We have cloned the cDNA encoding human GDP-mannose 4,6-dehydratase, the first enzyme in the pathway converting GDP-mannose to GDP-fucose. The message is expressed in all tissues and cell lines examined, and the cDNA complements Lec13, a Chinese Hamster Ovary cell line deficient in GDP-mannose 4,6-dehydratase activity. The human GDP-mannose 4,6-dehydratase polypeptide shares 61% identity with the enzyme from *Escherichia coli*, suggesting broad evolutionary conservation. Purified recombinant enzyme utilizes NADP\(^+\) as a cofactor and, like its *E. coli* counterpart, is inhibited by GDP-fucose, suggesting that this aspect of regulation is also conserved. We have isolated the product of the dehydratase reaction, GDP-4-keto-6-deoxymannose, and confirmed its structure by electrospray ionization-mass spectrometry and high field NMR. Using purified recombinant human GDP-mannose 4,6-dehydratase and FX protein (GDP-keto-6-deoxymannose 3,5-epimerase, 4-reductase), we show that the two proteins alone are sufficient to convert GDP-mannose to GDP-fucose *in vitro*. This unequivocally demonstrates that the epimerase and reductase activities are on a single polypeptide. Finally, we show that the two homologous enzymes from *E. coli* are sufficient to carry out the same enzymatic pathway in bacteria.

Fucose is found as a component of glycoconjugates such as glycoproteins and glycolipids in a wide range of species from humans to bacteria. For example, fucose is a component of the capsular polysaccharides and antigenic determinants of bacteria, while in mammals fucose is present in many glycoconjugates, the most widely known being the human blood group antigens. Fucose-containing glycoconjugates have been implicated as playing key roles in embryonic development in the mouse (1) and more recently in the regulation of the immune response, specifically as a crucial component of the selectin ligand sialyl Lewis X (reviewed in Refs. 1 and 2). In all cases, fucose is transferred from GDP-fucose to glycoconjugate acceptors by specific transferases. Thus, defects in GDP-fucose biosynthesis will affect all fucosylation within the cell. Recently, individuals deficient in the biosynthesis of GDP-fucose have been identified (3, 4) and suffer from the immune disorder leukocyte adhesion deficiency type II (LADII). These patients fail to synthesize fucosylated blood groups, and their leukocytes do not express the fucose containing carbohydrate sialyl Lewis X. The patient’s leukocytes do not extravasate normally, which leads to recurrent infections.

In his pioneering work in the early 1960s, Ginsberg (5, 6) elucidated the enzymatic pathway converting GDP-mannose to GDP-fucose. Later, Yurchenco and Atkinson (7) showed that this was the primary biosynthetic route to GDP-fucose. As shown in Fig. 1, GDP-mannose is converted to GDP-fucose by GDP-mannose 4,6-dehydratase via the oxidation of mannose at C-4 followed by the reduction of C-6 to a methyl group, yielding GDP-4-keto-6-deoxymannose. The reaction has been reported to proceed with transfer of a hydride from C-4 to C-6 (8) by a tightly bound cofactor, thought to be NADP\(^+\) or NAD\(^+\), which is regenerated during the reaction. This intermediate is then epimerized at C-3 and C-5 to yield GDP-4-keto-6-deoxy-glucose and finally reduced by NADH or NADPH at C-4 to produce GDP-fucose. In the initial studies, it was not certain if the last two steps, the epimerizations and reduction, were performed by one enzyme or two. One potential regulatory mechanism in the pathway was first revealed in the studies of Kornfeld and Ginsberg (9), who demonstrated that GDP-mannose 4,6-dehydratase was inhibited by the final product in the biosynthetic pathway, GDP-fucose.

Recent studies have addressed several open questions about the enzymes in this pathway. Two studies have shown that GDP-mannose 4,6-dehydratase utilizes NADP\(^+\) and not NAD\(^+\) as a cofactor. Yamamoto et al. demonstrated this with the enzyme from *Klebsiella pneumoniae* (10) and more recently Stura et al. detected NADP\(^+\) bound to the *Escherichia coli* enzyme (11). This requirement for NADP\(^+\) differentiates GDP-mannose 4,6-dehydratase from the two other well characterized sugar nucleotide 4,6-dehydratases, dTDP-glucose 4,6-dehydratase and CDP-glucose 4,6-dehydratase, both which require NAD\(^+\) (12, 13). Additionally, recent studies have addressed the question of whether the epimerase and reductase activities are present in one protein or are two separate proteins as is the case in dTDP-rhamnose biosynthesis (14, 15). Serif and co-workers (16) suggested that the 3,5-epimerase and

---

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1744 solely to indicate this fact.

† To whom correspondence should be addressed: Small Molecule Drug Discovery, Genetics Institute, Inc., 87 Cambridgepark Dr., Cambridge, MA 02140. Tel.: 617-498-8936; Fax: 617-498-8993; E-mail: fsullivan@genetics.com.

---

\(^{*}\) The abbreviations used are: LADII, leukocyte adhesion deficiency type II; hGM, human GDP-mannose 4,6-dehydratase (EC 4.2.1.47); GMD, *E. coli* GDP-mannose 4,6-dehydratase; hFX, human FX protein (GDP-4-keto-6-deoxymannose 3,5-epimerase, 4-reductase); WCAG, *E. coli* GDP-4-keto-6-deoxymannose 3,5-epimerase, 4-reductase; PFR, polynucleotide chain reaction; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; ESI-LC-MS, electrospray ionization-liquid chromatography-mass spectrometry; ESI-MS, electrospray ionization-mass spectrometry; EST, expressed sequence tag; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

\(^{1}\) Additional studies have addressed the question of whether the epimerase and reductase activities are present in one protein or are two separate proteins as is the case in dTDP-rhamnose biosynthesis (14, 15). Serif and co-workers (16) suggested that the 3,5-epimerase and
4-reductase activities were present on a single polypeptide when they purified small amounts of the enzyme from pig thyroids (16). This was confirmed by Tonetti et al. (17) when they cloned the human protein FX. Sequencing the gene revealed homology to the bacterial sugar nucleotide reductases. Using antibody depletion experiments, purified protein, and cell extracts as a source for the GDP-4-keto-6-deoxymannose, they demonstrated that FX combined both the epimerase and reductase activities in one polypeptide.

To understand better the human enzymes involved in this pathway, their role in selectin-mediated cell adhesion, and the LADII defect, we have undertaken the molecular cloning of the human gene encoding GDP-mannose 4,6-dehydratase. Furthermore, utilizing purified recombinant enzymes expressed in E. coli, we have reconstituted the GDP-fucose biosynthetic pathway in vitro. We demonstrate that two enzymes, GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxymannose 3,5-epimerase, 4-reductase, are sufficient to synthesize GDP-fucose from GDP-mannose, confirming earlier studies suggesting that both epimerase and reductase activities are encoded in a single polypeptide. Additionally, we show that human GDP-mannose 4,6-dehydratase has a strict specificity for NADP⁺ over NAD⁺. Using the homologous E. coli enzymes, GDP-mannose 4,6-dehydratase (GMD) and GDP-4-keto-6-deoxymannose 3,5-epimerase, 4-reductase (WCAG), we demonstrate that the same is true in bacteria. We also show that human GDP-mannose 4,6-dehydratase is subject to feedback inhibition by GDP-fucose and that this, along with its differential levels of gene expression, provides potential mechanisms for regulating its activity.

**Materials and Methods**

**Data Base Searching and Sequence Alignments**—The National Center for Biotechnology Information (NCBI) EST data base was searched with BLAST on the NCBI server. The human and E. coli dehydratase peptides were aligned with the program GAP of the Genetics Computer Group analysis package. Amino terminal peptides of the GDP-mannose 4,6-dehydratase from different species were aligned using the GeneWorks program from IntelliGenetics, Inc.

**Cloning Human GDP-mannose 4,6-Dehydratase**—A cDNA library was constructed from HL-60 cells as described by Sako et al. (18). This plasmid-based cDNA expression library was assembled into 19 pools, each representing 100,000 to 100,000 individual clones/pool. The pools were screened by PCR using DNA primers based on both the mouse EST (accession number W29220) and the E. coli sequence: 5′-TGATGAGCCAGAGCACTTTGCTATGCTAC-3′ and 5′-CAGAGA-GTCCACTTCAGTCGGTCGGTAGTA-3′. Two pools gave the expected 200-base pair fragment. The 200-base pair PCR product was reamplified by PCR, random primer-labeled with 32P, and used to identify positive clones from the two libraries by colony hybridization. This approach yielded plasmid pMT-hGMD containing the cDNA of human GDP-mannose dehydratase in a eukaryotic expression vector.

**Cloning of Human GDP-4-keto-6-deoxymannose 3,5-Epimerase, 4-Reductase**—A similar approach was taken to isolate the human epimerase reductase genes using following oligonucleotides based upon the published sequence (17): 5′-GTGACATGGGTAACCCCAGGGATCCATGC-3′ and 5′-TGCCCATCTCCTAGTGTGAAGTGTCGTGTGGC-3′. The positive pools were probed with the 32P-labeled PCR primers. This yielded plasmid pMT-hFX, containing the cDNA of human GDP-mannose epimerase-reductase in a eukaryotic expression vector.

**Transfection of Lec13 and Cell Staining**—The Chinese hamster ovary (CHO) cell line Lec13, was obtained from Professor P. Stanley at Albert Einstein College of Medicine. This line was first transfected with pMT-Neo-STIV, a vector expressing human fucosyltransferase IV and the Neo gene, and then transfected with the plasmid pMT-hFX. Expression by immunofluorescence after staining with CD15 antibody (Immunotech) and goat anti-mouse fluorescein isothiocyanate secondary antibody (Boehringer Mannheim).

**In Vitro Assays of CHO Cell Extracts**—Mutant CHO cells transfected with human dehydratase cDNA, human epimerase-reductase cDNA, or wild type CHO cells were lysed under nitrogen pressure in 0.75 ml of 25 mM Hepes, pH 7.4, 100 mM NaCl, 10 mM EDTA, 10 mM DTT for 5 min in a Parr Bomb at 900 p.s.i. on ice. The cell debris was pelleted at 50,000 × g for 1 h, and soluble extracts were assayed in 25 mM Hepes, pH 7.4, 100 mM NaCl, 15 mM MgCl₂, 10 mM DTT, 10 μM GDP-mannose, with 100,000 cpm of 14C-labeled GDP-mannose for 2 h at 37 °C. The reactions were stopped by boiling for 5 min followed by centrifugation for 5 min at 15,000 rpm in a microcentrifuge. Unlabeled GDP-mannose and GDP-fucose were added as standards. GDP-mannose, GDP-fucose and the 4-keto-6-deoxy intermediate were separated as described by Yamamoto et al. (10) except the amide-80 column (Tosohas) was run in 66% acetonitrile and 7.5 mM citric acid/NaHPO₄ buffer, pH 4.0. The 14C-labeled sugar nucleotides were detected with a flow through scintillation counter Beta-1 (Packard) run with a solid scintillant cell. The unlabeled sugar nucleotides were detected at 254 nm.

**Expression and Purification of Human Enzymes from E. coli**—The human dehydratase and epimerase-reductase genes were cloned by PCR into the EcoRI and HindIII sites of vector pSEThGMD and pSEThFX. The inserts in the resulting vectors were sequenced in their entirety. The resulting dehydratase fusion protein had the sequence MRSHSHHHHHSRSMTGGGQMRGRLYDDEDDKPSPPSAGTM-EF added to amino acid 20 of the predicted sequence, the position homologous to the start of the E. coli enzyme. For the epimerase-reductase, the same 43-amino acid fusion peptide was added to amino acid 20 of the predicted sequence, the position homologous to the start of the E. coli enzyme. For the epimerase-reductase, the same 43-amino acid fusion peptide was added to amino acid 20 of the predicted sequence.
acid 2 of the published sequence. The two expression vectors were transformed into *E. coli* strain BL21/DE3 and the bacteria were grown in LB media containing ampicillin and chloramphenicol. Both the dehydratase-expressing cells and epimerase-reductase-expressing cells were grown at room temperature to an *A*<sub>600</sub> of 0.5 and induced with 0.3 mM IPTG for 4 h at 20 °C. Two-dimensional total correlation spectroscopy, nuclear Overhauser effect spectroscopy, and heteronuclear multiple-quantum coherence were performed with standard Varian pulse sequences.

**Cloning of *E. coli* GMD and *E. coli* WCAG**—The *gmd* and *wcaG* genes were cloned from the *E. coli* K-12 genome using PCR oligonucleotides over their entire lengths, and both contain an extended con- codon 255 and the first nucleotide in codon 256 changed from a CG in *(Fig. 3)*. The human protein more closely aligns with the initiating methionine of the *H. sapiens* genome.”

**Molecular Cloning of Human GDP-mannose 4,6-Dehydratase**—To clone human GDP-mannose 4,6-dehydratase, we first performed a TBLASTN search of the NCBI EST data base using the sequence of the *E. coli* enzyme. This identified a mouse EST, accession number W29220, with a high degree of homology to the *E. coli* enzyme. We designed two oligonucleotide primers based on conserved amino acid sequences present in both the *E. coli* sequence and the partial sequence of the mouse gene. Using the oligonucleotides as primers, we obtained a 200-base pair PCR fragment from a human promyelocytic cell line HL-60 cDNA library. This fragment was then used as a probe to isolate two apparently full-length cDNA clones for the putative human GDP-mannose 4,6 dehydratase, the sequence of which is shown in *(Fig. 2). There are two potential initiator methionines located at nucleotides 76 and 130 of this sequence. As shown in *(Fig. 3A)*, the downstream methionine of the human protein more closely aligns with the initiating methionine of the *E. coli* protein. However, alignment of the human sequence to the recently cloned arabidopsis GDP-mannose 4,6-dehydratase gene and a putative *C. elegans* translation product *(Fig. 3B)* suggests that translation of the human protein initi- ates at the first methionine, at nucleotide 76, and that dehy- dratases from nonbacterial sources contain an amino-terminal extension. The human and *E. coli* proteins are 18% identical in their two lengths, and both contain an extended con- sensus sequence, GXXGXXG, identifying the ββββ fold found in many NAD<sup>+</sup> - and NADP<sup>+</sup>-binding proteins *(22)*. This sequence is found between amino acids 9 and 15 of the *E. coli* enzyme. *(Fig. 4)* shows that the human dehydratase gene encodes a single mRNA transcript of about 1.7 kilobase pairs that is expressed in all tissue and cell types examined, albeit at varying levels.
The mRNA levels are highest in pancreas followed by small intestine, liver, colon, and prostate and lowest in ovary, brain, lung, spleen, and peripheral blood lymphocytes. Likewise, a varied level of expression was seen in the human cell lines examined (Fig. 4 C).

GDP-mannose 4,6-Dehydratase cDNA Complements the Dehydratase Defect in Lec13—To demonstrate the isolated cDNA encoded GDP-mannose 4,6-dehydratase activity, we transiently transfected it into Lec13, a CHO cell line that previously has been identified as lacking GDP-mannose 4,6-dehydratase activity (23). To monitor the GDP-mannose 4,6-dehydratase activity, we first stably transfected Lec13 with human fucosyltransferase IV (19), an enzyme that utilizes GDP-fucose to synthesize the Lewis X (CD15) epitope. This gave a readily identifiable cell surface marker dependent upon the biosynthesis of GDP-fucose. Restoration of dehydratase activity in the mutant cells should restore GDP-fucose biosynthesis and produce Lewis X antigen on the cell surface. As demonstrated in Fig. 5 A, culture of these Fuc-T IV-expressing Lec13 cells in media containing fucose allowed the synthesis of GDP-fucose through the salvage pathway and generated CD15-positive cells. Transient transfection of the same cell line with the vector expressing the human GDP-mannose 4,6-dehydratase, in media lacking fucose, also causes the cells to stain positive for Lewis X, demonstrating that the dehydratase gene complements the CHO cell defect (Fig. 5 B). By contrast, transfection with the same vector expressing the human epimerase-reductase gene, again in media lacking fucose, shows no staining by CD15 (Fig. 5 C). As further evidence, lysates of Lec13 cells transiently transfected with human GDP-mannose 4,6-dehydratase cDNA readily converted 14C-labeled GDP-mannose to GDP-fucose (Fig. 6 B), whereas lysates of Lec13 cells transfected with the human epimerase-reductase cDNA in the same expression vector did not (Fig. 6 A).

Based on this data, we conclude that the cDNA encodes GDP-mannose 4,6-dehydratase.
Fig. 4. Northern analysis of human GDP-mannose 4,6-dehydratase gene expression. Autoradiogram of Northern blots probed with 32P-labeled human GDP-mannose dehydratase-specific probe is shown. The 1.5-kilobase pair EcoRI fragment of plasmid fragment pMT-HOMD, containing the complete hGMD cDNA insert, was gel-purified, random primer-labeled, and used to probe poly(A)+ RNA blots from CLONTECH (human I (catalog number 7760-1); human II (catalog number 7759-1); and human cancer cell line (catalog number 7757-1)) under high stringency conditions. A and B show expression in human tissues, and C shows expression in human cell lines. Analysis of the β-actin messages are shown at the bottom.

Fig. 5. Transfection with human GDP-mannose 4,6-dehydratase cDNA complements the defect in Lec13 cells. The top parts of panels A–C show fluorescent micrographs of Fuc-T IV-transformed 9E9A LT2.9 Lec13 cells stained with CD15 antibody and fluorescein isothiocyanate-conjugated secondary antibody. The lower parts show phase contrast micrographs of the same fields. A, the cells fed fucose, as a positive control, stain positive for Lewis X, the product of the Fuc-T IV gene and GDP-fucose. B, the cells transfected with the cDNA for human GDP-mannose 4,6-dehydratase also stain positive for Lewis X, demonstrating the complementation of the dehydratase defect in Lec13. C, the cells transfected with the cDNA for human FX do not stain at all. Fuc-T IV activity in 9E9A LT2.9 cells was unstable and gradually decreased with passage in culture. Thus, not all of the cells in panel A stain positive. The experiment shown above was done on the same day using the same starting cells to make the comparison in CD15 staining meaningful.

Fig. 6. In Vitro assay of dehydratase and epimerase-reductase activity in wild type CHO and transfected Lec13 CHO cell lines. HPLC analysis of reactions of 14C-labeled GDP-mannose with cell extracts are shown. A, 125 μg of extract of 9E9A LT2.9 Lec13 cells transfected with epimerase-reductase cDNA. B, 25 μg of extract of 9E9A LT2.9 Lec13 cells transfected with dehydratase cDNA. C, 125 μg of extract of CHO Dukx cells (wild type for GDP-mannose 4,6-dehydratase). The positions of 14C-labeled GDP-mannose and GDP-fucose standards are shown above.
Chromosomal Localization of Human GDP-mannose 4,6-Dehydratase and FX Gene—To determine if the two genes for GDP-fucose biosynthesis are linked on the human genome we mapped their location using fluorescence in situ hybridization. Full-length cDNA inserts encoding the human dehydratase (pMT-hGMD) and epimerase reductase (pMT-hFX) genes were used to probe a human genomic PAC (hGMD) or P1 (hFX) libraries (24, 25) (Genome Systems, Inc., St. Louis, MO). Two genomic clones were obtained for each probe. We confirmed that the genomic clones contained the hGMD and hFX genes by subcloning and sequencing (data not shown). The hGMD and hFX genes were mapped using two-color fluorescence in situ hybridization utilizing the genomic clone for each gene and a centromere-specific probe (26) (Genome Systems, Inc., St. Louis, MO). The two genes are not linked in the human genome. The human dehydratase gene was localized to the p terminus of chromosome 6, an area corresponding to band 6p25. A total of 80 metaphase cells were analyzed, with 64 exhibiting specific labeling. In a similar fashion, human epimerase-reductase was mapped to the q terminus of chromosome 8, an area corresponding to band 8q24.3. A total of 80 metaphase cells were analyzed, with 70 exhibiting specific labeling (data not shown).

**Human GDP-mannose Dehydratase and Epimerase-Reductase**

**FIG. 7.** SDS-PAGE of recombinant enzymes purified from *E. coli*. A, hGMD and hFX. B, GMD and WCAG. Molecular masses of markers, in kDa, are shown in the center. Gels are stained with Coomassie Blue.

**FIG. 8.** *In vitro* assay of human and *E. coli* dehydratase and epimerase reductase activity. HPLC analysis of reactions of 14C-labeled GDP-mannose with purified recombinant enzymes are shown. A, 1 μg of human dehydratase plus 100 μM NADP⁺; B, 1 μg of human dehydratase plus 1 mM NAD⁺; C, 1 μg of human dehydratase plus 100 μM NAD⁻; D, 1 μg of *E. coli* dehydratase plus 1 mM NADP⁺; E, 1 μg of *E. coli* dehydratase plus 1 mM NAD⁺, followed by 1 μg of *E. coli* epimerase-reductase plus 100 μM NADPH; F, 1 μg of *E. coli* dehydratase plus 1 mM NAD⁺, followed by 1 μg of *E. coli* epimerase-reductase plus 100 μM NADPH. The arrows show the position of 14C-labeled standards GDP-mannose and GDP-fucose.
Cloning of Human GDP-mannose 4,6-Dehydratase

8199
tase Are Sufficient to Convert GDP-mannose to GDP-fucose—To further characterize GDP-mannose 4,6-dehydratase, and to reconstitute GDP-fucose biosynthesis in vitro, we needed to obtain purified proteins for both the dehydratase and epimerase-reductase enzymes. To this end, we expressed both the human GDP-mannose 4,6-dehydratase and GDP-4-keto-6-deoxy-mannose 3,5-epimerase 4-reductase in E. coli as fusion proteins. The fusion proteins were purified (Fig. 7A), and their identities were confirmed by sequencing the first 15 amino acids of each peptide (data not shown). The human dehydratase protein migrated on SDS-PAGE near the position expected based upon its calculated molecular mass (42.7 kDa), but the epimerase-reductase migrated more slowly than expected (38.3 kDa), as previously reported by Tonetti et al. (17). We confirmed that recombinant hFX had the mass predicted from its cDNA sequence by ESI-LC-MS (data not shown).

To characterize the reactions of the purified dehydratase and epimerase-reductase, we incubated the enzymes with 14C-labeled GDP-mannose and identified the reaction products by HPLC and paper chromatography. As shown in Fig. 8A, purified GDP-mannose 4,6-dehydratase converts 14C-labeled GDP-mannose to a new species that runs at the position reported for GDP-4-keto-6-deoxymannose (10). We confirmed the identity of GDP-4-keto-6-deoxymannose by descending paper chromatography. As shown in Fig. 1, the expected monosaccharides resulting from reduction of GDP-4-keto-6-deoxymannose by borohydride and cleavage from the nucleotide with acid would be rhamnose and 6-deoxytalose. Fig. 9A (filled triangles) shows that when the reaction products obtained by incubating GDP-mannose, human dehydratase, and NADP+ were reduced, cleaved, and run on paper, four spots resulted. The major species, running at 16 cm, co-migrates with an unlabeled standard for rhamnose. This component also co-migrates with rhamnose in solvent systems II and III (data not shown). Unlike the human dehydratase, human dehydratase, and NADPH catalyzed 14C-labeled GDP-mannose was GDP-4-keto-6-deoxymannose. NMR analysis of the isolated product of human dehydratase and epimerase-reductase by descending paper chromatography. 14C-Labeled reaction products were reduced with NaBH4, and the resulting sugar was cleaved with acid and spotted on Whatman 3MM paper developed in water-saturated methyl ethyl ketone for 24 h. The paper was cut into strips, and 1-cm sections of each strip were counted. A, the reactions contained GDP-mannose, human dehydratase, and NADP+, followed by human epimerase-reductase plus NADPH (open squares) or GDP-mannose, human dehydratase, and NADP+ (filled triangles). B, the reactions contained GDP-mannose, E. coli dehydratase, NADP+, E. coli epimerase-reductase, and NADPH (open squares) or GDP-mannose, E. coli dehydratase, and NADP+ (filled triangles). The position of 14C-labeled GDP-mannose and GDP-fucose that had been treated identically to the reaction mixtures is shown above labeled as mannose and fucose, respectively. The position of unlabeled free rhamnose is also shown at the top of the trace.

Characterization of GDP-4-keto-6-deoxymannose by ESI-MS

and High Field NMR—To confirm that the reaction product of human dehydratase and GDP-mannose was GDP-4-keto-6-deoxymannose, we isolated the product and analyzed it by mass spectrometry and NMR. The major peaks in the ESI-MS spectrum of the isolated intermediate were at 586.1 and 292.6, corresponding to [M – H]− ion and [M – H]+, respectively for GDP-4-keto-6-deoxymannose. A partial spectrum is shown in Fig. 10, where the [M – H]− peak at 586.1, the [M + Na]+ peak at 608.1, and the [M + 2Na]+ peak at 630.1 for GDP-4-keto-6-deoxymannose are clearly visible. A [M – H]− peak for residual GDP-mannose at 604.0 is also present in the spectra. A combination of one-dimensional homonuclear, two-dimensional homonuclear, and two-dimensional heteronuclear NMR experiments revealed that the isolated reaction product was a mixture of at least two related sugar nucleotides. The observed chemical shifts and coupling constants are listed in Table I. From these data, we have identified the two major species as GDP-4-keto-6-deoxymannose and GDP-3-keto-6-deoxymannose. NMR analysis of the isolated product of E. coli dehydratase also showed a similar mixture of GDP-4-keto-6-
deoxymannose and GDP-3-keto-6-deoxymannose.

E. coli GDP-mannose Dehydratase and Epimerase-Reductase Are Sufficient to Convert GDP-mannose to GDP-fucose—To address whether the bacterial enzymes catalyze the same reactions as the human dehydratase and epimerase-reductase, we cloned, expressed, and purified the E. coli enzymes (Fig. 7B). We examined the reactions of the E. coli enzymes in the same way we characterized the human enzymes. Fig. 8E shows that incubation of E. coli dehydratase with GDP-mannose and NADP$^+$ resulted in production of the reaction intermediate GDP-4-keto-6-deoxymannose. This reaction product produced only two spots in paper chromatography after reduction and cleavage. One migrated with the rhamnose standard at 17 cm, and one migrated faster at 37 cm (Fig. 9B, filled triangles). The spot seen at 25 cm in the reaction with human dehydratase is missing (Fig. 9, compare A and B (filled triangles)). The E. coli dehydratase used NADP$^+$ as a cofactor and could not substitute NAD$^+$ at concentrations up to 1 mM (Fig. 8, D and E).

As with the human enzymes, E. coli dehydratase and epimerase-reductase were sufficient to convert GDP-mannose to GDP-fucose. Incubation of GDP-mannose with E. coli dehydratase and E. coli epimerase-reductase in the presence of NADP$^+$ and NADPH converted the GDP-mannose to GDP-fucose (Fig. 8F). We confirmed the identity of GDP-fucose by paper chromatography (Fig. 9B, open squares). The E. coli epimerase-reductase can use NADH but not as efficiently as NADPH (data not shown).

We performed a preliminary characterization of the two dehydratases, and the results are shown in Table II. The E. coli enzyme has a significantly higher $K_m$ for GDP-mannose than the human enzyme but also has a significantly higher $V_{max}$. Additionally, we monitored inhibition of the dehydratase by GDP-fucose, which has been proposed as a mechanism to regulate the enzyme's activity. Both human and E. coli dehydratases were inhibited by GDP-fucose with IC$_{50}$ values lower than the IC$_{50}$ values for inhibition by GDP, suggesting a specific effect and a potential role in regulation of the enzyme’s activity. Quite unexpectedly, both enzymes are stimulated by NADPH at micromolar concentrations, although this co-factor does not

| Table I Chemical shifts and J coupling for GDP-4-keto-6-deoxymannose and GDP-3-keto-6-deoxymannose |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | $^{13}$C       | $^1$H          | $^1$H          | $^{13}$C       | $^1$H          | $^1$H          | $^{13}$C       | $^1$H          | $^1$H          |
| Base            | 140.15         | 8.11           | 140.15         | 8.11           |
| Ribose          | 89.12          | 5.93           | 89.12          | 5.96           | 6.0            |
| 2'              | 75.97          | 4.81           | 75.97          | 4.86           |
| 3'              | 72.85          | 4.51           | 72.85          | 4.53           |
| 4'              | 86.29          | 4.35           | 86.29          | 4.35           |
| 5'              | 67.69          | 4.21           | 67.69          | 4.21           |
| Mannose         | 98.09          | 5.59           | 98.87          | 5.45           | 1.6, 7.7       |
| 1'              | 77.53          | 4.46           | 72.99          | 4.01           | −1             |
| 2'              | 75.09          | 4.82           |                |                |
| 3'              | 72.99          | 4.34           | 70.88          | 3.94           | <1             |
| 4'              | 70.88          | 4.70           | 72.88          | 4.07           | 6.6            |
| 5'              | 72.88          | 4.70           | 72.88          | 4.07           | 6.6            |
| CH$_3$          | 15.18          | 1.23           | 13.63          | 1.23           |
play a role in catalysis. It is not clear if this stimulation by NADPH is relevant to the in vivo regulation of the enzyme.

**DISCUSSION**

We have cloned the gene encoding human GDP-mannose 4,6-dehydratase using homology between the *E. coli* enzyme and a mouse EST. The cloned gene complemented the previously identified GDP-mannose 4,6-dehydratase defect in the CHO cell line Lec13, demonstrating that the cDNA encodes a functional protein. The dehydratase gene shows high levels of identity between bacteria and human and indeed across the spectrum of species examined (for a comparison of dehydratases from a variety of bacterial and nonmammalian sources see Bonin et al. (28). The message for human GDP-mannose 4,6-dehydratase is expressed in all tissues examined, albeit at varying levels. The varying levels of expression of the dehydratase message suggest the enzyme may be regulated at the level of transcription, and in fact there is evidence for developmental regulation in rat and nereids (29, 30). It also appears that, in both humans and *E. coli*, GDP-fucose biosynthesis is regulated by feedback inhibition of the dehydratase by GDP-fucose, the final product in the pathway. This mechanism of inhibition was noted for *Aerobacter aerogenes* by Kornfeld and Ginsberg (9) and was suggested for the porcine enzyme by Serif and co-workers (33).

The cloning of the human dehydratase gene, along with the recent cloning of the human epimerase-reductase by Tonetti et al. (17) has allowed us to reconstitute GDP-fucose biosynthesis in vitro using purified, recombinant enzymes. Thus, we have definitively shown that two enzymes, a dehydratase and an epimerase-reductase, are sufficient to convert GDP-mannose to GDP-fucose. In doing so, we demonstrated, using purified recombinant proteins, that in humans and in *E. coli*, both 3,5-epimerase and 4,6-reductase activities are present in a single protein. This confirms the earlier studies of Serif and co-workers (16) on the enzyme purified from porcine thyroids and the recent work of Tonetti et al. (17) with the human FX protein. Additionally, we find that human dehydratase has a strict cofactor requirement for NADPH+ for which NADH cannot substitute. This is consistent with earlier reports demonstrating that GDP-mannose 4,6-dehydratase from *K. pneumoniae* requires NADPH+ (10) as well as the recent work of Sturla et al., who reported that purified *E. coli* dehydratase contains NADP+.

We have characterized the product of the dehydratase reaction, GDP-4-keto-6-deoxymannose. This product was first reported by Ginsberg (6) for the bacterial enzyme and later by Overton and Serif (27) for the porcine enzyme. Using purified dehydratase, we have analyzed the reaction products by both HPLC and paper chromatography and find results consistent with the expected product. For unambiguous structural confirmation, we isolated and analyzed the intermediate by ESI-MS and high field NMR. Mass spectrometric analysis yielded a molecular mass consistent with GDP-4-keto-6-deoxymannose but no evidence of any other GDP-mannose derivatives of different masses. High field NMR revealed the presence of two compounds, one being the expected product GDP-4-keto-6-deoxymannose and the other the related GDP-3-keto-6-deoxymannose. This was the case for the product of both the human and *E. coli* dehydratases. The presence of both the 4-keto- and 3-keto-6-deoxy-sugars was also seen in the dTDP-4-keto-6-deoxyglucose pathway where a 4,6-dehydratase converts dTDP-glucose to dTDP-4-keto-6-deoxyglucose (31, 32). The biological significance of both GDP-4-keto-6-deoxymannose and GDP-3-keto-6-deoxymannose intermediates is unclear, although apparently both are epimerized and reduced to GDP-fucose by the epimerase-reductase (Fig. 8, C and F). It is possible that the 3-keto-sugar arose during work-up of the isolated reaction product. However, the isolated, unlabeled intermediate also was converted to GDP-fucose by purified human epimerase-reductase (data not shown).

Cells from two patients having LADII do not fucosylate their cell surfaces (4) and as such lack both blood group antigens and the sialyl Lewis X and related epitopes that function as selectin ligands. The molecular basis of this disorder is still unknown. As with Lec13, this phenotype can be rescued in cell lines derived from these patients by culturing them in the presence of fucose, suggesting that GDP-fucose transport and the complement of fucosyltransferrases are intact in these cells. This would imply that the defect in the LADII patients lies in the pathway converting GDP-mannose to GDP-fucose, i.e. either the dehydratase or epimerase-reductase. With the human genes for both enzymes now cloned, we can determine if either is responsible for the LADII phenotype. There are suggestions from *E. coli* that there may be an additional gene that plays a role in GDP-fucose biosynthesis in vivo. Sequencing of the capsular polysaccharide operon in *E. coli* led to the identification of an open reading frame (*wcaH*) immediately downstream of the dehydratase, *gmd*, and epimerase-reductase genes, *wcaG* (21). This putative protein has been assigned to the GDP-fucose biosynthetic pathway, yet it is clearly not necessary for conversion of GDP-mannose to GDP-fucose in vitro. *WcaH* may play a role in GDP-fucose biosynthesis in vivo or play another, yet unidentified, role in capsular polysaccharide biosynthesis. The cloning of the human GDP-mannose 4,6-dehydratase gene provides a valuable tool to address outstanding questions in the regulation, biosynthesis, and role of GDP-fucose in vivo.

**Acknowledgments**—We thank Professor Pamala Stanley of the Albert Einstein Collage of Medicine for the gift of the Lec13 cell line; Drs. Diane Sako and Monique Davies of Genetics Institute for the gifts of plasmids pMTNeoFV1 and pEDPyLT, respectively; Drs. Elliott Nickbarg and Robert Gassaway of Genetics Institute for ESI-LC-MS analysis and peptide sequencing; Kevin Bean and Mark Proia of Genetics Institute for DNA sequencing and library screening. We thank Drs. John Lowe and Peter Smith of University of Michigan for sharing data on the LADII cell lines prior to publication. We thank Drs. Simon Jones and John Knopf of Genetics Institute for critical reading of the manuscript.

**REFERENCES**

1. Feizi, T. (1991) Trends Biochem. Sci. 16, 84–86
2. Kansas, G. S. (1996) Blood 88, 3259–3267
3. Frydman, M., Etzioni, A., Edidit-Markus, T., Avidor, I., Varsano, I., Shechter, Y., Orlin, J. B., and Gershoni-Baruch, R. (1992) Am. J. Med. Genet. 44, 297–302
4. Etzioni, A., Frydman, M., Pollack, S., Avidor, I., Phillips, M. L., Paulson, J. C., and Gershoni-Baruch, R. (1992) N. Engl. J. Med. 327, 1789–1792
5. Ginsberg, V. (1960) J. Biol. Chem. 235, 2366–2371
6. Ginsberg, V. (1961) J. Biol. Chem. 236, 2389–2393
7. Yurchenco, P. D. and Atkinson, P. H. (1977) Biochemistry 16, 944–953
8. Oths, P. J., Mayer, R. M., and Floss, H. G. (1990) Carbohydr. Res. 198, 91–100
9. Kornfeld, R. H., and Ginsburg, V. (1966) Biochem. Biophys. Acta 117, 79–87
10. Yamamoto, K., Katayama, I., Onoda, Y., Inami, M., Kumagai, H., and Tochikura, T. (1993) Arch. Biochem. Biophys. 300, 694–698

2. P. Smith and J. Lowe, personal communication.
11. Sturla, L., Bisso, A., Zanardi, D., Benatti, U., De Flora, A., and Tonetti, M. (1997) FEBS Lett. 412, 126–130
12. Glaser, L., and Zarkowski, H. (1971) in The Enzymes (Boyer, P. D., ed) Vol. 5, pp. 465–497, Academic Press, New York
13. Yu, Y., Russell, R. N., Thorson, J. S., Liu, L., Liu, H. (1992) J. Biol. Chem. 267, 5868–5875
14. Melo, A., and Glaser, L. (1968) J. Biol. Chem. 243, 1475–1478
15. Jiang, X.-M., Neal, B., Santiago, F., Lee, S. J., Romana, L. K., and Reeves, P. R. (1991) Mol. Microbiol. 5, 695–713
16. Chang, S., Duerr, B., and Serif, G. (1988) J. Biol. Chem. 263, 1693–1697
17. Tonetti, M., Sturla, L., Bisso, A., Benatti, U., De Flora, A. (1996) J. Biol. Chem. 271, 27274–27279
18. Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Abern, T. J., Furie, B., Cumming, D. S., and Larsen, G. R. (1993) Cell 75, 1179–1186
19. Kumar, R., Potvin, B., Muller, W. A., and Stanley, P. (1991) J. Biol. Chem. 266, 21777–21783
20. Chaplin, M. F. (1986) in Carbohydrate Analysis: A Practical Approach (Chaplin, M. F., and Kennedy, J. F., eds) pp. 1–37, IRL Press Ltd., Oxford
21. Stevenson, G., Anadrianopoulos, K., Hobbs, M., and Reeves, P. R. (1996) J. Bacteriol. 178, 4885–4893
22. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) J. Mol. Biol. 187, 101–107
23. Ripka, J., Adamany, A., and Stanley, P. (1986) Arch. Biochem. Biophys. 249, 533–545
24. Ioannou, P., Amemiya, C., Garees, J., Dkroisel, P., Shizuya, H., Chen, C., Batzer, M., and de Jong, P. (1994) Nat. Genet. 6, 84–89
25. Shepherd, N., Pfotzigner, B., Coulby, J., Ackerman, S., Vaidyanathan, G., Sauer, R., Balkenhol, T., and Sternberg, N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2629–2633
26. Stokke, T., Collins, C., Kuo, W., Kowbel, D., Shadravan, F., Tanner, M., Kallioniem, A., Kallioniem, O., Pinkel, D., Deaven, L., Gray, J. (1995) Genomics 26, 102–117
27. Overton, K., and Serif, G. S. (1981) Biochem. Biophys. Acta. 675, 281–284
28. Bonin, C. P., Potter, J., Vanzin, G. F., and Reiter, W.-D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2085–2090
29. Ruggiero-Lopez, D., Biol, M. C., Louisot, P., and Martin, A. (1991) Biochem. J. 279, 801–806
30. Bulet, P., Hoflack, B., Porchet, M., and Verbert, A. (1984) Eur. J. Biochem. 144, 255–259
31. Stein, A., Kula, M., Elling, L., Verseck, S., and Klaffke, W. (1995) Angew. Chem. Int. Ed. Engl. 34, 1748–1749
32. Naundorf, A., and Klaffke, W. (1996) Carbohydr. Res. 285, 141–150
33. Broschat, K. O., Chang, S., and Serif, G. (1985) Eur. J. Biochem. 153, 397–401
Molecular Cloning of Human GDP-mannose 4,6-Dehydratase and Reconstitution of GDP-fucose Biosynthesis in Vitro
Francis X. Sullivan, Ravindra Kumar, Ronald Kriz, Mark Stahl, Guang-Yi Xu, Jason Rouse, Xiao-jia Chang, Amechand Boodhoo, Barry Potvin and Dale A. Cumming

J. Biol. Chem. 1998, 273:8193-8202. doi: 10.1074/jbc.273.14.8193

Access the most updated version of this article at http://www.jbc.org/content/273/14/8193

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 12 of which can be accessed free at http://www.jbc.org/content/273/14/8193.full.html#ref-list-1