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USE OF SITE-DIRECTED MUTAGENESIS TO IDENTIFY THE GALACTOSYLTRANSFERASE BINDING SITES FOR UDP-GALACTOSE+

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Site-directed mutagenesis was utilized to identify binding sites for UDP-galactose in galactosyltransferase (EC 2.4.1.22). Mutant cDNAs were generated by a procedure based on PCR, and the mutated enzymes were expressed in E.coli cells. The mutant enzymes were purified by Ni-NTA Sephadex, and the degree of purification was judged by SDS-PAGE. Purified mutant GTs, F305L, P306V, N307S, N308S, showed dramatic decreases in activities in comparison with the activity of the wild-type GT. Enzyme kinetic analysis revealed that the Km values of F305L, P306V, N307S and N308S for UDP-galactose were, respectively, 9-, 11-, 50- and 20-fold higher than the Km of wild-type GT, but the Km values for manganese were not significantly different from that of the wild-type GT. The quartet mutant F305L/P306V/N307S/N308S showed no activity. From the results of this study it is concluded that amino acids, Phe-305, Pro-306, Asn-307 and Asn-308, in GT are most probably involved in GT catalysis or are located close to the UDP-galactose binding region but are not involved in the binding of manganese.

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Abbreviations: ANUP, 4-azido-2-nitrophenyluridylyl pyrophosphate. CNBr, cyanogen bromide. EDTA, ethylenodiamino-tetraacetic acid. GT or galactosyl transferase, UDP-galactose N-acetylglucose β1, 4-galactosyltransferase. IPTG, isopropyl-beta-D-thiogalactopyranoside. Km, Michaelis kinetic constant. NADH, nicotinamide adenine dinucleotide (reduced form). PCR, polymerase chain reaction.

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Galactosyltransferase (EC 2.4.1.22) is an enzyme that transfers galactose from UDP-galactose to terminal N-acetylglucosamine in glycoproteins and glycolipids (1). The enzyme exists in a soluble form in body fluids such as milk, colostrum and serum. The soluble form of the enzyme is derived from the membrane bound form by proteolytic cleavage (2, 3) at Arg-77 (4). The catalytic domain, or soluble form, of GT is composed of 323 amino acid residues (5). Galactosyltransferase (UDP-galactose N-acetylglucose β1, 4-galactosyltransferase) cDNA was the first human glycosyltransferase cDNA to be isolated (6). The cDNA was cloned into the pIN-III ompA derived vector, pIN-GT, and was subsequently used to express an active form of the enzyme (4). Currently a number of newly isolated glycosyltransferases are being used in expression vector systems for site-specific mutagenesis experiments that can give information about the specific amino acids used by galactosyltransferase in substrate recognition (5,7).

Previous classical types of enzyme kinetic studies produced a great deal of fundamental information about galactosyltransferase (8,9,10,11), but had limitations with respect to their ability to identify the specific amino acids involved in substrate binding. Previously photoaffinity substrate labeling studies were done and revealed the approximate intramolecular location of the UDP-galactose binding site (12). The photoaffinity label, 4-azido-2-nitrophenyl uridylyl pyrophosphate (ANUP) covalently bound to a CNBr cleaved fragment of galactosyltransferase consisting of amino acids Asp-276 through Met-328 (4). Hence this previous work served to identify the target region of GT where specific amino acid mutations were to be made. Previous kinetic studies of the mutated GT showed also that Tyr-284, Tyr-309 and Trp-310 are critically involved in the N-acetylglucosamine binding, and Tyr-309 is involved in UDP-galactose binding as well (5). The present paper further defines the location of the UDP-galactose binding site region.

MATERIALS AND METHODS

Construction of Mutated Galactosyltransferase cDNA Fragments and Their Insertion into the pIN-GT Expression Vector.

General DNA recombinant techniques were carried out as described in Short Protocols in Molecular Biology (13). Two restriction sites, Not I and Hind III, in previously reported the pIN-GT vector, were used for the cloning purposes (5). A quartet mutant, F305L/P306V/N307S/N308S, with a 6-histidine tail was made using

the three step PCR method described by Higuchi, et al. (14). Also a silent Eco RV mutation, near the site of additional desired mutations, was made by the same three step PCR method. As shown in Figure I, the F305L, P306V, N307S and N308S mutations were made with two PCR steps. The conditions used make PCR products were 1 min. at 94° C, 1 min. at 60-65° C and 2 min. at 72° C using Vent DNA polymerase. The amplification was carried out for 40 cycles. The final PCR products, containing the mutated galactosyltransferase DNAs, were purified using Promega Wizard DNA purification kits and subsequently treated with restriction enzymes to form the two required ends so that the DNA fragment would be cloned into the corresponding restriction sites of pIN-GT expression vector. The mutations that were made are shown in Table I and had 6-histidine tails as showed in the same table.

Expression of Wild Type and Mutated Galactosyltransferase in JM 109 E. coli Cell.

JM 109 E.coli cells were used to express the wild type and mutated galactosyltransferase using the method described by Aoki et al. (5). After mutation the DNAs were ligated into the pIN-GT vector, the pIN-GT mutation vectors were used to transform to JM 109 E. coli cells by means of electroporation. The wild-type and mutated GTs were expressed in JM 109 E. coli cells under the vector control of the *lpp* promoter and *lac* promoter-operator. E. coli JM 109 cells harboring the constructed plasmids were thus able to produce the enzyme upon induction with isopropyl β-D-thio-galactopyranoside (IPTG). The transformed cells were incubated overnight at 37° C in LB (Luria-Bertani) medium containing ampicillin (100μg/ml) and then transfered to the larger cultures and allowed to grow under the same conditions until reaching the mid-log growth phase which required 2-3 hours. IPTG was then

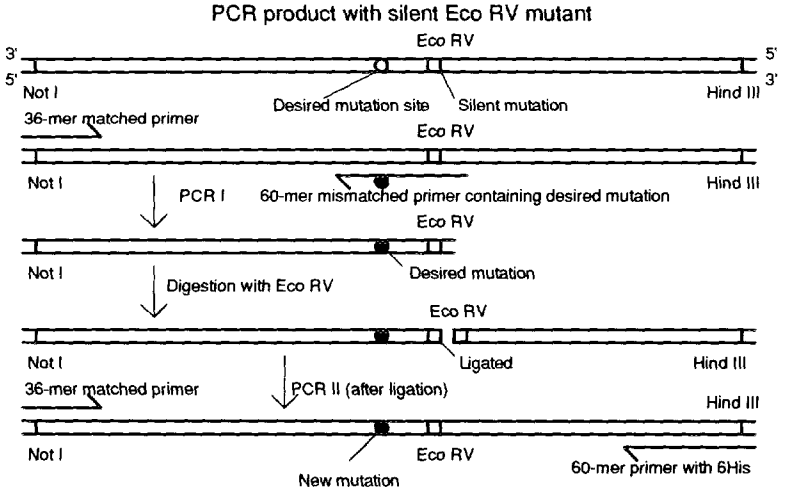

Figure I. Use of the Eco RV silent mutant to make F305L, P306V, N307S and N308S mutations. PCR I produced a DNA fragment that contained Not I, Eco RV and desired mutation. The PCR I product and the Not I-Hind III silent Eco RV mutant were digested with Eco RV. After purification of the Eco RV-Hind III fragment of the silent mutant, it was ligated to the digested PCR I product at the Eco RV site and used as a template for PCR II. The PCR II produced a DNA segment that contained the desired mutation with Not I and Hind III ends which were produced after Not I and Hind III digestion. The Not I-Hind III DNA segment containing the new mutation was subsequently ligated into the pIN-GT vector.

Table I. Wild-type and mutation GT sequences and mutation primers. PCR primer sequences are shown below and made as indicated in footnotes. Bases in bold font indicate mutations. Mutated amino acids are underlined.

GTs	DNA and amino acids sequences
Wild*	901 · base pair · 939
	5'-ACC ATC AAT GGA TTT CCT AAT AAT TAT TGG GGC TGG GGA-3'
	Thr Ile Asn Gly Phe Pro Asn Asn Tyr Trp Gly Trp Gly·····6His
	301 302 303 304 305 306 307 308 309 310 311 312 313
F305L/P306V/N307S/N308S†	
	888 · base pair · 948
	5'-CAA · · · · · AAT GGA **TTG GTT AGT AGT** TAT TGG · · · · · · · · · GAT-3'
	Gln · · · · · · Asn Gly **Leu Val Ser Ser** Tyr Trp · · · · · · · · · Asp·····6His
	296 · · · · · · 303 304 305 306 307 308 309 310 · · · · · · · · · 316
F305L‡	910 · base pair · 959
(F->L)	5'-GGA **TTG** CCT AAT AAT TAT TGG GGC TGG GGA · · · · · · · · · ATC TT-3'
	Gly **Leu** Pro Asn Asn Tyr Trp Gly Trp Gly · · · · · · · · · · Ile····· ·6His
	304 305 306 307 308 309 310 311 312 313 · · · · · · · · · · 319
P306V‡	910 · base pair · 959
(P->V)	5'-GGA TTT **GTT** AAT AAT TAT TGG GGC TGG GGA · · · · · · · · · ATC TT-3'
	Gly Phe **Val** Asn Asn Tyr Trp Gly Trp Gly · · · · · · · · · · Ile·····6His
	304 305 306 307 308 309 310 311 312 313 · · · · · · · · · · 319
N307S‡	910 · base pair · 959
(P->V)	5'-GGA TTT CCT **AGT** AAT TAT TGG GGC TGG GGA · · · · · · · · · ATC TT-3'
	Gly Phe Pro **Ser** Asn Tyr Trp Gly Trp Gly · · · · · · · · · · Ile·····6His
	304 305 306 307 308 309 310 311 312 313 · · · · · · · · · · 319
N308S‡	916 · base pair · 959
(N->S)	5'-CCT AAT **AGT** TAT TGG GGC TGG GGA · · · · · · · · · · · · ATC TT-3'
	Pro Asn **Ser** Tyr Trp Gly Trp Gly · · · · · · · · · · · · · · Ile·····6His
	306 307 308 309 310 311 312 313 · · · · · · · · · · · · · · · · 319

* Wild type GT DNA sequence contained a 6-histidine tail.

† Two complementary primers were used containing nucleotide sequences 888—>948.

‡ Primer sequence was complementary to that shown above.

added to culture to induce the synthesis of the fusion proteins, and the E.coli cultures were subsequently incubated for an additional 6 hours. Extraction of the galactosyltransferase from the cell utilized the method described by Henco (15). Mutant plasmids were extracted from the E.coli cells utilizing to Promega Plasmid Miniprep Kits. Existence of the desired GT cDNA mutations was confirmed by sequencing using the dideoxynucleotide termination method (16).

Purification of Wild and Mutation Galactosyltransferase with Ni-NTA-Sephadex Chromatography.

Ni-NTA Sephadex (Qiagen Inc.) chromatography was used to purify the galactosyltransferase mutants which contained the 6-histidine tails added to the C-terminal of the wild and mutated galactosyltransferases. The cell extract that contained the GTs were purified about 4-fold by one step Ni-NTA-Sephadex affinity chromatography (Table II) and were subsequectly used to measure Km for UDP-galactose and manganese. The Ni-NTA Sephadex affinity columns were prepared by equilibration with 1.9 M ammonium sulfate in 10 mM Tris buffer. The crude enzyme, treated with the same salt concentration and buffer, was applied to the Ni-NTA column and eluted from the column with a low salt buffer (50 mM ammonium sulfate in 10 mM Tris buffer). The galactosyltransferase eluate with the highest activity and lowest amount of protein was concentrated by ultrafiltration and either used immediatly or stored at -20° C.

Table II. Purification of GT by one step Ni-NTA Sephadex affinity chromatography

step	volume ml	protein conc. µg/ml	activity *u/min/ml	total activity	Yield %	sp.act. u/min/mg	fold purify
extract	80	5.5	0.142	11.36	100	25.8	--
Ni-column	40	2.5	0.263	10.52	96.2	105.3	4.1

*u=unit. 1 unit equals: the amount of UDP that is produced and needed to convert 1mM NADH + H to NAD/min.

Galactosyltransferase Activity Assay.

Galactosyltransferase was assayed using the pyruvate kinase, lactate dehydrogenase coupled reaction in which the change in A340, due to a decrease in the concentration of NADH, was followed spectrophotometrically as described by Wong et al.(17).

Kinetic Studies of the Wild and Mutated Galactosyltransferases.

The Km values for the UDP-galactose and manganese are obtained from the initial reaction velocities measured at various concentrations of one substrate determined in the presence of fixed, saturating concentration of other two substrates (18). The GraphPad InPlot computer program Version 4.03 (GraphPad Software Inc.) was used to calculated Km values from the initial velocity and substrate concentrations.

RESULTS

Comparison of the Michaelis Constants of Wild Type and Mutated GTs.

In order to further investigate the structurally related functions of GT, we measured the enzyme-kinetic properties of wild-type GT and its mutants in which residues 305, 306, 307 and 308 were substituted with the amino acids shown in Table I. The wild-type enzymes showed higher activity than the mutated GTs, and the mutants F305L, P306V, N307S and N308S showed dramatic decreases in enzyme activities as shown in Table III which also shows the Michaelis constants (Km) for UDP-galactose and manganese. The results indicate, therefore, that amino acid residues Phe-305, Pro-306, Asn-307 and Asn-308 in galactosyltransferase are most

Table III. The Km of wild and mutated GTs for UDP-galactose and manganese

GTs	Km(UDP-Gal)	Km(UDP-Gal) increased fold	Km (Mn^{2+} mM)	Activity % of wild GT
wild	69 µM	--	0.29	100
F305L	610 µM	9	0.29	22.6
P306V	750 µM	11	0.29	16.5
N307S	3490 µM	50	0.28	11.4
N308S	1380 µM	20	0.29	16.4
LVSS	NA*	--	NA	0

* NA=No activity.

probably involved in GT catalysis or are closely located to the binding sites for UDP-galactose binding and are not involed in the binding of manganese.

DISCUSSION

This study used PCR to make mutant galactosyltransferase cDNAs which were inserted into the pIN-III ompA vector which was in turn used to transform E.coli cells resulting in the expression of mutant forms of GT. This method is general in that it should be usable to put almost any deletion, insertion, or substitution anywhere along the entire length of a DNA segment. In this study the use of PCR to introduce mutations into the DNA sequence needed either two or three steps. PCR was performed using mismatched primers to introduce mutations into the galactosyltransferase DNA sequence. Two primary PCR reactions produced two overlapping DNA fragments, both bearing the same mutation, which was introduced into the region of overlap via primer mismatch. The overlap in sequence allowed the two fragments to recombine in two possible ways after their mixture, denaturation, and renaturation. Only one of these combinations was able to produce a structure with a recessed 3'-OH end that could be extended by the DNA polymerase reaction to result in a complete duplex fragment. These extended segments could then serve as templates for the secondary reamplification of the combined sequences (19). By means of a silent mutation, a new restriction site we made that was very close to the region of mutation. This introduced DNA mutation resulted in a sequence that therefore needed only a two step PCR to produce the desired mutations as shown in Figure I.

In order to identify the galactosyltransferase substrate, UDP-galactose, binding site, the Phe-305—>Asn-308 region of the GT peptide chain was selected to make mutations for the following reasons. *Firstly*, this is a hydrophobic area of the enzyme as predicted by the method of Kyte and Doolittle (20), and a hydrophobic pocket may be involved in GT substrate binding since a number of other enzyme catalyzed reactions require a hydrophobic environment. *Secondly*, this area is more likely to have a β-sheet rather than an α-helix secondary structure as predicted by the method of Kyte and Doolittle (4,20), and substrate binding sites are believed to occur more

frequently in the former than in the latter type of secondary structure. *Thirdly*, in a prior study by Aoki. et. al. (5), mutations were made at 309, 310 and 312, and these mutations were found to affect GT activity. A previous study by Aoki et al. (5) showed that the mutant Tyr-309 has Ka and Kia values that are 30-fold larger than those of the wild-type, and hence those the results suggested Tyr-309 is likely involved in the UDP-galactose binding. *Fourth*, the Phe-305—>Asn-308 region of the molecule lies within the peptide sequence labeled with the UDP-galactose analog photoaffinity label ANUP as reported by Aoki. et. al. (5).

The rationale for choosing specific amino acid to replace the original amino acids was as follows: 1) Phe-305 was replaced by leucine, because phenylalanine and leucine both are hydrophobic amino acids, and hence the substitution is more likely to change substrate hydrogen bonding rather than substrate binding related to hydrophilicity; 2) Pro-306 was replaced by valine, because both proline and valine have small side chains which means that after the valine side chain replaces that of proline there should still enough space for the new amino acid side chain; 3) Asn-307 and Asn-308 were replaced by serines, because asparagine and serine are similar in that they are both are small and polar amino acids; 4) wild-type kinetics data differences in mutants that have less drastic changes in amino acid side chain would be expected to produce changes that are more closely related to specific substrate binding rather than to changes in peptide secondary structure.

The above results show that Km values of the mutation N307S and N308S are 50 and 20-fold, respectively, higher than that of wild-type. We, therefore, conclude that the side chains of Asn-307 and Asn-308 constitute binding sites for the substrate, UDP-galactose, because loss of the asparagine side chain results in decreased enzymatic activity. Due to the more hydrophilic character of the serine side chain as compared to that of asparagine, the former probably does not contribute to the formation of a hydrophobic pocket. After serine replaces the asparagine, the hydrophobic pocket structure probably becomes more hydrophilic and may result in a microenvironment that is less favorable for the GT reaction. There appears to be a similar requirement for the Phe-305 and Pro-306 side chains which are also hydrophobic.

REFERENCES

1. Ram, B. P. and Munjal, D. D. (1985) CRC Critical Reviews in Biochemistry. 17: 257-311.
2. Smith, C. A. and Brew, K. (1977) Journal of Biological Chemistry. 252:7294-7299.
3. Strous, G. J., Van Berkhof, P., Willamsen, R., Gueze, H. J. and Berger, E. G. (1985) J. Cell Biol. 97:723-727.
4. Masri, K. A., Appert, H. E. and Fukuda, M. N. (1988) Biochem. Biophys. Res. Commun. 157:657-663.
5. Aoki, D., Appert, H. E., Johnson, D., Wong, S. S. and Fukuda, M. N. (1990) EMBO J. 9:3171-3178.
6. Appert, H. E, Rutherford, T. J, Tarr, G. E, Weist, J. S, Thomford, N. R and McCorquodale, D. J. (1986) Biochem. Biophys. Res. Commun. 139:163.
7. Wang, Y., Wong, S. S., Fukuda, M. N., Zu, H., Liu, Z., Tang, Q. And Appert, H. E. (1994) Biochem. Biophys. Res. Commun. 204:701-709.
8. Berliner, L. J. and Robinson, R. D. (1982) Biochem. 21: 6340-6343.
9. Berliner, L. J., Davis, M. E., Ebner, K. E., Beyer, T. A. and Bell, E. (1984) Mole. Cell. Biochem. 62:37-42.
10. Clymer, D. S., Geren, C. R. and Ebner, K. E. (1976) Biochem. 15:1093-1097.
11. Takae, K. And Ebner, K. E. (1984) Current Topics in Cellular Regulation. 24:51-62.
12. Lee, T. K., Wong, L. J. C. And Wong, S. S. (1983) J. Biol. Chem. 258:13166-13171.
13. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmae, J. G., Smith, J. A. and Struhl, K. (1992) [Short Protocols in Molecular Biology] John Wiley & Sons, New York. pp. 8.18-8.25.
14. Higuchi, R., Krummel, B. And Saiki, R. K. (1988) Nucleic acids research. Vol.16. number 15. pp7351-7367.
15. Henco, K. (1991) [The QIA Expressionist] Qiagen Inc. Chatsworth, CA. pp.43-46.
16. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proceedings of the National Academy of Sciences of the USA. 74:5463-5467.
17. Wong, S. S., Malone, T. E. and Lee, T. K. (1983) Biochem. Biophys. Acta. 745: 90-96.
18. Cleland, W. W. (1963) Biochim. Biophys. Acta. 67:104-137.
19. Higuchi, R., Krummel, B. and Saiki, R. K. (1988) Nucleic acids research. Vol.16. number 15. pp7351-7367.
20. Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. 157:105-132.