI-33 bound by Pst I-Rsa I, Pst I-Eco RI and Bam HI-Eco RI RE sites were subcloned into the M13 bacteriophage vectors mp8 and mp9,

and mp18 and mp19 (7) (Figures 23 and 24). JM109 (300 ul of an overnight culture in M9 media) was transformed by approximately 400 ng of the plasmid subclone, and 10 ul IPTG (24 mg/ml) and 50 ul X-gal in dimethylformamide was added, after heat shocking the transformation reaction at 42^{o}C for 2 min. The cells were plated in 0.7% YT-agarose (8 g tryptone/5 g NaCl/5 g yeast extract) onto 1.5% YT-plates. White colonies were isolated and single-stranded bacteriop[hage was isolated according to the Pharmacia procedure supplied with their M13-dideoxy sequencing kit. Clone insert sizes were estimated by electrophoresing intact bacteriophage through an 0.8% agarose gel containing ethidium bromide (DIGE; Pharmacia). Modified dideoxy-sequencing reactions (158) were performed with materials from Pharmacia Fine Chemicals.

FIGURE 23. Subcloning Fragments of pI-33 into the M13 Vectors mp8 and mp9 Prior to Dideoxy sequencing.

The whole plasmid, pI-33, was digested with Eco RI and Bam HI and subcloned into Eco RI/Bam HI digested M13 mp8 and mp9. The identity of the cDNA inserts could be decided by sizing by DIGE or by the restriction enzyme site Pst I. B: Bam HI; E: Eco RI; P: Pst I restriction enzyme sites.

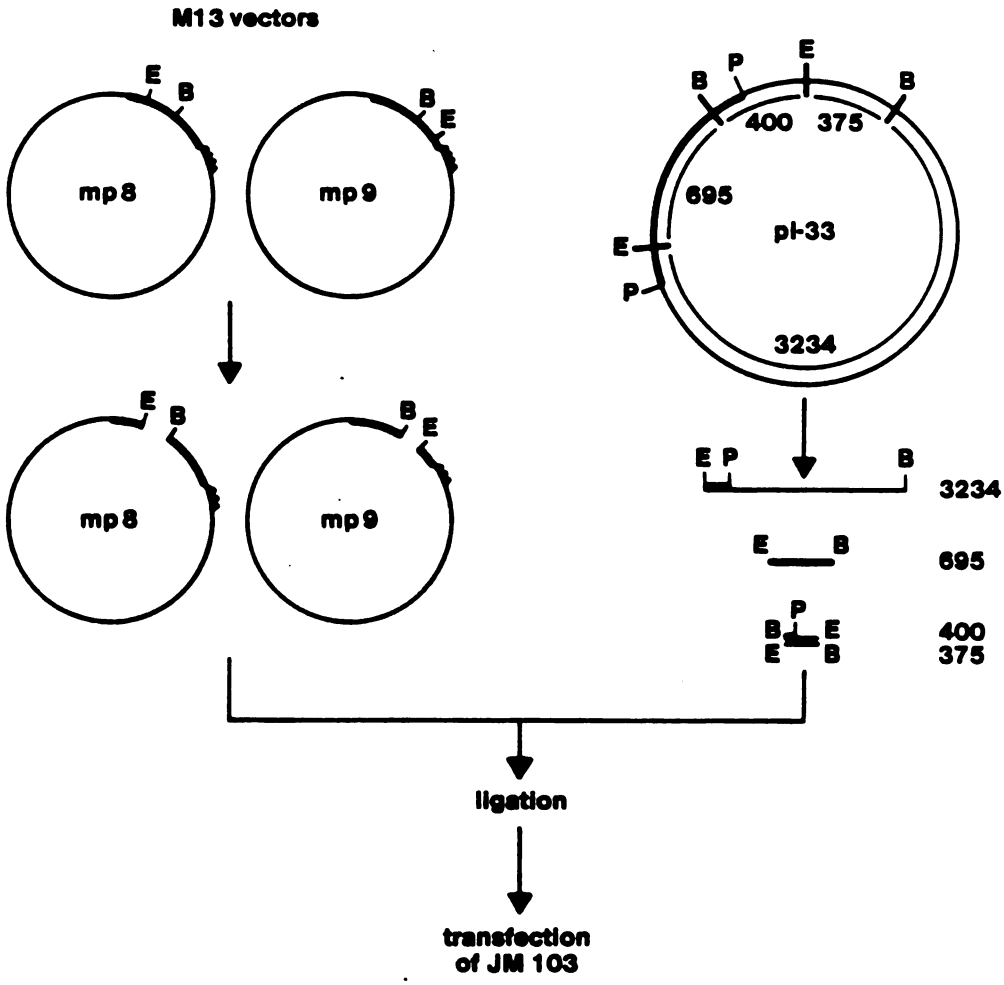
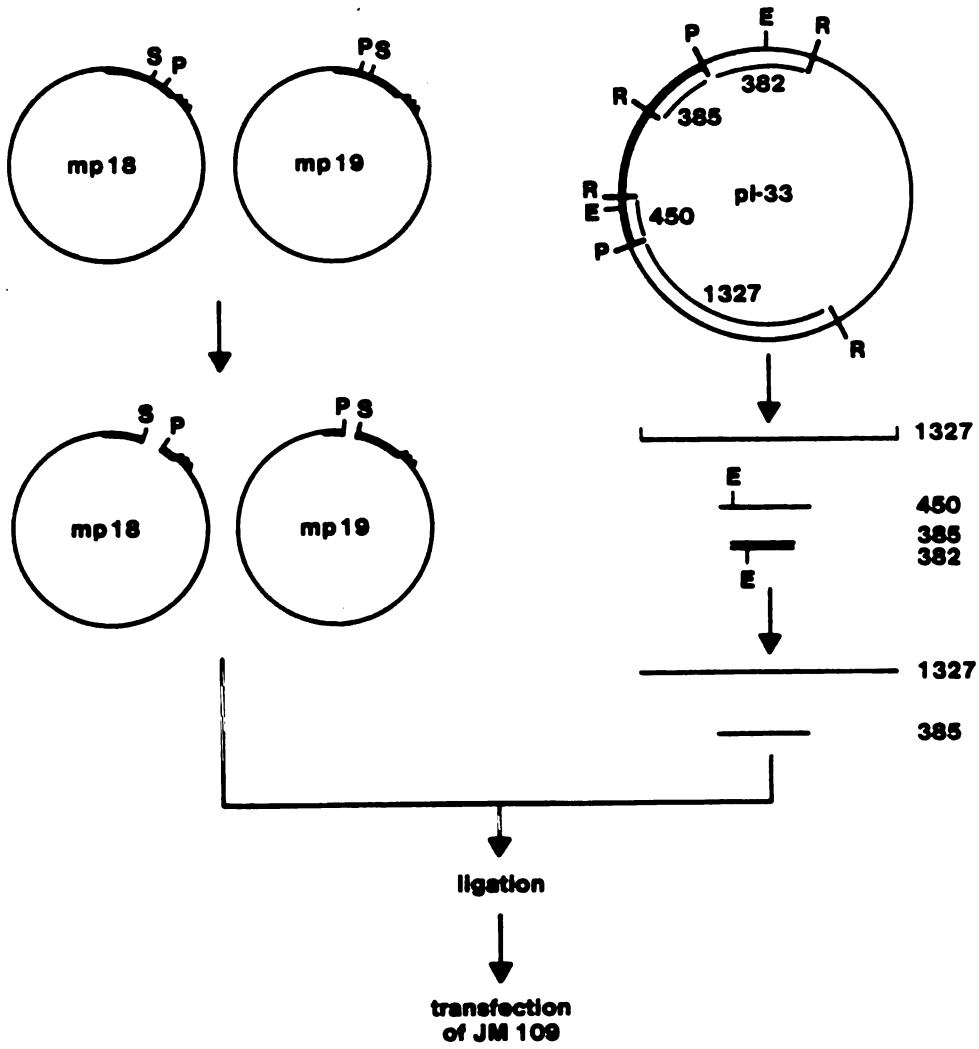

M13 vectors
mp8
mp9
mp8
mp9
E
B
B
E
P
400
375
695
pI-33
3234
E P
B
3234
E B
695
P
B E
E B
400
375
ligation
transfection
of JM 103

FIGURE 24. Subcloning Fragments of pI-33 into the M13 Vectors mp18 and mp19 prior to Dideoxy Sequencing.

Whole plasmid, pI-33, was double digested with Rsa I and Pst I, then digested with Eco RI. Pst I-Rsa I fragments could then be subcloned into the complementary Pst I and Sma I RE sites in the M13 bacteriophages mp18 and mp19. E: Eco RI; P: Pst I; R: Rsa I; S: Sma I. Recombinant bacteriophages could be identified on the basis of the insert sizes.


S P
mp18
P S
mp19
P E R
382
R
385
R
E
450
P
pI-33
1327
R
1327
E
450
385
382
E
1327
385
ligation
transfection
of JM 109

RESULTS

Wallace et al. (210, 225) showed that stringent hybridization criteria can be used to select a single correct sequence from a mixture of oligonucleotides whose sequences represent all possible codon combinations predicted from a particular peptide sequence. By increasing the temperature of hybridization and using a high salt solution, the duplexes with single mis-matches can be eliminated.

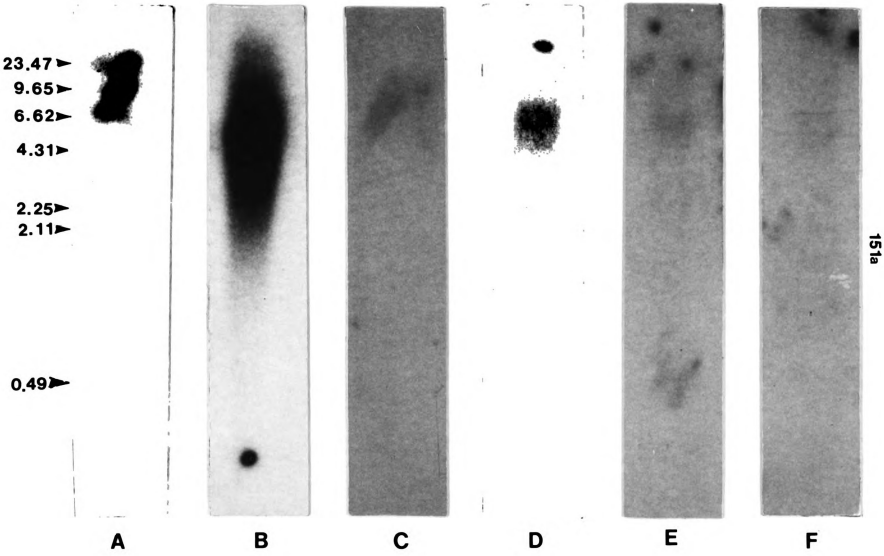
Since the probe designed from the alpha-gal B sequence was actually a mixture of 256 different 18-mers, it was necessary to determine if the probe would selectively hybridize to human DNA and if duplexes were thermally stable. This was accomplished by hybridizing the ^{32}P-labelled 18-mer to a blot prepared from Eco RI digested human placental DNA at a low temperature and washing at progressively higher temperatures.

Figure 22 shows the results of hybridizing the 18-mer to the blot at 12°C and washing at 12°C, 20°C, 37°C and 42°C. The 12°C wash (B) shows a high degree of hybridization extending from the high molecular weight DNA to approximately 1.5 kb. with the greatest concentration of radioactivity from 7 kb to 4 kb. As the wash temperature increased (C-F), the area and intensity of radioactivity decreased, indicating more specific hybridization of the probe to genomic DNA. At 42°C (F), a faint blot is seen at approximately 4 kb. Because washing may not remove all the initially binding probe, this was repeated by hybridizing human DNA to the probe at 42°C and similar results were seen.

The differences observed in the thermal stability of hybridization suggested that the non-selective hybridization to human DNA could be virtually eliminated at higher temperatures by eliminating mis-matched or

FIGURE 22. Hybridization of the 18-mer to Human Genomic DNA.

Human placental DNA was digested with Eco RI, electrophoresed through a 0.7% agarose gel and Southern blotted to nitrocellulose. The 18-mer was hybridized at 12°C and washed at different temperatures.
Lane A: Lambda Hind III washed at 12°C
Lane B: Human placental DNA washed at 12°c
Lane C: Lambda phage Hind III fragments washed at 20°C
Land D: Human placental DNA washed at 20°C
Lane E: Human placental DNA washed at 37°C
Lane F: Human placental DNA washed at 42°C


23.47
9.65
6.62
4.31
2.25
2.11
0.49
A
B
C
D
E
F

semi-homologous pairing, even with the very mixed 18-mer. In order to determine if the 18-mer was selective for human DNA, a second blot experiment was performed with DNA from various sources. DNA from human placenta, a transformed human cell line (T3), two chicken sources, and E. coli was digested with Eco RI and, in a separate digestion, with Bam HI. As the temeperature of hybridization increased, hybridizationto lambda DNA and E. coli decreased dramatically and was eliminated at 42°C. Hybridization to the other DNAs also decreased, with the T3 and placental DNA retaining the most radioactivity.

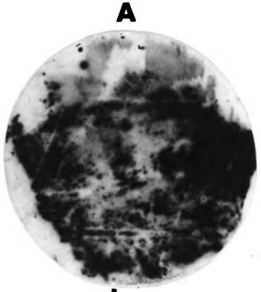
These results showed that hybridization to lambda and E. coli could be eliminated and that hybridization to human DNA was preferred by the 18-mer. In another experiment, plasmid DNA, pBR322, was digested and blotted, then hybridized to the 18-mer. Above 30°C, no hybridization was seen.

The human fetal liver cDNA library presumably contains sequences homologous to the DNA sequences in the genes coding for alpha-gal B and A. Using the conditions of stringency determined in the blotting experiments, colonies containing cDNAs homologous (at 42°C) or semi-homologous (at 37°C) to the 18-mer were isolated.

The library was screened as described at low (20°C), medium (37°C), and high (42°C) stringencies. The hybridization decreased as the temperature increased, as expected (Figure 25). Of the 200,000 colonies screened, 111 were positive at high stringency. These were selected and re-screened and a total of 131 positive clones were selected, some having more intense hybridization signals than others. (In the secondary screening, more than one colony was selected from several plates when the colony sizes or autoradiographic signals seemed different, or when all clones on the secondary plate were positive. This resulted in the isolation of more clones than were selected from the primary screening.)

Figure 25. Screening the Human Fetal Liver cDNA Library: Differential Hybridization of the 18-mer.

The 18-mer was hybridized to the cDNA library on filters at A: 20°C, low stringency; B: 37°C, medium stringency and C: 42°C, high stringency. Colonies identified by the arrows were positive at medium and high stringencies on duplicate filters (second filter not shown).


A
B
C
low
med
high

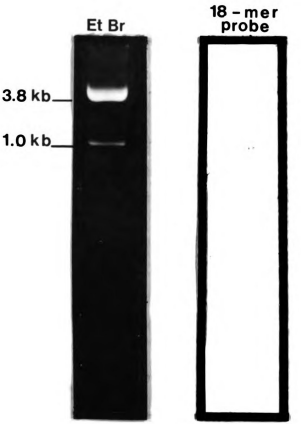
The DNAs from these clones were isolated, digested with various restriction endonucleases (RE), Southern blotted and hybridized to the 18-mer at 42 ^{o}C and 37^{o}C in 1 M salt as described. One clone had a 1 kb cDNA insert which stably hybridized up to 56^{o}C, and thus was thought to be a partial clone of alpha-gal B (Figure 26). A number of smaller clones hybridized with the 18-mer at 37^{o}C. This instability suggested only partial homology with one of the oligonucleptide sequences in the 18-mer mixture; thus, they were not likely candidates for alpha-gal B clones, but could possibly be homologous to alpha-gal A (considering the similarity of the protein sequence at the N-termini of the two enzymes).

The 1 kb clone, designated pI-33, was characterized by determining the recognition sites of a number of restriction enzymes (RE) (Figures 27 and 28). The 18-mer probe hybridizes to a specific 200 nucleotide (nt) long fragment bounded the RE sites of Rsa I and Bam HI, nearly 185 nucleotides from the Pst I end of the cDNA insert (Figure 29).

The isolated plasmid was digested as described in Methods (Figure 30), and sequenced by the Maxam-Gilbert method. The DNA sequence of the fragment was obtained from close to the Bam HI site beyond the Rsa I site and through the Hinf I and Dde I sites expected by the map (Figure 29).

Figure 26. Plasmid pI-33: cDNA Insert Size

Plasmid pI-33 was digested with Eco RI and electrophoresed through a 0.7% agarose gel and stained with ethiduim bromide (EtBr). The gel was Southern blotted and hybridized in 6X SSC at 50°C as described. The cDNA insert is 1.1 kb.

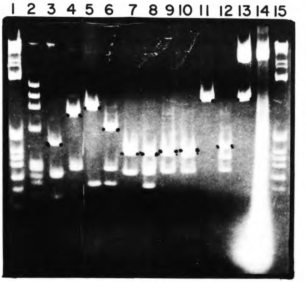


pI-33

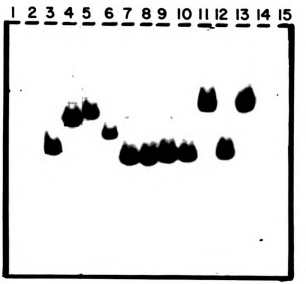
Figure 27. Hybridization of Restriction Enzyme Cut pI-33 cDNA Insert with the 18-mer Probe.

(A) The cDNA insert was isolated from pI-33 and digested with the restriction enzymes indicated.
(B) The gel was Southern blotted and hybridized to the 18-mer at 42^{o}C.

A. Ethidium Bromide Stained Gel



B. Autoradiogram of Southern Blot

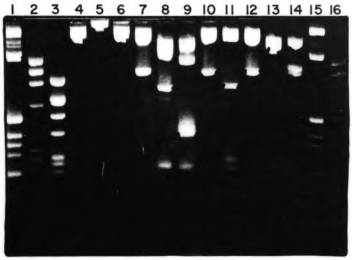


Key:

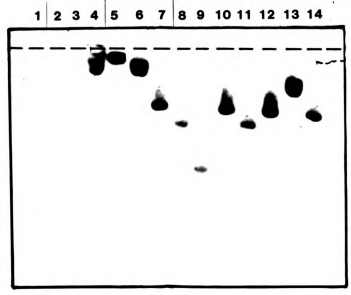
1) Hind III λ DNA, Hinf I pBR322 DNA size marker
2) Alu I cut pBR322
3) Alu I
4) Eco R I
5) Bam H I
6) Bam H I + Eco R I
7) Rsa I
8) Rsa I + Eco R I
9) Hinf I
10) Hinf I + Eco R I
11) Bgl I
12) Dde I
13) Pst I (whole pI-33)
14) Pst I (whole pI-55 contol)
15) Hind III λ, Hinf I pBR322 size markers

Figure 28. Hybridization of the 18-mer to Restriction Enzyme Digested Whole Plasmid pI-33.

A. Ethidium Bromide Stained Gel



B. Autoradiogram of Southern Blot



Key:

size markers
1) Hind III cut λ DNA, Hinf I cut pBR 322 DNA
2) ΦX Hae III pBR322 cut DNA
3) Alu I cut pBr 322
4) Cla I
5) Pvu I
6) Pvu II
7) Pst I
8) Pst I + Eco RI
9) Rsa I
10) Eco RI
11) Eco RI + Bam HI
12) Bam H I
13) Bgl I
14) Pst I + Bgl I
15) Taq I
16) Size markers Hind III A

Figure 29. Restriction Enzyme Map of the cDNA Insert from PI-33.

The isolated pI-33 cDNA insert was digested with restriction enzymes as described. Those cutting the cDNA insert are indicated. The 18-mer probe hybridizes to the Rsa I- Bam HI fragment indicated.

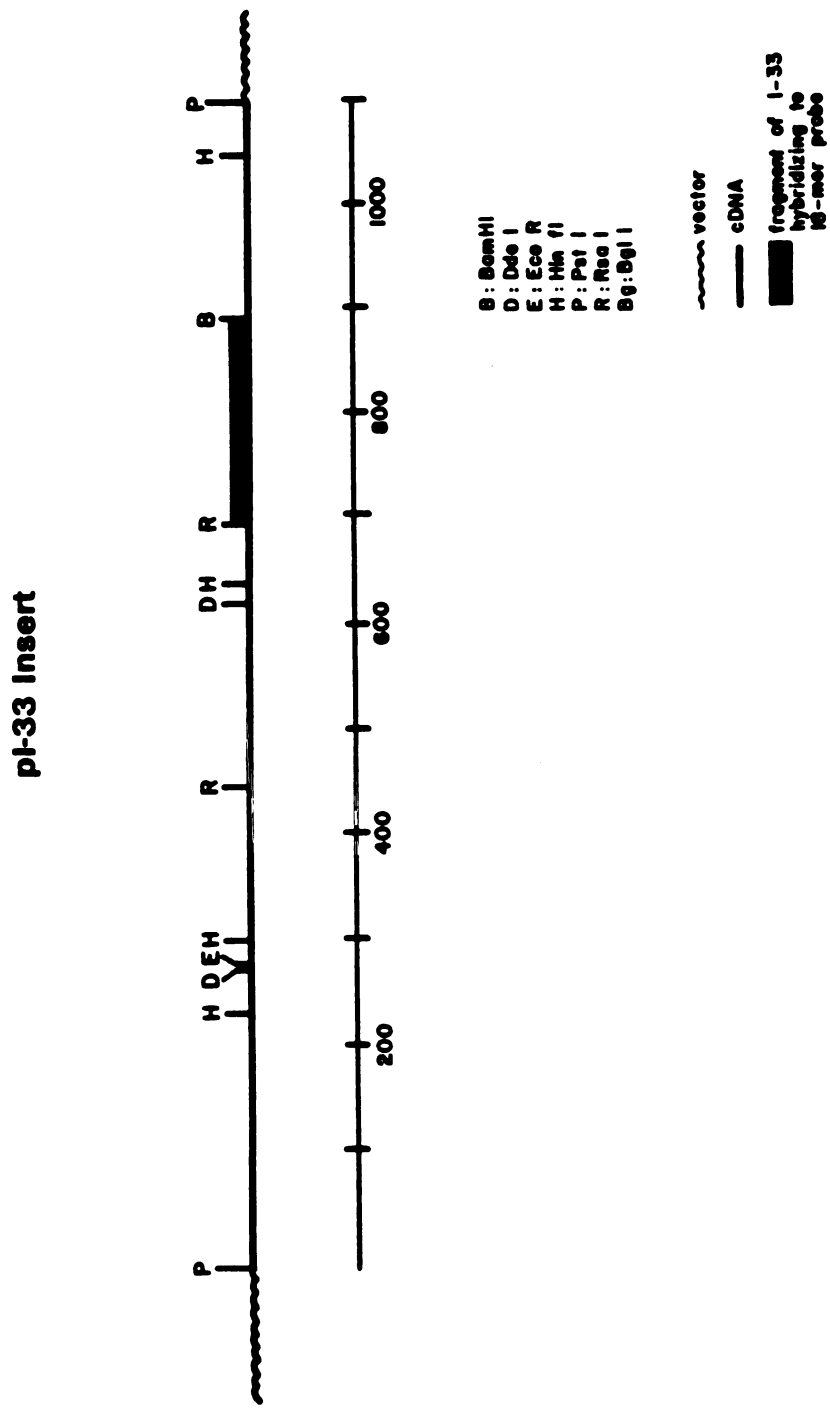
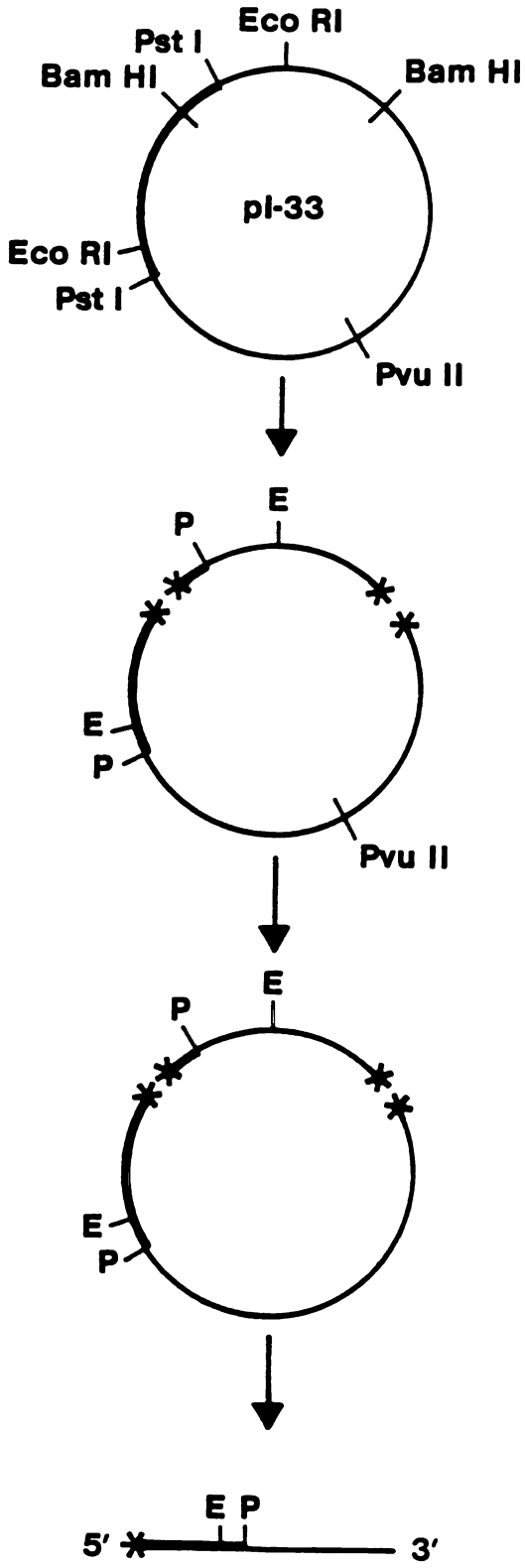

pI-33 Insert
P
H D E H
R
D H
R
B
H
P
200
400
600
800
1000
B: BamHI
D: Dde I
E: Eco R
H: Hin fI
P: Pst I
R: Rsa I
Bg: Bgl I
vector
cDNA
fragment of I-33 hybridizing to 48-mer probe

FIGURE 30. Maxam-Gilbert Sequencing Strategy

The plasmid, pI-33, was digested with Bam HI, kinase labelled (*) and redigested with Pvu II. The Bam HI-Pvu II fragment containing the majority of the cDNA insert was isolated and sequenced by the Maxam-Gilbert reactions. Alternatively, the cDNA fragment (Pst I-Pst I) was isolated, cut with Bam HI, and kinase labelled. Any 3' label at the Pst I site was removed by digesting the cDNA with Eco RI, then isolating the Bam HI-Eco RI fragment containing the majority of the cDNA insert. B: Bam HI; E: Eco RI; P: Pst I sites.


Eco RI
Pst I
Bam HI
Bam HI
pI-33
Eco RI
Pst I
Pvu II
E
P
E
P
Pvu II
E
P
E
P
E P
5′
3′

tThere were four spots where the probe mixture is 73% homologous with the DNA, the most homology found in the fragment.

In order to determine the DNA sequence across the Bam HI RE site, fragments of pI-33 bound by Pst I-Rsa I, Pst I-Eco RI, and Bam HI-Eco RI RE sites were subcloned into the M13 bacteriophage vectors (Figures 23 and 24). The cloning was largely successful as determined by the large number of white plaques appearing after transfection of E. coli strain JM 109.

The majority of these clones contained the same size insert as determined by direct gel electrophoresis through an 0.8% agarose gel. Representative plaques were picked and the single stranded bacteriophage was sequenced by the Sanger dideoxy chain-terminating reactions. Sequences obtained confirmed the sequence determined by the Maxam-Gilbert method. No further homology between the 18-mer and the cDNA sequence was seen.

Although there is an open reading frame, there was no colinearity between the nucleotide sequences of the pI-33 fragment and any known amino acid sequence of alpha-gal B or A (Figure 31).

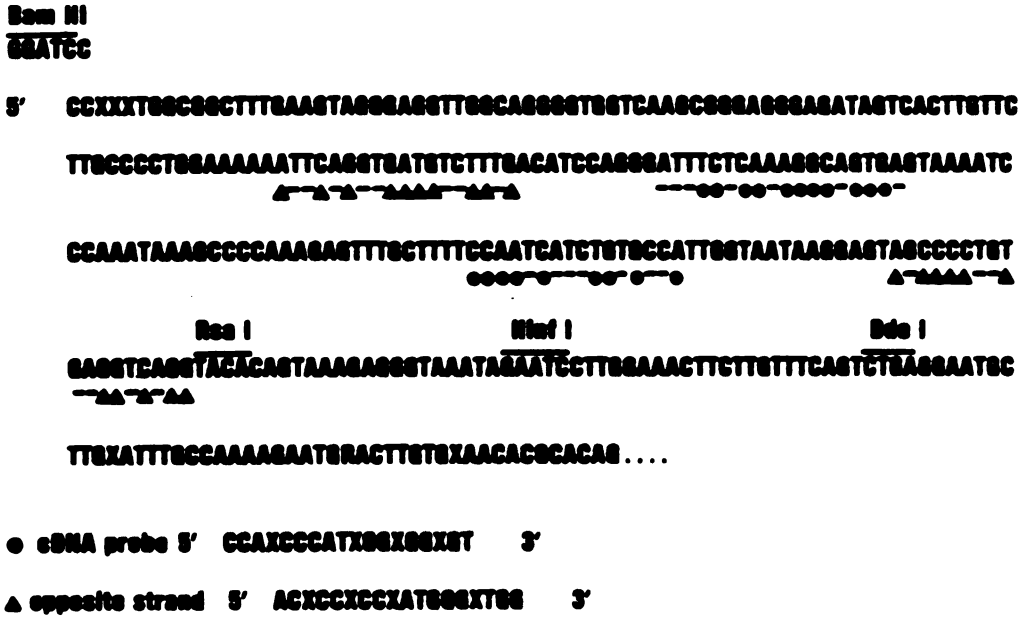
Figure 31. DNA Sequence of the Bam HI-(Rsa I)-Pst I cDNA from pI-33

The cDNA sequence was determined by Maxam-Gilbert Sequencing and confirmed by dideoxy sequencing from close to the Bam HI site through the Rsa I, Hinf I and Dde I sites.

"X" in the nucleotide sequence indicates a nucleotide which could not be determined;

+ or x indicates nucleotides exactly matching the 18-mer sequences and - indicates mismatches.

There are four places indicated where the 18-mer matches the cDNA sequenc with 73% homologous bases.


Bam HI
GGATCC
5'
Rsa I
Hinf I
Bde I
● cDNA probe 5' CCAXCCCATXGGXGGXGT 3'
▲ opposite strand 5' ACXCCXCCXATGGGXTGG 3'

At that time, eight other peptides from alpha-gal B were obtained and sequenced (219) (Figure 7). The amino acid sequence of two peptides could be translated into two distinct oligonucleotide mixtures of considerably less redundancy than the 18-mer mixture (Figure 22). The Protein Sequence Query (PSQ) and the Nucleic Acid Query (NAQ) banks were searched for protein and DNA sequences exactly matching and matching with the exception of any one nucleotide the regions of homology in the pI-33 fragment, the 18-mer, and a 17-mer and 14-mer mixture corresponding to the two peptide sequences. The banks listed only one viral protein matching the 14-mer with one nucleotide mismatch. Since using the 17-mer and 14-mer as probes seemed to offer a better chance of isolating an alpha-gal B cDNA, these sequences were synthesized by Applied Biosystems.

Specificity of the probe mixtures and stringency were determined using human placental and liver DNA, mouse DNA, E. coli, lambda, and plasmid pKT218 DNA. These probes proved unsatisfactory for screening the fetal liver library: the 14-mer hybridized as strongly to E. coli DNA as to human DNA even at high stringencies, and the 17-mer hybridized to pKT218. Whether this hybridization was due to homologous sequences between the DNA and probe mixtures, high G/C hybridizing to the G/C

tailing of the plasmid or secondary structures is not known.

The method of Wood *et al.* using base composition-independent hybridization in tetramethylammonium chloride (221) was tried in order to eliminate hybridization with the 14-mer and 17-mer. However, hybridization to the *E. coli* and plasmid DNA could not be eliminated preferentially over human DNA.

The original plasmids hybridizing to the 18-mer at high stringency were isolated, digested to separate the cDNA insert from the plasmid DNA, electrophoresed and Southern blotted. The filters were then hybridized to the 17-mer mixture at high stringency, but none of the cDNA inserts showed any hybridization to the probe.

In lieu of more protein sequence that could be translated into different probes, or obtaining or synthesizing a new project was set aside. At this time we had obtained a cDNA expression library and had produced specific anti-alpha-gal B IgGs. We continued our isolation attempts by screening the expression library with the anti-B IgGs.

C. IMMUNOSCREENING A LAMBDA gt11 cDNA EXPRESSION LIBRARY

Improvements in the purification of alpha-gal B made it possible to raise very specific polyclonal antibodies in rabbits. This, in association with the development of the lambda gt11 expression vector (223), opened the possibility of a third method of isolating the gene for alpha-gal B.

The lambda gt11 exression vector-host system makes it possible to identify foreign antigens in E. coli by overcoming major problems associated with the expression and accumulation of foreign proteins in the bacterium. Foreign DNA is placed under the control of an E. coli promotor which is efficiently recognized and used by the bacterial translation machinery. By fusing the foreign DNA to a selectively inducible bacterial gene that produces a stable protein, and using bacterial hosts deficient in specific proteases, the problem of excessive accumulation of foreign proteins which may be harmful to the cell is eliminated, as are the problems of unstable foreign gene products and degradation by bacterial proteases.

The lambda phage expression vector is designed so that foreign DNA may be inserted into the beta-galactosidase structural gene lacZ under the control of the lac operator. The expression of the foreign DNA is repressed by the lacI gene product and the foreign DNA can be propagated lytically. Rapid induction of the fused gene is accomplished by the addition of isopropyl-beta-D-thio-galactoside (IPTG) to the culture medium. The presence of the lon mutation permits the accumulation of a relatively stable beta-galactosidase fusion protein.

Two other requirements for the immunologic detection of expressed antigens are monospecific antibodies which are able to recognize the primary (or unprocessed) sequence of the antigen (usually polyclonal, although monoclonal antibodies have been used successfully (130), and genomic or cDNA which includes multiple copies of the coding sequence for the desired protein.

This section describes the isolation of cDNA clones which are recognized by monospecific, polyclonal antibodies against mature and denatured alpha-gal B. The clones have been identified by immunological recognition and detection by enzyme conjugated-double antibody systems.

MATERIALS

Tissue culture and bacterial culture media was from Gibco, Grand Island, NY. Restriction enzymes were from BRL, New England Biolabs, Boehringer Mannheim or Promega Biotech and were used as suggested. SDS-Page markers and other reagents for SDS-PAGE were from Bio-Rad, as were DNA markers (Lambda Hind III digestions and Phi X Hae III). Nitrocellulose was from Schleicher and Schuell. Ultrapure agarose and NuSieve agarose were from FMC. BSA, Tris-Cl and other reagents were purchased through general chemical suppliers. Ribi Probe MPL + TDM emulsion was from RIBI Labs. A-gel Chromatography matrix was from Chemicon Inc. Horseradish peroxidase goat-anti-rabbit affinity purified IgG was purchased from Bio-Rad. HRP substrate, 4-chloro-1-napthol was from either Aldrich Chemical or from BioRad. Alkaline phosphatase-conjugated goat-anti-rabbit affinity purified IgG was from Promega Biotech. Substrates for the alkaline phosphatase-conjugated IgGs were from Promega Biotech in a kit form, or were purchased from Sigma Chemicals.

METHODS

Cell Lines and Bacterial Strains. E. coli strains are described in ref. 223. Y1090 was used for propagation of the library (E coli lacU169 proA$^+$ lon araD139 strA

supF[trpC22]::TN10] (pMC9) (amp^r). Y1089 is a protease deficient E coli used for the isolation of fusion proteins: E. coli lacU169 $proA^+$ lon araD139 strA hlflA[chr::Tn10] (pMC9) (amp^r). Y1088 is a DNase deficient E. coli used for DNA isolation: E. coli lacU169 supE supF $hsdR^-$ $hsdM^-$ metB trpR tonA21 proC::TN5 (pMC9) (amp^r). BNN103 (lambda gt11) is a lysogenic strain of E. coli C600, containing an incorporatd, inducible lambda gt11 phage genome and was used as a beta-galactosidase-fusion protein control. The genotype of lambda gt11 is lac5 (shindIII lambda 2-3) sri lambda3^o cI857 srIlambda4^o nin5 srI lambda5^o Sam100. The plasmid pMC9 is pBR322-$lacI^Q$ which confers ampicillin resistance (amp^r) to the E. coli strains Y1090, Y1088, and Y1089.

Chang liver cells are an "apparently normal" (not virally transformed, but immortal), human liver cell line (American Type Culture Collection [ATCC] CCL 13). HepG2 and Hep3B are uncharacterized, immortal, human hepatoma cell lines containing hepatiti virus particles (ATCC HB8064 and HB8056). The FS-4 fibroblast line is an apparently normal human foreskin fibroblast cell line obtained from the MIT Cell Culture Center.

SDS-Polyacrylamide Gel Electrophoresis. SDS-PAGE was performed essentially by the procedure of Laemmli (104).

The stacking gel was normally 3% acrylamide (30:0.8::acrlyamide:bis), pH 6.8 and the separating gels were 10% or 12%, pH 8.8.

Silver Staining. The silver staining procedure of Wray et al. (222) was used after the gel was soaked in 50% reagent-grade methanol for at least 8 hr.

Western Transfer. Proteins separated by electrophoresis were transferred to nitrocellulose (45 um Schleicher and Schuell) using the Bio-Rad Trans-Blot cell as directed by the manufacturer. The gel and nitrocellulose were pre-equilibrated in transfer buffer (25 mM Tris base/192 mM glycine/20% reagent grade methanol, approx. pH 8.3) for at least 30 min, but no longer than 1 hr. The transfer was run at 30V for at least 6hr, followed by 70V for 2hr. Filters were used immediately.

"Dot-blotting". Proteins (and DNA) were applied to nitrocellulose through a multi-well vacuum manifold from Schleicher and Schuell according to the manufacturer's directions.

Immunization of Rabbit with alpha-gal B. Homogeneous alpha-gal B was suspended in RIBI MPL-TDM emulsion, 0.1 mg/ml, and 1 ml was injected into a 12-week-old rabbit at multiple sites on the back. Three booster shots (1 ml

each) were given of the same suspension on days 14, 28, and 35. The rabbit was bled on day 42 and, after clotting at room temperature, serum was obtained by centrifugation at 2500 x g. This crude anti-serum was used as the basic material for the immunological detection and screening.

Purification of Anti-alpha-gal B IgG. The IgG fraction of the anti-serum was selected on a Protein A-Sepharose 4B affinity chromatography matrix (Sigma Chemicals). The crude serum was applied to the column, which was then extensively washed with 0.01M NaP, pH 8.2. The IgG fraction was eluted with a step-wise gradient of 0.1M citrate buffer, pH 6.0, 4.5, and 3.0. One ml fractions were collected, titrated to pH 7.4, and tested for the ability to immunoprecipitate enzyme activity. Active fractions were pooled and concentrated.

Antigenic determinants to E. coli and coliphage proteins were removed by absorbing the IgG fraction to cyanogen bromide-activated Sepharose 4B beads (120) to which E. coli and induced coliphage proteins were attached (223). E. coli strains Y1090, Y1089, and BNN103 (lambda gt11) were grown in separate 500ml cultures in LB media. The media for Y1090 and Y1089 was supplemented with 50 ug/ml ampicillin. The cultures were grown at 32°C until the OD_{600} reached 0.3, then the cultures were heat induced at

42°C for 20 min. IPTG (10 mM) was added and the cultures were incubated at 37°C for 3 hr. The cultures were pelleted by centrifugation at 3000 x g, resuspended in 10 ml DNase buffer (50 mM Tris-Cl, pH7.9/0.2 mM EDTA/10% (v/v) glycerol/2.5 mM $MgCl_2$/0.15 M NaCl/5 mM phenylmethylsulfonyl fluoride), frozen-thawed three times in a CO_2-EtOH bath, incubated at 37°C for 10 min with 10 ug DNase, then centrifuged at 8000 x g for 30 min. The protein suspension was incubated with the CNBr-activated Sepharose 4B beads (120), with inversion overnight at room temperature. The protein-bead matrix was then equilibrated with TBS and 1ml matrix was mixed with each mg IgG plus 0.2% sodium azide for 18 hours at room temperature. The unadsorbed IgGs were washed from the matrix with TBS, concentrated in an Amicon concentrator with an XM50 filter, and immediately purified over an alpha-gal B column.

Final purification of anti-alpha-gal B IgG involved binding pure alpha-gal B to A-gel (Chemicon International, Inc.) and purifying specific anti-alpha-gal B IgGs by affinity chromatography. Alpha-gal B (1 mg denatured with SDS, BME and boiling, and 1 mg undenatured) was dissolved in 0.1 M sodium carbonate buffer, pH 9.6, at a concentration of 1 mg/ml and added to a washed gel cake (approx. 5 ml) pre-equilibrated with 0.1 M sodium carbonate, pH 9.6. After shaking the

suspension gently overnight at room temperature, 10 ml of 0.1 M glycine was added and mixing was continued for 2 hrs. The gel was washed with 10 vol of 0.1 M acetic acid followed by 0.1 M Tris-Cl, pH 9.6, and, finally, equilibrated with sodium acetate buffer, pH 8.2. The anti-alpha-gal B IgGs (2mg/ml TBS) were passed through the column and eluted with a stepwise gradient of 0.1 M citrate buffer, pH 6.0, 4.5, and 3.0, after washing the column thoroughly with the equilibration buffer, and fractions were quickly neutralized. Anti-alpha-gal B activity was found in all fractions of the pH 6.0 and 4.5 washes and the first third of the 3.0 fractions. Thses fractions were pooled and concentrated to approximately 1 mg protein/ml.

Protein and Enzyme Sources. The specificity of the anti-serum was determined by immunoprecipitating enzyme activity and immunodetection ofprotein from crude tissue and cell homogenates, as well as pure enzymepreparations. E. coli strains Y1090 and Y1089 (223) were grown in Luria-Bertani (LB) media supplemented with 50 ug ampicillin/ml at 37°C until stationary phase was reached. BNN103 (lambda gt11) was grown in LB media at 32°C until the OD_{600} was 0.3 (doubling time ~40 min). Then the phage proteins were induced by incubating the culture at 42°C for 20 min, then incubating the culture at 37°C for 2-3 hr.

Human Chang liver cells and human FS-4 fibroblasts were grown in Minimal Essential Medium (MEM) supplemented with 10% calf and fetal calf serum, respectively. HepG2 and Hep3B human hepatoma cell lines were grown in OptiMEM media supplemented with 0.4% beta-mercaptoethanol (BME) and 5% fetal calf serum. All cells were harvested by trypsinizing (0.02% trypsin in Hanks salts) for 2 min., washed from the plates with phosphate-buffered saline (PBS), pelleted at 1500 x g, frozen-thawed, suspended in PBS at 10^9 cells/ml and sonicated with five 5-second blasts of a Brinkman sonicator. Mouse and human liver, and human placenta samples were minced and homogenized in PBS (1 mg/ml). Samples were centrifuged at 15,000 x g before the enzyme assay, SDS-PAGE, or blotting.

Immunoprecipitation of Activity. IgGSorb (a commercial preparation of Staph A protein) was prepared according to the manufacturer's directions, then 50 ul was pelleted by centrifugation at 1500 x g and the pellet was incubated with 50 ul antiserum at 37°C for 2 hr, followed by an 8-16 hr incubation at 0°C. After a second centrifugation to remove unreacted serum, the source of protein or enzyme (pure enzyme, cell or tissue lysates) and immune complex were incubated at 37°C for 30 min, then at 0°C for 8-16 hr with the IgGSorb/antiserum complex.

Immunocomplexed alpha-gal B was removed by centrifugation and, after washing the pellet with 20 ul PBS, both the pellet and supernatant fraction were assayed for alpha-gal A and remaining alpha-gal B activity.

Immunodetection of Antigens. This immunodetection method was used for the detection of antigens transferred from polyacrylamide gels (Western blot), dot-blotted to nitrocellulose, and for screening assays of the expression library. The protein-complexed nitrocellulose filter (0.45 u) was blocked in 5% bovine serum albumin (BSA)/Tris-buffered saline (TBS; 0.17 M NaCl, 0.01 M Tris-HCl, pH 7.5) for at least 3 hr. After three brief rinses in TBS, the filter was incubated with the purified anti-alpha-gal B IgG at a 1:100 dilution (approximately 0.03 mg/ml final purified IgG protein concentration) in 5% BSA/TBS for at least 6 hr at room temperature. The filter was again rinsed three times for five minutes each time in TBS, then incubated with a second, affinity purified and enzyme conjugated goat anti-rabbit IgG antibody (IgG H+L chains) for three hours at room temperature.

Two different commercially prepared enzyme-linked second antibodies were used separately to detect antigens: goat-anti-rabbit horseradish peroxidase-conjugated, affinity purified IgG (Bio-Rad), GAR-HRP, and an alkaline

phosphatase-conjugated goat anti-rabbit IgG (Promega Biotech), GAR-AlkP. Horseradish peroxidase activity was detected by incubating the double antibody-bound filter with 4-chloro-1-napthol and hydrogen peroxide substrate solution. 4-Chloro-1-napthol (60 mg) was dissolved in 20 ml cold, distilled methanol. Immediately before use, 60 ul fresh hydrogen peroxide was mixed with 100 ml TBS kept at 30°C. The hydrogen peroxide solution was immediately mixed with the 4-chloro-1-napthol solution, then incubated with the damp antibody-bound filter. Antigens were identified by the purple color. Alkaline phosphatase activity was detected according to the manufacturer's directions using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Positive antigens were identified by purple bands or spots.

Screening the lambda gt11 Expression Library with the Monospecific IgG. The procedure used was a modification of the method of Young and Davis (223). A human hepatoma cDNA expression library was provided by J.R. deWet and J.S. O'Brien (36) as a coliphage suspension, along with the appropriate host E. coli strains.

Briefly, the phage was diluted, added to 2 ml of a fresh overnight of Y1090 grown in Luria-Bertani media, pH 7.5 supplemented with 50 ug ampicillin/ml (LB+amp) which was

pelleted and resuspended in 500 ul $MgSO_4$, allowed to absorb to the host cell, and plated in 8ml LB-M+amp top agarose (LB plus 10 mM $MgSO_4$ and 50 ug amp/ml) at a density of 10^6 phage per 150mm LB-M+amp agarose plate. After incubating the plates at 42°C for 2 hr, each plate was overlaid with a nitrocellulose filter (BA85, 137 mm, Schleicher and Schuell) that had been soaked in 10 mM IPTG and air-dried, marked and incubated for three hours at 37°C. The filters were gently pulled from the plates and rinsed briefly in TBS. Duplicate filters were laid on the plates which were incubated for an additional two hours. Plates were stored inverted at 4°C until the chromogenic detection was complete. Approximately 10^7 phage were screened in this manner.

The filters were rinsed briefly in TBS, air-dried until damp, but not wet, placed in a chamber saturated with chloroform vapor for 15 min, and rinsed briefly in a solution of 0.01% SDS/150 mM NaCl/5mM PMSF/25 mM Tris-Cl, pH 7.4 for 30 min to remove bacterial debris, then blotted with Whatman 3MM paper saturated with 5 mM $MgCl_2$ to remove the SDS. After a brief rinse with TBS, the filters separately were immersed in 5% BSA/TBS and blocked for at least three hours.

In the screening procedure, the purified monospecific IgG were incubated at 37°C for one hr with 100 ug human liver

alpha-gal A and 100 ul of E. coli Y1090 and induced BNN103 (lambda gt11) stock protein suspensions (500 ml overnight cultures of each strain were pelleted, resuspended in 5 ml TBS and frozen/thawed three times. Stock suspensions were kept frozen at -20°C. The IgGs were diluted 1:100 in 5% BSA/TBS, pH 7.5, and incubated at least six hours with the filters at room temperature. The filters were then washed separately three times for 5 min each in TBS, then incubated individually with alkaline phosphatase-conjugated goat anti-rabbit affinity purified IgG (Promega Biotech) at a 1:7500 dilution as suggested. The remainder of the detection procedure was as described above and as suggested by the manufacturer. The color reaction, producing a dark purple color, is due to the substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-phosphate.

Selection of Positive Clones. Colonies which were positive by the chromogenic detection method described were picked from the plate, diluted, replated at a lower density (approx. 10^3 plaques/100 mm plate, with 85 mm nitrocellulose filters), and rescreened until all plaques on a plate from one phage were positive.

As a negative control, certain clones were replated as described for the library screening and duplicate filters were pulled from each plate. One filter was screened

with IgGs as for normal screening and one filter was incubated with IgGs which were preabsorbed with 0.5 mg pure alpha-gal B. The filters were then incubated with the alkaline phosphatase antibody as described.

Characterization of Positive Clones. Phage from positive plaques were isolated by removing an agar plug containing the plaque and suspended in sterile tubes containing 1 ml of 10 mM Tris-Cl, pH 7.5/10 mM $MgCl_2$ (SM) and 50 ul chloroform. Phage were allowed to diffuse from the agar plug overnight at room temperature and the suspension was stored at 4°C.

Expression of Fusion Proteins. A fresh overnight of E. coli Y1089, grown in LB with 10 mM $MgSO_4$ and 50 ug/ml ampicillin, was pelleted and resuspended in an equal volume of 10 mM $MgSO_4$. Phage from a resuspended plaque (100 ul from a 1 ml stock) was added to 50 ul of the bacteria, and the cells were incubated with gentle shaking at 32°C for 20 min. The cells were then diluted to different densities and replated on LB-amp plates (usually a 1:1000 dilution was necessary to obtain separated colonies) and incubated 3-6 hr at 32°C. Colonies were selected and streaked onto duplicate LB-amp plates. One plate was incubated at 32°C and one plate at 42°C. Colonies which grew at 32°C, but not at 42°C were selected as lysogens.

Three ml cultures of LB-amp were inoculated with selected lysogens and were grown 3-4 hr until the OD_{600} was approximately 0.3. Bacteria (1.5 ml of the fresh culture) was added to 40 ml LB-amp broth and incubated until the OD_{600} was again 0.3. The culture was then heat-induced at 44°C for 15 min, and then split into two 20 ml cultures. One culture was further induced by the addition of IPTG to 1 mM and the cultures were incubated with shaking at 37°C for 2-3 hr. The cultures were then pelleted at 8000 x g for 10 min at 25°C and resuspended in 1% original volume (200 ul) DNase buffer (50 mM Tris-Cl, pH 7.9/0.2 mM EDTA/10% glycerol/2.5 mM $MgCl_2$/0.15 M NaCl/5 mM PMSF) and frozen-thawed in a CO_2-EtOH bath. DNase was added (1 ug) and the suspension was incubated at 37°C for 15 min. The protein suspension was then frozen at -80°C until further use.

Approximately 1 ul of the suspension was sufficient for silver-stained electrophoresis gels, and for dot-blots to detect antigens using the horseradish peroxidase-conjugated double antibody detection system described above.

Isolation of Phage DNA from Plate Lysates. The plate lysate method used is a modification of E.F. Fritsch (118). Briefly, 50 ul of the phage suspension was mixed

with 50 ul of a fresh overnight of Y1088 grown in LB with 10 mM $MgSO_4$ and 50 ug/ml ampicillin, incubated at 32°C for 30 min, mixed with 2.5 ml 0.7% top LB-amp agarose and plated on 1.5% fresh LB-amp plates. The plates were incubated at 37°C for approximately 6 hr until the phage plaques were nearly confluent. The agar was scraped from the plate and suspended in 5 ml 10 mM Tris-Cl, pH 7.5/10 mM $MgSO_4$ with 200 ul chloroform. The tubes were shaken at room temperature overnight, centrifuged at 3000 x g and the supernatant (avoiding the chloroform) was transferred to a second tube. The suspension was again centrifuged at 8000 x g for 10 min at 4° to remove bacterial debris. The supernatant was incubated with DNase I and RNase A (1 ug/ml each) for 30 min at 37°C, then 1 ul diethylpyrocarbonate (DEPC)/ml SM was added and the suspension was incubated for 30 min at 37°C with shaking. An equal volume ice-cold 20% (w/v) polyethylene glycol 8000/2M NaCl was added and incubated at 0°C for 1 hr, the solution was centrifuged at 10,000 x g for 20 min, and the supernatant was drained gently. SM was added (0.5 ml) and the pellets were resuspended by vortexing, then centrifuged for 5 min at 4°C and 10,000 x g to remove bacterial debris. The supernatant was transferred to a fresh Eppendorf tube and 1/50 vol 10% SDS and 0.5 M EDTA was added and incubated at 68°C for 15 min. One vol of proteinase K (100 ug/ml in 0.01 M Tris-Cl, pH 7.9) was added and the suspension was incubated at

37°C for 30 min. The DNA was then extracted with phenol, phenol:chloroform, chloroform two to three times each, then precipitated with ethanol.

The resulting DNA (usually 1/5th of the total preparation) was digested with Eco RI restriction buffer and electrophoresed on agarose gels (1% ultrapure agarose plus 1% NuSieve agarose (FMC) in TBE) in order to size the DNA insert.

RESULTS.

The lambda gt11 library obtained from J. R. de Wet was constructed from cDNA copied from poly A^+ RNA isolated from a human hepatoma line, Li-7, which was grown in Balb/c athymic mice. The original hepatoma was determined to be 90% human in origin and 10% mouse from invading vascular tissue. The library contains approximately 8×10^6 independent clones with 75% of the phage carrying inserts.

The specificity of the alpha-gal B antibodies was tested by immunoprecipitating alpha-gal B activity from human FS4 fibroblasts, human Chang liver (normal) cells, two human hepatoma cell lines, HepG2 and Hep3B, human liver, human placenta and mouse liver. As seen in Table 6, the antibodies were most efficient in immunoprecipitating the

Table 6. Immunoprecipitation of Alpha-Gal B Activity.

Approximately 60 U alpha-gal B activity was immunoprecipitated from each enzyme source with the momo-specific anti-alpha-gal B IgG preparation.

Table 6. IMMUNOPRECIPITATION OF ALPHA-GAL B ACTIVITY.

Enzyme source	Specific Activity (nmol/min x mg)	Activity Remaining preimmune	Activity Remaining immune	% Activity Precipitated
Human liver	1.2	60	0	100
Human placenta	1.1	62	48	20
Mouse liver	0.69	58	56	0.3
Chang liver cells	0.10	61	60	0
FS-4 cells (fibroblast)	0.8	60	42	30
HepG2 cells	2.1	60	3	95
Hep3B cells	0.9	59	10	83

activity from human liver (100 %) and the human hepatoma cell lines. (Chang liver cells have little alpha-gal B activity as measured by the 4-MU-alpha-gal +/- GalNac assay and only 25% of the normal levels by p-NP-GalNAc assay). No activity was precipitated from the mouse liver and only slight and not always reproducible amounts were precipitated from human placenta and FS4 cell line.

The library was screened essentially by the method of Young and Davis (223), the major modifications being the chromogenic detection methods, the preparation of the library filters and the selection of monospecific IgGs. In order to ascertain whether our antibodies were sensitive and specific enough, the same protein sources were dot-blotted onto nitrocellulose along with media from the cell lines and lysates of BNN103, Y1090, and Y1089, E. coli derivatives which do not have alpha-gal B activity. Alpha-gal B, alpha-gal A and E. coli beta-galactosidase (Sigma) were included as controls. Whole serum, pre-immune serum, anti-alpha-gal A IgGs and selected, monospecific alpha-gal B IgGs were used as the first antibodies and horseradish peroxidase-conjugated affinity purified IgGs were used as the second antibody in the HRP chromogenic detection method described above.

The monospecific alpha-gal B IgGs detected protein in human liver and human placenta, but not mouse liver.

Cross-reactive protein was seen in the hepatoma cell lines and in spent medium from the Chang cells, but not in the Chang cell lysate. The background color was high (slightly positive) with lysates of induced BNN103 (lambda gt11) and Y1090, but after pre-absorbing the IgGs with protein from these cells this color was essentially eliminated. Alpha-gal A and beta-gal were not detected with the anti-B IgGs, but alpha-gal A from coffee beans (Sigma) and watermelon (isolated in this lab: M.J. Thome and K.J. Hawes) were both positive (Figure 31).

Alpha-gal A IgGs were not selected with an alpha-gal A affinity matrix. Alpha-gal A protein was detected in human liver, human placenta, FS4 fibroblasts (although activity cannot be precipitated from cell lysates), Chang liver cells, the hepatoma cell lines, and spent media from the Chang liver cells. No cross-reactive protein was precipitated from the mouse liver, or in spent media from the fibroblasts. Cross-reactivity was seen with alpha-gal B and beta-gal from E. coli. Positive reaction to BNN103 and Y1090 was eliminated after preabsorbing the IgGs with cell protein (Figure 31).

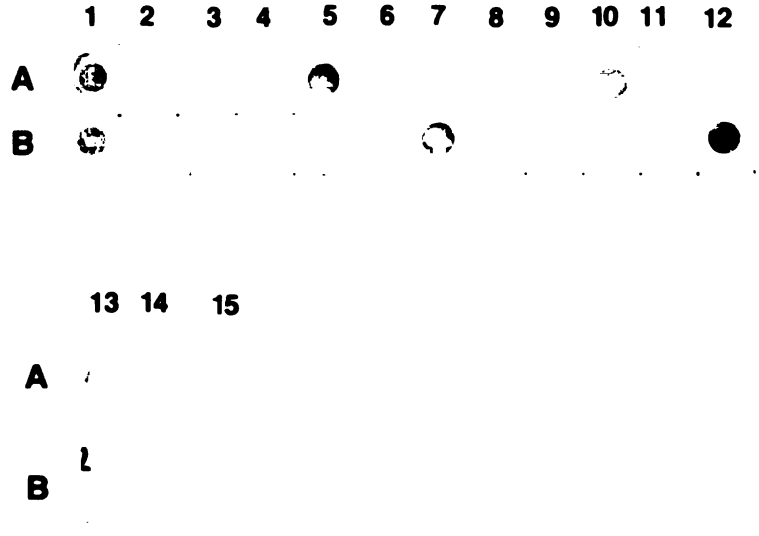
These results show that the selected IgGs were specific for alpha-gal B and that cross-reactivity could be eliminated by preabsorbing with E. coli proteins. When the IgGs were preabsorbed with alpha-gal B, the positive

Figure 31. Chromogenic Immunodetection of Anitgens.

Crude enzyme was dot-blotted to nitrocellulose and detected with the goat-anti-rabbit horseradish peroxidase conjugated IgG (GAR-HRP). The amount of protein applied was normalized to the amount alpha-gal B activity present. Spent media from Chang liver cells and FS-4 cells (200 ul) was blotted although there was no detectable activity. BNN103(lambda gt11) and Y1090 have no detectable enzyme activity; therefore, 0.05 mg protein was applied to the blot.

Protein/Enzyme Source	Immunodetection anti-A IgG	anti-B IgG
1) human liver	+ *	+ *
2) Chang liver cells	+	-
3) FS-4 fibroblasts	+	+
4) fibroblast spent medium	-	-
5) human placenta	+ *	+ *
6) mouse liver	- *	- *
7) HepG2	+	+
8) BNN103(lambda gt11)	+(-)	+(-)
9) Y1090 *E. coli*	+(-)	+(-)
10) Chang liver spent medium	+	-
11) alpha-gal A (5 ng pure)	+	-
12) alpha-gal B (5 ng pure)	-	+
13) beta-gal (*E. coli*)	+	+(-)
14) alpha-gal (coffee beans)	+	+
15) alpha-gal (watermelon)	+	+

(*) color appears darker in black-and-white due to protein staining the nitrocellulose


1 2 3 4 5 6 7 8 9 10 11 12
A
B
13 14 15
A
B

reaction was eliminated for alpha-gal B and alpha-gal A from coffee beans and watermelon. Interestingly, when the IgGs were preabsorbed with alpha-gal A from coffee beans and watermelon, the color reaction was diminished when reacted with alpha-gal B protein, but not eliminated.

With these results in mind monospecific alpha-gal B was preabsorbed with the E. coli lysates, alpha-gal A from human, coffee beans and watermelon and beta-gal before screening the library. After the initial pre-adsorpbtion and one round of screening (1 plate per 5 ml IgG in BSA) no further adsorption was necessary to reduce background color due to cross-reactive proteins, while 0.1 ng of alpha-gal B was still positive.

The alkaline phosphatase conjugated goat anti-rabbit IgG was used for the actual screening of the library. We found this color reaction to be more sensitive in the plaque detection. Although the general background color was high necessitating the more elaborate washing conditions than are generally used, a better differential (and more permenant color) could be determined between background and positive plaques. Approximately 10^7 plaques were screened and 102 were chosen as positives in the first screening. Phage were screened at lower densities in subsequent rounds until the plate from one

phage was homogeneously positive (Figures 32 and 33). Thirteen phage were eventually selected for further characterization based on their continual hybridization with the IgGs.

The fusion proteins from these thirteen phage were induced and the resulting lysate was dot-blotted and screened by the chromogenic detection with horseradish peroxidase conjugated goat anti-rabbit IgGs. This switch was made because more protein was applied to the dot-blots (total approximately 100 ng) than was present in a bacterial plaque and the GAR-HRP is less sensitive for the cross-reactive proteins. Cross-reactivity to coffee bean and watermelon alpha-gal A can be completely eliminated with the HRP system, but not with the Alk-Phos system, thereby possibly eliminating some chances of false positives.

Eleven of the phage showed some color reaction in the dot-blots. Nine were selected as possible true positives since the color reaction of the induced phage lysates were substantially darker than in the uninduced state: 1-1-1, 1-1-2, 2-3-1, 2-3-3, 4-2-1, 8-2-1, 17-5-2-1, 19-1-6, and N-1-1 (Figure 34).

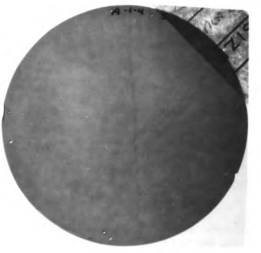
The phage were replated and duplicate filters were pulled then incubated with the IgG preparation, or with the

Figure 32. Examples of Library Filters Screened by the Alkaline Phosphatase Conjugated Double Antibody System With anti-Alpha-Gal B IgG Primary Antibodies.

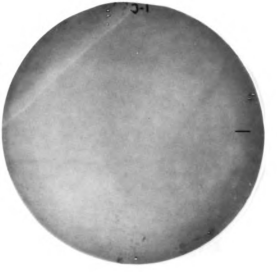
Filters were pulled from plates of phage that were plaque-purified at least once and replated at similar densities. Plaques that were positive on duplicate filters were chosen as positive phage.

A, B, and C are from secondary screenings of phage that are no longer positive after rescreening (Phage 19-1-4, J-1 and N-3).

A



B



C

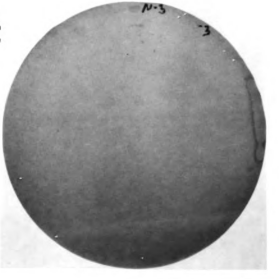
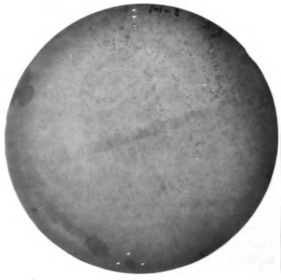


Figure 33. Examples of Library Filters Screened by the Alkaline Phosphatase Conjugated Double Antibody System With anti-Alpha-Gal B IgG Primary Antibodies.

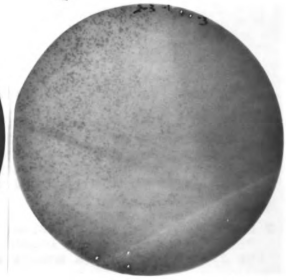
Filters were pulled from plates of phage which were plaque-purified at least once and replated at similar densities. Plaques that were positive on duplicate filters were rescreened as positive phage.

D, E, F, and G are examples of phage which remained positive after four rounds of rescreening (1-1-2, 2-3-3, 19-1-2, and N-1-3).

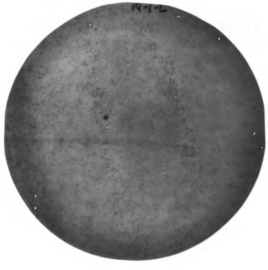
D



E



F



G

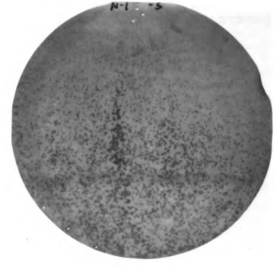


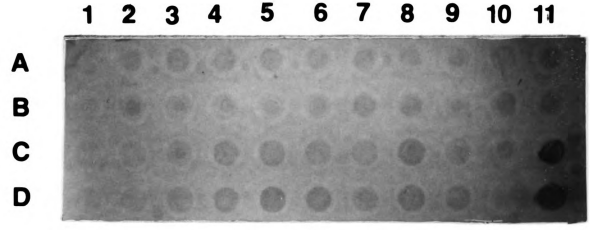
Figure 34. Dot-blots of Induced and Uninduced Positive Phage.

Induced and uninduced phage were applied to nitrocellulose and antigenically positive fusion proteins were identified with the monospecific anti-alpha-gal B IgGs and horseradish peroxidase-conjugated goat-anti-rabbit second antibody system.

Rows A and C contain induced phage lysates and rows B and D contain the corresponding uninduced phage. C-10 and D-10 are BNN103(lambda gt11) preparations and C-10 and D-11 are alpha-gal B (5 ng and 10 ng).

A-2, A-3, A-4, A-5, A-6, A-7, A-9, C-2, and C-3 were considered positive when compared to thee uninduced preparation. All samples were normalized for protein content.

Photographic reproduction has greatly decreased the contrast between positive and negative dots. The contrast on original filters is sharper, but fades rapidly under strong lights needed for photography.


1 2 3 4 5 6 7 8 9 10 11
A
B
C
D

alpha-gal B preabsorbed IgGs. There was a definite elimination of the alkaline phosphatase color reaction with several of the clones: 17-5-2-1, N-1-1, 19-1-6; the color intensity was reduced in the reaction with the other clones, although the background was high due to the dense plating (Figure 35).

Induced phage proteins were electrophoresed (3% stacking, 12% separating SDS-PAGE). One half of the gel was silver stained and the duplicate lanes of the other half were Western blotted and the fusion proteins were detected by the HRP system (Figures 36 and 37). On one gel, 17-5-2-1 was positive: a fusion protein of approximately 122,000. Other Western blots were not as conclusive as several lysates contained bands in both the uninduced and induced lanes, or several smaller bands which were also immunoreactive in the same lanes (Figure 37).

Most of the fusion protein bands observed were approximately 120,000 to 125,000 MW in a 12% gel, which indicate fusion proteins 6,000-11,000 larger than the *E. coli* beta-gal gene of 114,000 apparent M_r. Therefore, the human peptide fragments were approximately 60-100 amino acids, or 180-300 nucleotides per cDNA.

Phage DNA was prepared as described. Early isolations did not include the proteinase K digestion; the lanes

Figure 35. Competitive Immunoselection of 17-5-2-1.

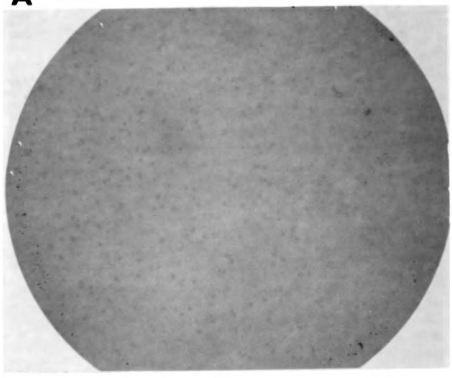
Approximately 10^3 phage from positive dot blots were plated and duplicate filters were pulled as for screening.

A) Plaque purified 17-5-2-1 incubated with anti-alpha-gal B.

B) Plaque purified 17-5-2-1 incubated with anti-alpha gal B after absorption with an excess of alpha-gal B.

Photographic reproduction has greatly decreased the contrast seen on original filters.

A



B

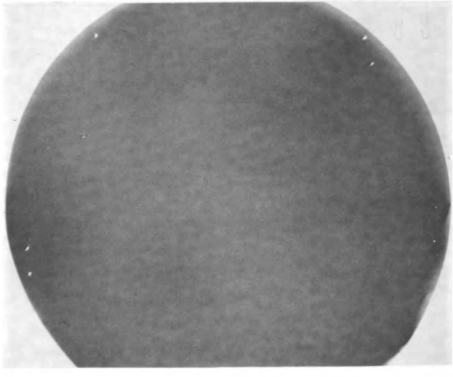


Figure 36. SDS-PAGE of Induced and Uninduced Positive Phage.

Lysates of induced and uninduced phage were electrophoresed through a 12% SDS-PAGE and silver stained.

Lane 1) Induced 17-5-2-1
Lane 2) Uninduced 17-5-2-1
Lane 3) Induced 8-2-1
Lane 4) Uninduced 8-2-1
Lane 5) Induced BNN103 (lambda gt11) control
Lane 6) Uninduced BNN103 (lambda gt11) control

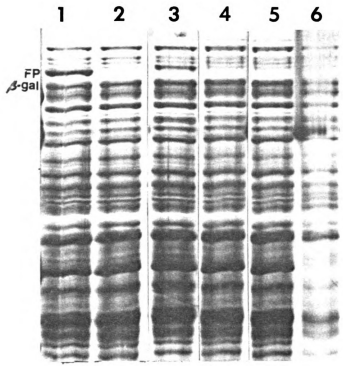

1
2
3
4
5
6
FP
β-gal

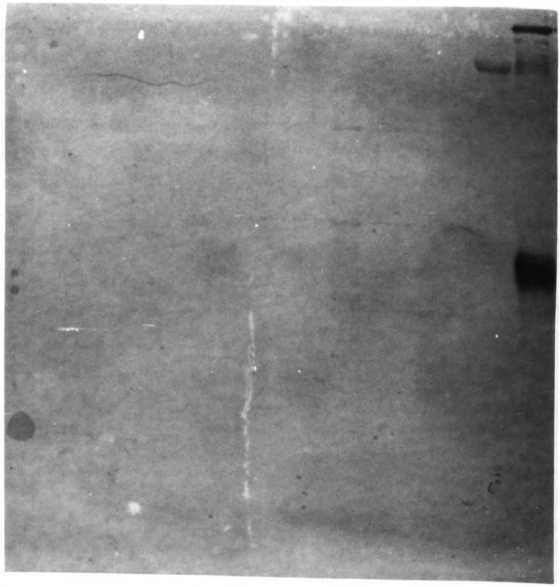
Figure 37. Western Blot of Induced Fusion Proteins.

Lysates of induced and uninduced phage were electrophoresed through a 12% SDS-PAGE, Western blotted, and antigens were immunodetected with anti-alpha-gal B IgG and the GAR-HRP chromogenic detection method.

Lane 1) Affinity purified human alpha-gal B
Lane 2) Induced phage 17-5-2-1

Other lanes contain induced and uninduced, dot-blot-positive phage. BNN103 (lambda gt11) and beta-galfrom E. coli were included as controls.

2 1



appeared smeared and the cDNA was not cut from the phage. Subsequent preparations, although much cleaner, were equally difficult, if not impossible to cut from the phage DNA. Decreases in the phage molecular weight could be discerned, and a faint cDNA band from 17-5-2-1 was seen at approximately 400 nt, but no conclusive sizes could be determined for any cDNA.

The fact that the cDNA could not be cut from the phage with Eco RI is not unusual for this library. J. R. deWet reported that perhaps 30% of the phage are missing at least one Eco RI site on either side of the cDNA insert. As yet, no other restriction enzymes have been used for characterizing this cDNA clone.

DISCUSSION

When embarked upon, the primary goal of this project was to isolate the gene for alpha-gal B and, if possible, for alpha-gal A. Although it seemed to be a "do-able" project with the most interesting parts being those studies to be undertaken once the genes were cloned, there was still much groundwork that needed to be done before the project could be started.

One of those minor considerations was the availability of a specific probe for alpha-gal A or B. Purification of the two enzymes was in progress, although not complete. Sequence information for the N-termini of the enzymes became available early in the project, but more was necessary if the DNA sequence was to be confirmed. The possibility of isolating the genes by immunoprecipitating nascent proteins on the polyribosomes or fusion proteins from expression libraries existed as long as specific antibodies could be produced.

Thus, although not an original goal of this project, the necessity of improving the purification scheme presented itself in order to obtain pure, homogeneous enzyme. As side efforts of the project, minor investigations involving the

biosynthesis and the identification of precursors were undertaken.

Others in this lab, notably K. J. Dean and F. E. Wilkinson, worked on the purification of alpha-gal A and B, resulting in the separation of the enzyme activities and the purification of A, with recovery of B as a side product. These preparations were pure enough to perform kinetic and substrate studies, and to obtain some primary sequence information.

However, the purification of alpha-gal B could not be reliably repeated with the intention of maximizing the yield of pure B. It was necessary to revise the scheme and to sacrifice the yield and purity of alpha-gal A in order to obtain homogeneous alpha-gal B.

The purification scheme presented here has a 55% yield of alpha-gal B with a specific activity of 5.7 umoles 4-MU-alpha-gal per mg protein per min that is completely inhibited in the standard assay with 50 mm GalNAc. This yield is calculated from the total amount of alpha-gal B present in a crude, 37% homogenate of human liver and is higher than yields previously reported (29, 30, 219).

The specific activity is also higher than Wilkinson's value of 4.1 U (U=umol 4-methylumbelliferyl-alpha-D-

galactopyranoside hydrolyzed per minute per mg protein in the presence of 50 mM N-acetylgalactosamine). Dean's value of 6.3 U was admittedly not completely inhibited by GalNAc, indicating that the A and B activities wee not completely separated.

The purification scheme uses traditional purification techniques as well as Wilkinson's affinity chromatography matrix. The notable differences in this procedure include the elimination of the DEAE-chromatography step, the reshuffling of the gel filtration step to the end of the procedure, and the inclusion of detergent in the gel filtration step.

The use of sodium cacodylate as the buffer throughout the purification of the enzyme seemed to stabilize the enzyme as well as increase the specific activity of the enzyme in cruder states. In 0.1 M sodium phosphate buffer, alpha-gal A has a specific activity in the crude homogenate of 0.02 mU and alpha-gal B has a specific activity of 0.04 mU. These values increase to 0.74 mU and 0.19 mU when sodium cacodylate buffer, 25 mM, pH 6.5 is used for the homogenization. Several other buffers were briefly examined including 10 mM and 25mM sodium citrate, pH 6.5, 25 mM sodium phosphate, pH 6.5 and water. Sodium cacodylate buffer had the greatest positive effect on the

specific activity and stability during further purification.

The elimination of the DEAE step had a great effect on the purification of alpha-gal B. While others essentially used this step to separate the two activities, we were confident that the affinity step could accomplish that objective. In our hands, the use of DE-52 (Whatman) as suggested by Wilkinson did not result in the separation of the two activities, nor did several other ion-exchange resins without substantial loss of enzyme activity with little or no increase in the specific activity or purification. Some of this loss might have been due to the interaction of the enzyme with the matrix itself, followed by denaturation as higher salt concentrations were used to wash the enzyme from the column. Substantial degradation was observed by SDS-PAGE when the enzyme was washed with buffer including 0.5 M NaCl or higher salt concentrations.

From the Con A step (after concentration and buffer exchange), the enzyme solution was applied directly to the affinity ligand by the batch method. Included in the wash buffer was 50 mM N-acetylglucosamine (GlcNAc). Neither alpha-gal A nor alpha-gal B is inhibited by GlcNAc (219 and unpublished observations). This was included in order to prevent any initial binding of enzymes or lectins which bind to substances having conformations similar to an

alpha-galactose unit from binding to the alpha-galactose-affinity ligand. This addition reduced the volume of buffer (and time) necessary for subsequent washing of the column, without interfering with the binding or elution of alpha-gal A or B.

The elution of enzyme from the affinity column was performed essentially as described by Wilkinson, except that 0.1 M NaCl was included in the wash and the enzymes were sequentially eluted from the column with GalNAc, then Gal, resulting in the elution of alpha-gal B first, then alpha-gal A. A second affinity step after removing the inhibitors by concentration and buffer exchange resulted in separate activities for the A enzyme pool and the B enzyme pool.

At this point, the enzymes were not as pure as in other procedures although the activities were completely separated. Gel filtration on Sephadex G-150 was chosen in order to eliminate some high molecular weight and a minor small molecular weight contaminant still found in both alpha-gal A and B as seen by SDS-PAGE. However, good separation was not accomplished until a small amount of detergent was included in the running buffer in order to eliminate trailing and smearing. Such smearing is common due to the presence of the carbohydrate structures on glycoproteins. The presence of the detergent did not

affect the activity of alpha-gal B over the elution period, and resulted in the separation of the desired contaminants and a second peak of 4-MU-alpha-gal activity at 45,000 apparent M_r.

Pure alpha-gal B (having p-NP-GalNAc activity and 4-MU-alpha-gal activity inhibited by GalNAc) was isolated at M_r 94,000 by gel filtration. The subunit molecular weight on SDS-PAGE was 45,000, and when the N-linked oligosaccharides were cleaved from the denatured enzyme with N-glycanase, the subunit M_r was 38,000, a reduction of 15.5%. Alpha-gal A had a M_r of 105,000 by gel filtration, and a subunit M_r of 46,000. These results are comparable to those of other investigators.

Future isolations of alpha-gal B and A should further investigate the effects of buffers and ionic strength on the isolation and stability of the enzymes. From observations made during these purification trials, it might also be beneficial to investigate the effect of detergents during extraction and measurement of activity during various assays. As can be seen, the yield of alpha-gal B activity increases to nearly 200% during initial pruification (crude homogenate to Con A), perhaps due to the separation of an inhibitor, or dissociation from a membrane or receptor which keeps the enzyme inactive during

synthesis and transport in vivo, as is seen with cathepsin D and alpha-glucosidase (146).

Several observations made during the purification are notable, although not completely investigated since this was not a "primary" goal of the project overall. In Wlkinson's preparations of both A and B, a small, tight band of approximately 110,000 was seen on SDS-PAGE and could not be eliminated. This band also appeared in our preparations prior to the Sephadex chromatography.

When these cruder preparations were digested with N-glycanase, the major, diffuse enzyme band at M_r 45,000 was reduced by approximately 15.5% to 38,000, and produced a tighter, more compact band which usually characterizes non-glycosylated or less glycosylated proteins.

The higher MW band was compact to begin with, but was also reduced by approximately 15% after N-glycanase treatment to M_r 93,000, indicating it, too, was N-glycosylated. Although this band was separated from the preparations by gel filtration, before the enzyme was used to immunize the rabbits, it was still immunoreactive when cruder preparations were used as controls and were blotted with mono-specific anti-alpha-gal B IgGs.

This band could represent a precursor of alpha-gal B, or a membrane bound precursor which is not completely dissociated by SDS and BME. This band appears similar to a band in alpha-gal A isolated by both Wilkinson's procedure, and this one. It, too, is approximately 110,000 and cleaved by N-glycanase, indicating a glycoprotein. These bands may represent the same protein that is eliminated from the affinity column by both GalNAc and Gal, or may be different proteins, but a similar form of precursor enzyme of both A and B. Alternatively, this protein could be a lectin (or different lectins) with Gal- or GalNAc- binding properties. Extensive washing of the affinity column prior to inhibitor elution of the two enzyme does not eliminate it from the preparations.

Likewise, the 45,000 M_r band that elutes from the gel filtration column during the purification of B might be a separate enzyme or ligand that has some affinity for alpha-galactose units, thus causing its elimination with GalNAc and alpha-gal B. However, it may merely represent a subunit of alpha-gal B that becomes dissociated by the inclusion of SDS in the running buffer, and retains activity toward alpha-galactosyl units, but because of its dissociation and resulting conformational change is no longer reactive toward GalNAc residues.

An alternative model also presents itself: alpha-gal B may not actually be a homodimer as is generally accepted. It may be comprised of two extremely similar subunits, one which has specificity toward alpha-galactose residues and one which confers GalNAc specificity when associated with the second subunit, but does not eliminate activity toward galactose. Indeed, when these preparations were incubated with the natural substrate of alpha-gal A, $GbOse_3Cer$, alpha-gal A hydrolyzed the substrate, but B did not in a two hour reaction (data not shown, but similar results can be seen in ref. 158). However, after an 18 hr incubation at 37°C and the same conditions, alpha-gal B does hydrolyze $GbOse_3Cer$ to some extent, while alpha-gal A has no activity left at all against the artificial substrates (also demonstrating its thermal instability compared to B). Again, this could be due to the much lower affinity B has toward alpha-gal residues rather than alpha-galNAc residues, or due to the fact that B is slowly degrading and losing its specificity in the long run. The activity is probably not due to residual A activity since A is quickly degraded above room temperature in two or three hours.

This is an aspect that certainly needs more attention. Other investigators have postulated that B is a precursor of A based on their observations that B is slowly converted to A upon storage (166, 167). Tager also postulated that

the residual A-like activity found in Fabry liver is actually a modified alpha-gal B.

The fact that the N-terminal sequencing of alpha-gal B resulted in single major residues indicating the same N-terminus on both subunits does not necessarily argue against the fact that alpha-gal B may be a heterodimer. The enzyme used in the N-terminal sequencing was stored in solution over the course of a year as several preparations were collected and pooled. Proteolytic degredation, as indicated by changes in specific activity, and changes in subunit M_r as visualized by SDS-PAGE, of alpha-gal B occurs quickly in the impure purification stages or less concentrated conditions of Wilkinson's procedure. Such proteolytic degredation may result in similar N-termini, or the loss of one subunit in respect to the other.

The N-termini of alpha-gal A and B are homologous for the first five residues and extensive homology is seen in residues (after the eighth) from human placental alpha-gal A (219, Figure 38). In addition, the N-terminus from watermelon alpha-galactosidase (reportedly a monomer of 45,000 M_r) isolated in this lab by M. J. Thome and K. J. Hawes using a modification of published procedures (83), also showed extensive homology and further similarity after N-terminal sequencing at the Michigan State University Macromolecular Facility (unpublished data, Fig 39). The

Figure 38. N-Terminal Sequences of alpha-Galactosidases

1) Human liver alpha-galactosidase A
2) Human placenta alpha-galactosidase A
3) Watermelon alpha-galactosidase
4) Human liver alpha-galactosidase B

1) Leu-Asp-Asn-Gly-Leu-Ala-Arg-Xa -Pro-Tyr/Thr-Met-Gly-Xa -Xa -Leu

2) Leu-Asp-Asn-Gly-Leu-Ala-Arg-Thr-Pro- Thr -Met-Gly-Trp-Leu-Xa -Trp-Glu-Arg-Phe-Xa -Gly-Asn

3) Leu-Gln-Asn-Gly-Leu-Ala-Xa -Thr-Ser- Gln -Met-Gly-Trp-Cys-Ser-Xa -Ile-Phe-Phe

4) Leu-Asp-Asn-Gly-Leu-Leu-Gln-Thr-Pro- Pro -Met-Gly-Trp-Leu-Ala-Trp-Glu-Arg-Phe

N-terminal similarity may be indicative of affinity for alpha-galactose units, with specificity toward GalNAc conferred by internal amino acid residues on one subunit, carbohydrate structures, or by the association of two either heterologous or homologous subunits.

Obviously, these postulations and observations need more investigation, and confirmation must be made by the complete sequencing of the proteins, or the isolation of the DNA, and further biosynthetic / precursor studies. Our interest from here was focussed on the isolation of the gene for alpha-gal B and addressing these questions from the DNA level.

Polysome immunoprecipitation was the first method used in the attempt to isolate the coding sequence for alpha-gal A. Specific antibodies against mature alpha-gal A were raised in rabbits and used as the "probe" in order to immunoprecipitate polyribosomes containing the nascent protein and the mRNA for alpha-gal A. It was our intention to isolate the specific mRNA, then to translate it into cDNA to be used in further studies. Our criteria for the identification of the coding sequence would be the <u>in vitro</u> translation of the mRNA into a protein immunoprecipitatable by the anti-alpha-gal A antibodies, DNA sequencing of the synthesized cDNA, and, possibly, the inability of the cDNA to hybrid select mRNA from Fabry patient cells.

Although this method was not successful in that alpha-gal A specific mRNA was not identified, translatable mRNA was isolated after immunoprecipitation of polysomes. Several steps of the procedure were successful, the only drawback being that this method did not result in enough mRNA to be useful for our purposes.

We were able to determine conditions of in vitro cell culture which increased the activity and perhaps the synthesis of alpha-galactosidase A in Chang liver cells. The addition of fresh media to cells in log phase along with cycloheximide and RNase inhibitor resulted in an increase of the specific activity and the relative activity of alpha-gal A per cell. This is a logical consequence since alpha-gal A is a degradative enzyme. As cells become more confluent and substrates and "waste" build up, a cascade could be induced which would lead to an increase in the synthesis of degratory enzymes. On the other hand, activity could increase as a result of a slower turnover of the enzyme, or due to proteolytic processing under stress conditions which causes more enzyme to become active.

The yield of mRNA was increased 30-fold when cycloheximide and RNase inhibitor were added to media and buffers. Polysomes are aggregates of the individual ribosomes actively synthesizing protein from a single mRNA strand

which connects them. Cycloheximide and RNasin maintain the integrity of polysomes and protect the RNA from degradation. Specifically, cycloheximide binds to the large ribosomal subunits and blocks peptide bond formation. This aggregation of ribosomes probably prevents mechanical breakage of the mRNA strand. RNasin inhibits the actions of RNase, an enzyme that causes the degradation of RNA and, therefore, the disaggregation of polysomes.

Despite initial success in increasing enzyme activity and perhaps inducing enzyme synthesis, and in RNA isolation, attempts to assay the RNA by <u>in vitro</u> translation were inconclusive. A wheat germ reticulocyte system was used to translate the mRNA into ^{14}C-leucine containing polypeptides, but the visual analysis of electrophoresed translation products by fluorography was indefinite despite initial indications that radioactivity was incorporated into TCA-precipitable material. Non-specific trapping of ^{14}C-leucine overexposed the fluorogram, and specific bands could not be identified. Because the amount of mRNA was so limited, a second gel could not be run and Western blotted in order to examine the translation products by immunodetection.

A rabbit reticulocyte translation system may prove to be more efficient at translating alpha-Gal A mRNA. The unglycosylated subunit molecular weight of alpha-gal A is

approximately 42,000-46,000 and the precursor possibly translated by the in vitro system is about 58,000 (20,21). While the wheat germ system will translate polypeptides of higher molecular weight, it translates proteins of approximately 35,000-39,000 MW most efficiently.

Using an oligo-dT column chromatography to isolate poly-A tailed mRNA would improve the efficiency of the translation system, eliminating untranslatable mRNA and ensuring more complete translation products.

It was important for this study, and futher studies, to determine if glycosylation is important for the activity of the enzyme and for its antigenicity, since proteins still being translated on the ribosome are not glycosylated until part of the nascent chain enters the ER and interacts with specific enzymes there. If glycosylation is necessary for enzymatic activity due to conformation or structural modification, the in vitro translation products would have reduced or undetectable activity. The absence of activity would not matter in this case unless the in vitro translation product could not be isolated and detected by immunoprecipitation. If the carbohydrates alone account for antigenicity, or for a major portion of the antigenicity, neither the polysomes nor the in vitro translation product will be immunoprecipitable. Erickson (40-43), Hasilik (71-74), and others have been able to

immunoprecipitate unglycosylated lysosomal hydrolases; therefore, by analogy, alpha-gal A and alpha-gal B should be immunoprecipitable by our antibody.

We were able to show that glycosylation of proteins in Chang liver cells was inhibited by tunicamycin B2, a homologue which inhibits glycosylation by inhibiting the transfer of the oligosaccharide from the dolichol precursor without inhibiting protein synthesis (39). Concentrations which killed or were proven to completely block glycosylation in other cell lines (39, 117) failed to inhibit cell growth or activity.

The results from the studies of incorporation of ^{3}H-mannose into proteins showed that the radioactive sugar was not as efficiently incorporated into Chang liver cells after extensive growth in tunicamycin as it was in control cells. Moreover, the specific activity and total activity of the enzyme were unaltered as compared to control cells, and 100% of the relative activity could be immunoprecipitated in the tunicamycin-treated cells as compared to the control cells, indicating that glycosylation was not necessary for activity or immunoprecipitation.

Polysome immunoprecipitation of alpha-gal B and A should be possible with the inclusion of several advances, namely using a greater number of cells to begin with (500 T-150 mm

flasks vs. 25 T-150 mm flasks), the inclusion of more RNasin throughout the procedure (since a greater availability has reduced the cost per unit), and the inclusion of an oligo-dT chromatography step which would isolate only the poly A-tailed mRNA, thereby reducing the total number of mRNA transcripts translated in an in vitro translation system.

The procurement of mRNA for alpha-gal A, then, was partially successful in that polysomal mRNA was isolated from Chang liver cells. We were able to modify conditions so that the yield of RNA increased substantially over the yields attained initially. However, the amount of immunoprecipitated mRNA was not enough to insure overall success. This approach was dropped after N-terminal sequence information became available for alpha-gal B and A, and further sequence information seemed inevitable. However, this would still seem to be a viable approach to the isolation of alpha-gal A mRNA from the Chang liver cells, and alpha-gal B mRNA using HepG2 cells (Chang liver cells have little alpha-gal B activity and little immunoreactive protein, although they function "normally") if other approaches are not successful in the future.

The second approach to the problem of isolating the genes for alpha-gal A and alpha-gal B involved using a synthetic oligonucleotide to screen a plasmid library containing cDNA

sequences complementary to mRNA of fetal liver. The basic rationale for using this approach relies on the observation that the mixed 18-nucleotide-long probe sequence is based on a peptide sequence in alpha-gal B that is semi-homologous to the alpha-gal A protein sequence in the corresponding position. Others have shown that it is possible to exploit the thermal stability of oligonucleotide/DNA duplexes in order to isolate perfectly matched sequences (210, 221, 226).

A major task, then, was defining the specificity of the 18-mer probe toward genomic DNAs. This was accomplished by hybridizing the probe to human DNA and washing at successively higher temperatures to increase the stringency, or else merely hybridizing the probe at higher temperatures. The results shown in Figure 21 indicate the probe hybridizes non-specifically to genomic DNA, but selectivity can be obtained at higher temperatures.

Thus, sufficient hybridization to human genomic DNA was accomplished by exploiting the thermal stability of the duplexes formed. There was still hybridization seen at 42^{o}C which should have been strict enough to prevent single base pair mismatches, based on the calculations of Wallace et. al. We decided to examine the specificity of the probe by repeating the hybridization with DNA from different organisms. Results showed that even at stringent

conditions, the probe hybridizes to DNAs other than human, but washing at successively higher termperatures again eliminates highly mismatched duplexes, and the 18-mer preferentially hybridized with high molecular weight human DNA.

From the Southern blot results, we determined conditions that would allow detection of cDNA sequences contained in a plasmid library which are semi-homologous and strictly homologous to the 18-mer. The human cDNA library was used for a number of reasons. First, since fetal liver synthesizes alpha-gal A and B (35), the mRNA should be represented in the cDNA inserts. Second, intervening sequences are not present in cDNA and, therefore, would not interfere with the hybridization of the 18-mer to the gene of interest by interrupting coding sequences. Third, this library was previously used to isolate the gene coding for phosphoglycerate kinase (PGK), an enzyme of similar size. The library was constructed using cDNAs greater than 0.4 kb; the minimum insert detected was 0.4 kb and the largest was 1.9 kb.Since the mRNA for alpha-gal A and alpha-gal B is approximately 1.3 kb, a full reverse transcript should be represented.

As seen in Figures 23, hybridization of the 18-mer to the cDNA library was selectively controlled by washing at higher temperatures as was possible with the genomic blots.

Hybridizing and washing at 20°C produced extremely high background. This was sufficiently reduced at 37°C so that clones could be selected by comparing autoradiograms of duplicate filters. Washing at 42°C (the temperature calculated to allow only perfect matches) reduced the number of clones to about one-half the original number.

Based on these observations, the cDNA library was screened first at 42°C and clones were selected. The filters were re-hybridized with the 18-mer at 37°C in order to select any colonies which may contain similar, but mis-matched cDNA. Colonies were picked from both sets of screening in order to pick both strictly homologous cDNA representing alpha-gal B and semi-homologous cDNA, possibly representing alpha-gal A cDNA.

After the screening was complete, a total of 131 clones were selected at high stringency and further screened by RE digestion and Southern blotting in order to identify cDNA inserts hybridizing to the 18-mer. Of all these clones, only cDNA insert was positive. In successive hybridizations, this insert stably hybridized at higher temperatures, up to 56°C, furthering our confidence that it was indeed homologous to the alpha-gal B probe.

However, sequencing the cDNA fragment from pI-33 which hybridized to the 18-mer revealed that there were four

regions of the 200 bp fragment which hybridized to the 18-mer, all 73% homologous assuming the redundant nucleotides were exactly matched. Whether the hybridization was an artifact of secondary structure in the probe, the cDNA, or an artifact of the size and nucleotide composition is not known. The sequence was confirmed by three rounds of Maxam-Gilbert sequencing resulting in overlapping information, as well as by dideoxysequencing fragments subcloned into M13 vectors.

Our hopes that we would be able to screen the library with a 14-mer and 17-mer made to two internal peptides were also dashed. Although a check of the Protein Sequence Query (PSQ) and Nucleic Acids Query (NAQ) data banks for protein and DNA sequences exactly matching and matching with the exception of any one nucleotide in the 18-mer, the 17-mer and the 14-mer revealed only one viral protein matching the 14-mer with one nucleotide mismatch, hybridization of the 14-mer and 17-mer showed that the 17-mer hybridized to the plasmid vector at the same stringency as human DNA, as did the 14-mer. When the stringency increased, no hybridization to human DNA was seen, and none of the cDNA inserts isolated from the original 131 plasmids hybridized to the 17-mer. Obviously, neither new probe was useful for screening the fetal liver cDNA library.

A second hybridization method was briefly examined: hybridization using tetramethylammonium chloride according to the prodedure of Wood *et. al.* (221). Hybridization under conditions which maximized the hybridization according to the size of the probe as well as the G/C content gave no encouraging results. *In lieu* of more protein sequence, or of a better, more suitable library, the third phase of the cloning trials was engaged.

We had shown that antibodies raised against mature alpha-gal A and B would precipitate the activity or recognize the protein structure of unglycosylated enzyme. In the purification studies we describe digestion of the purified, mature, human liver alpha-gal B with N-glycanase, an enzyme which cleaves N-linked oligosaccharides from glycoproteins. Monospecific IgGs against alpha-gal B identify the deglycosylated enzyme on Western blots. When glycosylation of proteins was prevented in Chang liver cells by tunicamycin B2, anti-alpha-gal A IgGs were able to precipitate 100% of the relative activity of alpha-gal A.

Further studies showed that the anti-alpha-gal B IgGs selectively precipitated enzyme from human liver or human liver cells, with some reactivity toward placental enzyme. There was no cross-reactivity with mouse protein, and any reactivity toward *E. coli* proteins could be easily eliminated.

Using this information, we have isolated nine phage from a human hepatoma-derived cDNA expression library which express a fusion protein that reacts with anti-alpha-gal B IgG. These phage have not been fully characterized, and the possibility that one or more may be a false positive exists.

No insert size has been conclusively determined for any of the cDNAs contained in the phage. This is not an unusual occurrence in expression libraries in general and this library in particular. It has been estimated by the investigator who made the library in conjunction with several who have isolated cDNAs from the library that as many as 30% of the phage carrying inserts may have lost one or more of the Eco RI sites on either side of the inserted cDNA, thus preventing separation of vector and cDNA (36; personal communication, J.R. deWet).

The phage which have been isolated all carry relatively small inserts. These inserts may carry relatively little information about the coding sequence for alpha-gal B, but may be useful as probes themselves in order to isolate larger cDNAs from this or another library.

The most reliable way to prove that any or all of these clones carry alpha-gal B coding sequences would be to

simply sequence the cDNA and match the DNA sequence to the corresponding protein sequence known for alpha-gal B. However, since there is only approximately 18% of the protein sequence now known, and these inserts are small, there is a possibility that the DNA sequence dose not match any of the sequenced peptides.

A second proof would be the ability of the isolated cDNAs to hybrid-select mRNA from a human source which could then be translated in in an *in vitro* translation system. If the translated product could be immunoprecipitated by anti-alpha-gal B IgGs, this would be a strong indication that the cDNA indeed contained alpha-gal B coding information. Until this sort of confirmation can be accomplished, the real identity of any of these clones cannot be firmly established.

Any of the isolation methods for a cDNA clone of alpha-gal B are still promising, with the most likely means being the immunoprecipitation of the fusion protein in the cDNA expression library. The second most viable means would probably be immunoprecipitation of the mRNA from polysomes. Of the ten lysosomal enzymes (or subunits) recently cloned, six have been isolated using the lambda gt11 expression system including alpha-fucosidase (49), alpha-galactosidase A (22), beta-glucocerebrosidase (174), human and mouse preprocathepdin B, alpha-mannosidase, and beta-

galactosidase (204). Two have been isolated by polysome immunoprecipitation, the alpha-chain of human beta-hexosaminidase (129), and monkey alpha-glucosidase (97). Human cathepsin B (45) and the beta-subunit for beta-hexosaminidase (136) were isolated by screening with oligonucleotide probes. All of the above investigators reported the isolation of false positives at some point in their search, and three actually published information about the clones before the mistakes were discovered (49, 60, 61).

The isolation of clones for lysosomal enzymes marks the beginning of the ability to identify the precise lesions in many lysosomal storage disorders. On a larger scale, answers to questions concerning gene regulation and translational control, biosynthesis and processing can be determined. Perhaps some of the most exciting possibilities include the synthesis of quantities of lysosomal enzymes in DNA and RNA expression vectors with normal and altered properties which will provide material to researchers for study and comparison, as well as for patients and for industrial uses in amounts never before available for this family of enzymes.

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