Co-purification of galactosyltransferases from chick-embryo liver

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Two galactosyltransferases with nearly identical M_r values were purified 5000–7000-fold from microsomal membranes of chick-embryo livers by using several affinity columns. One enzyme transfers galactose from UDP-galactose to form a β -(1→4)-linkage to GlcNAc (*N*-acetylglucosamine) or AsAgAGP [asialo-agalacto-(α_1 -acid glycoprotein)]. The other enzyme forms a β -(1→3)-linkage to AsOSM [asialo-(ovine submaxillary mucin)]. Both enzymes were solubilized (85%) from a microsomal pellet by using 1% Triton X-100 in 0.1 M-NaCl. The supernatant activities were subjected to DEAE-Sepharose chromatography and four affinity columns: UDP-hexanolamine-Sepharose, α -lactalbumin-Sepharose, GlcNAc-Sepharose and either AsAgAGP-Sepharose or AsOSM-Sepharose. The AsAgAGP enzyme [(1→4)-transferase] and the AsOSM enzyme [(1→3)-transferase] behave identically on the DEAE-Sepharose and UDP-hexanolamine-Sepharose columns, and similarly on the α -lactalbumin-Sepharose column. Final separation of the two enzymes, however, could only be achieved on affinity columns of their immobilized respective acceptors. Both purified enzymes migrate as a single band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after silver staining, and both have an apparent M_r of 68000. The enzymes were radioiodinated and again subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Radioautographic analyses showed only one, intensely radioactive, band. Activity stains performed for both transferases after cellulose acetate electrophoresis indicate that, with this system too, both activities have identical mobilities, and co-migrate, as well, with the major, silver-stained, protein band. Kinetic studies with the purified enzymes show that the K_m value for GlcNAc, for the (1→4)-transferase, is 4 mM; for the (1→3)-transferase the K_m value for AsOSM is 5 mM, in terms of GalNAc (*N*-acetylgalactosamine) equivalents. Both enzymes have a K_m value of 25 μ M for UDP-galactose.

Glycosyltransferases catalyse the synthesis of all glycosidic bonds; the total number of these enzymes is unknown, but must be very large. The transferases can clearly recognize even subtle differences among acceptor molecules (Owens & Mackenzie, 1982; Betteridge & Watkins, 1983). Some investigators have suggested that the transferases exist as families of related proteins (Roseman, 1970; Roth, 1973). Although a small, but increasing, number of membrane-bound transferases has been purified extensively (Smith & Brew, 1977; Bouchilloux, 1979; Fraser *et al.*, 1980; Mendicino *et al.*, 1982; Weinstein *et al.*, 1982;

Thomson, 1983; Sheares & Carlson, 1983; Strous *et al.*, 1983; Kaplan & Hechtman, 1983), in no case have two or more functionally and biologically related galactosyltransferases been purified and compared. The present paper describes the purification to apparent homogeneity of two, initially membrane-bound, galactosyltransferases from 13-day chick-embryo livers.

Experimental

Tissue

Embryonated chicken eggs were purchased from Truslow Farms (Chestertown, MD, U.S.A.). Eggs were incubated in a commercial egg incubator at 37°C in water-saturated air. After 13 days of incubation, livers were removed immediately and

Abbreviations used: GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; AsAgAGP, asialo-agalacto-(α_1 -acid glycoprotein); AsOSM, asialo-(ovine submaxillary mucin); SDS, sodium dodecyl sulphate.

placed in a solution of ice-cold 0.9% NaCl, and frozen at -20°C . All subsequent purification steps were performed at 4°C . When 10g (wet wt.) was accumulated, the livers were thawed and rinsed twice with 0.9% NaCl, homogenized with five or six strokes, in a Teflon-pestled Potter-Elvehjem homogenizer, in 10vol. of homogenizing buffer (0.25M-sucrose in 10mM-Tris/HCl buffer, pH 7.4, containing 1mM-EDTA). The homogenate was filtered and centrifuged at 7700g for 30min. The resulting supernatant was then centrifuged at 105000g for 60min in a 50 Ti rotor. The pellets were suspended, pooled and stirred for 20min in 10ml of homogenizing buffer containing 1% Triton X-100 and 0.1M-NaCl. The solubilized proteins, referred to below as the microsomal detergent extract, were separated from the insoluble debris by centrifugation at 105000g for 30min, again in a 50 Ti rotor.

Materials

UDP-D-[6- ^3H]galactose (16.3Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.) and analysed periodically for purity by using high-voltage paper electrophoresis in 1% sodium tetraborate. UDP-hexanolamine-Sepharose was kindly given by Dr. Robert Hill (Duke University, Durham, NC, U.S.A.), and α_1 -acid glycoprotein was generously supplied by Dr. M. Wickerhauser (American Red Cross Blood Services Laboratories, Bethesda, MD, U.S.A.). Unlabelled UDP-galactose, and all other reagents, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Purification of galactosyltransferases

Bio-Gel A-1.5m. The concentrated detergent extract was applied to a column (1.5cm $\times$ 56cm) of Bio-Gel A-1.5m equilibrated with 50mM-sodium cacodylate buffer, pH 7.0, containing, as do all subsequent solutions, 0.1% Triton X-100. The column was calibrated with Blue Dextran 2000, bovine serum albumin and Phenol Red.

Step 1: DEAE-Sepharose. The microsomal detergent extract was concentrated by ultrafiltration on a YM-10 filter. The concentrate, generally 2ml, was applied to a DEAE-Sepharose CL-6B ion-exchange column (1.3cm $\times$ 22cm) equilibrated with 50mM-sodium cacodylate buffer, pH 7.0, containing 0.1M-NaCl. The column was eluted with the same buffer; over 95% of the galactosyltransferase activities was recovered in the eluate.

Step 2: UDP-hexanolamine-Sepharose. Fractions 13–23 from the DEAE-Sepharose column in Step 1 were pooled and concentrated to less than 1 ml by ultrafiltration as above. One-ninth volume of 250mM-MnCl₂ was added to the concentrate,

which was then applied to a UDP-hexanolamine-Sepharose column (0.8cm $\times$ 8.5cm) equilibrated with 50mM-sodium cacodylate buffer, pH 7.0, containing 25mM-MnCl₂. Bound enzymes were eluted with the column buffer containing 1mM-UDP and 1M-NaCl, but without MnCl₂.

Step 3: α -lactalbumin-Sepharose. The UDP-hexanolamine-bound fractions were concentrated as above to 1–2ml. One-ninth volume of 0.1M-GlcNAc and 0.1M-MnCl₂ was added to the concentrate, which was then applied to a lactalbumin-Sepharose column (1.6cm $\times$ 8cm) equilibrated with 50mM-sodium cacodylate buffer, pH 7.0, containing 10mM-GlcNAc, 10mM-MnCl₂ and 1M-NaCl. The bound fractions were eluted with the column buffer lacking GlcNAc and MnCl₂ but containing 0.5M-KSCN.

Step 4: GlcNAc-Sepharose. The α -lactalbumin-bound fractions, described in step 3, were dialysed to remove KSCN, and again concentrated to less than 2ml. One-ninth volume of 10mM-sodium cacodylate buffer, pH 7.0, containing 0.1M-MnCl₂ and 10mM-UMP was added to the concentrate. The solution was then applied to the GlcNAc-Sepharose column (1.6cm $\times$ 11cm) equilibrated with 10mM-sodium cacodylate buffer, pH 7.0, containing 10mM-MnCl₂, 1mM-UMP, and 1M-NaCl. The bound fractions were eluted with 10mM-GlcNAc and 1M-NaCl in the same cacodylate buffer.

Step 5: AsAgAGP-Sepharose. The fractions that initially bound to the GlcNAc-Sepharose column, described in step 4, were again concentrated to less than 2ml by ultrafiltration. One-ninth volume of 10mM-sodium cacodylate buffer, pH 7.0, containing 0.1M-MnCl₂ and 10mM-UMP was added to the concentrate, which was then applied to an AsAgAGP-Sepharose column (1.6cm $\times$ 8cm) equilibrated with 10mM-sodium cacodylate buffer, pH 7.0, containing 10mM-MnCl₂, 1mM-UMP and 1M-NaCl. The bound fractions were eluted with 10mM-sodium cacodylate buffer, pH 7.0, containing 10mM-GlcNAc and 1M-NaCl.

Step 6: AsOSM-Sepharose. The pass-through fractions from the GlcNAc-Sepharose and AsAgAGP-Sepharose columns described in step 4 and 5 were concentrated as above. One-ninth volume of 10mM-sodium cacodylate buffer, pH 7.0, containing 0.1M-MnCl₂, 10mM-UMP, 1M-NaCl and 0.1M-GlcNAc was added to the concentrate. The concentrate was then applied to an AsOSM-Sepharose column (1.6cm $\times$ 11cm) equilibrated with 10mM-sodium cacodylate buffer, pH 7.0, containing 10mM-MnCl₂, 1mM-UMP, 10mM-GlcNAc and 1M-NaCl. The bound galactosyltransferase activity was eluted with 10mM-sodium cacodylate buffer, pH 7.0, containing 0.5M-KSCN and 1M-NaCl.

Affinity columns

The Sepharose-based affinity gels used in steps 3–6 were synthesized by using CNBr-activated Sepharose according to the method of March *et al.* (1974). Sialic acid and galactose were sequentially removed from the α_1 -acid glycoprotein by (a) mild acid hydrolysis (Spiro, 1960) and (b) treatment with jack-bean β -galactosidase partly purified in this laboratory by using the methods of Li *et al.* (1975). Ovine submaxillary mucin was prepared from submaxillary glands dissected from sheep supplied by a local slaughterhouse. The mucin was purified by the method described by Tettamanti & Pigman (1968), and was desialylated by mild acid hydrolysis (Spiro, 1960). The increase in galactose-acceptor activity of both compounds was tested routinely with microsomal detergent extracts and UDP-[3 H]galactose.

Transferase assays

All transferase assays were conducted in a final volume of 50 μ l containing the following reagents: 23 μ M-UDP-[3 H]galactose (390 mCi/mmol), 3 mM-AMP, 0.1% Triton X-100, 20 mM-Mes, pH 6.5, the appropriate enzyme fraction, and MnCl_2 and acceptors according to the galactosyltransferase being assayed. For the (1 $\rightarrow$ 4)-transferase, the final MnCl_2 concentration was 15 mM and the acceptor was GlcNAc at 10 mM or AsAgAGP at 250 μ g. For the (1 $\rightarrow$ 3)-transferase, the MnCl_2 concentration was 10 mM and the acceptor was 10 mM-GalNAc equivalents of AsOSM. Mixtures were incubated at 37°C in a shaking water bath for times that are given for each experiment. In accordance with published methods (Roth *et al.*, 1971), reactions were terminated by the addition of 0.47 M-EDTA in 10% sodium tetraborate. The resulting mixtures were applied to Whatman 3MM paper, after which the paper was saturated with 1% sodium tetraborate and subjected to high-voltage electrophoresis (3000 V/23 cm) for 30 min. After the electrophoretograms were dried, the area around the origin was cut from each lane and the radioactivity determined.

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide gels (7.5 or 10%) were performed at a constant current of 30 mA/14 cm, according to the method of Weber & Osborn (1969). Gels were stained with silver by using procedures described by Willoughby & Lambert (1983).

Isoelectric focusing

The detergent extract from 2 g (wet wt.) of liver was mixed with Ultradex, Ampholyte (pH 3–10), and ferritin as a marker. Flat-bed isoelectric

focusing was performed until the ferritin was focused, at which time the gel slurry was sliced into 5 mm strips. The strips were suspended in 10 mM-sodium cacodylate buffer, pH 7.0, and centrifuged lightly to pellet the gel. The supernatant transferase activities were assayed as described above. pH values were determined with a pH-meter.

Detection of transferase activity on electrophoretograms

The coupled-enzyme method for the detection of glycosyltransferases (Pierce *et al.*, 1980) was utilized. Transferases were subjected to cellulose acetate electrophoresis in 0.1 M-sodium cacodylate buffer, pH 7.5. The electrophoretograms were overlaid with Whatman 3MM paper saturated with the coupled-enzyme mixture and incubated in a 37°C water-saturated atmosphere for 1 h. In the presence of the appropriate acceptor, the coupled enzymes produce 2 mol of NADH per mol of galactose transferred by a galactosyltransferase. The enzyme location was determined by drying the Whatman indicator papers and examining them under u.v. light.

Sugar and protein assays

Sialic acid was assayed by using the periodate/resorcinol reagent (Jourdan *et al.*, 1971); galactose was assayed by using the phenol/ H_2SO_4 reagent (Dubois *et al.*, 1956); hexosamine was measured by using Ehrlich reagent (Svennerholm, 1956). To assay GalNAc and GlcNAc in ovine submaxillary mucin and α_1 -acid glycoprotein, the acid hydrolysates were subjected to amino acid analysis with a Dionex amino acid analyser. More than 95% of the hexosamine in α_1 -acid glycoprotein was glucosamine, whereas more than 97% of the hexosamine in ovine submaxillary mucin was galactosamine. Protein was determined by the Lowry method with bovine serum albumin in the appropriate Triton concentration as a standard.

Product characterization

Galactosylated GlcNAc, AsAgAGP and AsOSM were prepared by incubating these acceptors separately with partly purified enzymes and UDP-[3 H]galactose under standard assay conditions. The product of the incubation with GlcNAc was eluted from the origin of a borate electrophoretogram, as described, and applied to a Bio-Gel P-2 column (0.8 cm $\times$ 59 cm) before and after treatment with either 0.1 M-NaOH at 37°C for 2 days (Mayo & Carson, 1970) or with the β -(1 $\rightarrow$ 4)-specific galactosidase from *Escherichia coli* (Sheares *et al.*, 1982). The galactosylated AsAgAGP thus prepared was treated with β -(1 $\rightarrow$ 4)-specific galactosidase from jack beans (Li *et al.*, 1975). The product of the incubation with AsOSM

was similarly isolated and treated either with the β -(1 $\rightarrow$ 4)-specific galactosidase from jack beans, which is active on large- M_r substrates, or with 0.1M-NaOH solution in the presence or in the absence of the reducing agent 1M-NaBH₄ (Sheares *et al.*, 1982). These reactants were also applied to the Bio-Gel P-2 column. The column was calibrated by using labelled mono- and di-saccharides.

Iodination

Purified enzymes were radioiodinated by the chloramine-T method of Hunter & Greenwood (1962). The proteins (<1 μ g) were oxidized at room temperature for 2 min in the presence of Na¹²⁵I (1 mCi). The reaction was terminated with sodium metabisulphite. The iodinated proteins were desalted with Sephadex G-25, and the three peak fractions for each of the transferases were pooled and used for analysis by SDS/polyacrylamide-gel electrophoresis.

Cleveland peptide analysis

Radioiodinated proteins were isolated by SDS/polyacrylamide-gel electrophoresis, and the area to which bovine serum albumin migrates was sliced and transferred directly to new wells containing *Staphylococcus aureus* V8 proteinase, in accordance with the method of Cleveland *et al.* (1977). After SDS/polyacrylamide-gel electrophoresis, the dried electrophoretograms were exposed to X-ray films to obtain radioautograms.

Results

Detergent extraction of galactosyltransferases

Four detergents (Triton X-100, Nonidet P-40, SDS and sodium deoxycholate) were used initially to extract transferases from the microsomal pellets. Each of the detergents were used at concentrations of 0.25%, 0.5%, 1% and 2%. SDS and sodium deoxycholate are very ineffective solubilizers of these activities, whereas Triton and Nonidet P-40, at 1%, each solubilizes nearly all the transferase activities. Because the addition of 0.1M salt enhances the extraction of both enzymes, and because Triton is slightly more effective than Nonidet P-40, 1% Triton X-100 in 0.1M-NaCl was used throughout for the initial solubilization of the transferases.

Effect of Mn²⁺ and pH on galactosyltransferases

The (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 3)-transferases have different optimal requirements for Mn²⁺: the (1 $\rightarrow$ 4)-transferase is most active at 15 mM, whereas the (1 $\rightarrow$ 3)-transferase is most active at 5–10 mM. These results correspond precisely to those previously obtained with rat liver enzymes (Strous *et al.*, 1980). Subsequent assays were therefore conducted with

15 mM-MnCl₂ for the (1 $\rightarrow$ 4)-transferase and with 10 mM-MnCl₂ for the (1 $\rightarrow$ 3)-transferase. The pH optima for both enzymes are broad and lie between pH 5 and 7. For all the following assays, the pH was maintained at 6.5 with 20 mM-Mes buffer. The Mn²⁺ and pH data were obtained with solubilized microsomal pellets as an enzyme source.

Properties of the (1 $\rightarrow$ 4)- and (1 $\rightarrow$ 3)-galactosyltransferases

The galactosylation of individual acceptors, as described in the Experimental section, was compared with the galactosylation obtained when acceptors were mixed in pair-wise combinations. In all cases, acceptor and UDP-galactose concentrations were saturating, and the assays were conducted for 30 min, well within the linear range of the assays. The data (Table 1) show that free GlcNAc and AsAgAGP are competitive substrates, whereas AsAgAGP and AsOSM are not. Similarly, free GlcNAc and AsOSM do not compete with one another. The results indicate that the (1 $\rightarrow$ 4)-transferase active site recognized both free GlcNAc and AsAgAGP, and that the (1 $\rightarrow$ 3)-transferase active site is different and recognizes, of the acceptors tested, only AsOSM.

To determine if the two activities could be separated by using isoelectric focusing, the detergent extract was subjected to flat-bed isoelectric focusing. Fig. 1 presents the results. Although the (1 $\rightarrow$ 4)-transferase (pI 6.24) and the (1 $\rightarrow$ 3)-transferase (pI 6.40) activities migrate differently from one another on the basis of their isoelectric points, the difference is not sufficient for separation. Nevertheless, the consistent difference in pI values

Table 1. Substrate-competition analyses of chick-embryo liver galactosyltransferases

Assays were performed as described in the text, with 14.6 μ g of protein from the DEAE-Sepharose purification step. Acceptor concentrations are GlcNAc, 10 mM, AsAgAGP, 250 μ g/50 μ l, and AsOSM, 250 μ g/50 μ l.

Acceptor	Activity (nmol of galactose transferred/30 min per μ g of protein)	
	Observed	Expected for two enzymes
GlcNAc	0.88 $\pm$ 0.02	
AsAgAGP	1.77 $\pm$ 0.06	
AsOSM	0.19 $\pm$ 0.01	
GlcNAc + AsAgAGP	1.45 $\pm$ 0.05	2.71
GlcNAc + AsOSM	1.03 $\pm$ 0.09	1.07
AsAgAGP + AsOSM	2.20 $\pm$ 0.03	1.97

Fig. 1. Flat-bed isoelectric focusing of galactosyltransferase activities in detergent extracts

The detergent extract was mixed with Ultradex, Ampholyte (pH 3–10), and ferritin as a marker. For full details see the text. ●, (1→4)-Transferase activity; ○, (1→3)-transferase activity; ▲, pH gradient; ○ (upper scale), protein.

between the (1→4)- and (1→3)-transferases suggests strongly that the two active sites are on two different molecular species.

Fig. 2 shows the results when three galactosyltransferase substrates, GlcNAc, AsAgAGP and AsOSM, are incubated with purified enzymes, isolated and subjected to enzymic and alkaline hydrolyses. Fig. 2(a) shows that galactosylated GlcNAc is sensitive to *E. coli* β -galactosidase, but not to alkaline hydrolysis. Figs. 2(b) and 2(c) show that galactosylated AsAgAGP, but not AsOSM, is sensitive to jack-bean β -galactosidase. Galactosylated AsOSM is sensitive to hydrolysis in alkaline borohydride (Fig. 2c). Also, the results show that alkaline hydrolysis in the absence of borohydride yields free galactose, consistent with prior reports (Sheares *et al.*, 1982) that (1→3)-glycosyl linkages are labile to simple alkaline hydrolysis. These data suggest that galactose is added to GlcNAc and AsAgAGP in a β -(1→4)-configuration, and to AsOSM in a β -(1→3)-configuration.

Purification of galactosyltransferases

Step 1: DEAE-Sepharose. The elution patterns of both the (1→4)- and the (1→3)-transferases from DEAE-Sepharose are shown in Fig. 3. For both enzymes, most activity is eluted relatively late, after the elution position of free glucose. No further activities are eluted even with 2M-NaCl. Neither the (1→4)- nor the (1→3)-transferase activities in the detergent extract bind to the Sepharose used as the supporting matrix for all the affinity columns in this study.

Step 2: UDP-hexanolamine-Sepharose. The major peak fractions of both enzyme activities from the DEAE-Sepharose (step 1) were applied to a UDP-hexanolamine-Sepharose column. More than 95% of both activities is retained. Both the retained activities were eluted with 1 mM-UDP in 1 M-NaCl. The two transferases were not separated on this column by elution with linear gradients of UDP or UDP-galactose.

Step 3: α -lactalbumin-Sepharose. All the frac-

Fig. 2. Characterization of the galactosylated products by Bio-Gel P-2 chromatography

In (a), with GlcNAc as an acceptor, the product was not treated (●), treated with *E. coli* β -galactosidase (○) or subjected to alkaline treatment (▲). In (b), the acceptor AsAgAGP was either untreated (●) or treated with jack-bean β -galactosidase (○). In (c), the acceptor AsOSM was untreated (●), treated with jack-bean β -galactosidase (○) or treated with NaOH in the absence (▲) or in the presence (■) of borohydride. In all cases, columns were calibrated with lactitol and glucose. For full details see the text.

tions eluted with UDP and NaCl were pooled, concentrated, and applied to the lactalbumin-Sepharose column. Almost all of the (1→4)-transferase activity is retained on this column, and about 85% of the (1→3)-transferase activity is similarly re-

tained. Both retained enzymes are eluted with 0.5M-KSCN. Kinetic analyses of the enzymes at this purification step show that both have a K_m towards UDP-galactose of about 25 μ M, and that the K_m value of the (1→4)-transferase activity toward GlcNAc is 4 mM, whereas that of the (1→3)-transferase activity towards AsOSM is 5 mM (results not shown).

Although α -lactalbumin is a demonstrated modifier protein for the (1→4)-galactosyltransferase, no previous data suggest an interaction between lactalbumin and the (1→3)-transferase. Fig. 4, however, shows that the chick-embryo liver (1→3)-transferase is as sensitive to lactalbumin as is the (1→4)-transferase.

Step 4: GlcNAc-Sepharose. All the activity eluted from the α -lactalbumin-Sepharose column was applied to the GlcNAc-Sepharose column. Almost all of the applied (1→4)-transferase activity is retained, whereas less than half of the (1→3)-transferase activity is retained. Elution with 10 mM-GlcNAc, as described in the Experimental section, yields 52% of the retained (1→4)-transferase activity and 72% of the retained (1→3)-transferase activity. These results are presented in Table 2.

Step 5: AsAgAGP-Sepharose. All the activity eluted from the GlcNAc-Sepharose (step 4) was applied to the AsAgAGP-Sepharose column. Of the applied (1→4)-transferase activity 86% is retained, whereas only 11% of the (1→3)-transferase activity is retained. Elution with 10 mM-GlcNAc, as described in the Experimental section, yields only 25% of the applied (1→4)-transferase, whereas (1→3)-transferase activity represents about 2% of the (1→4)-transferase activity (Table 3).

Step 6: AsOSM-Sepharose. The pass-through fractions from the GlcNAc-Sepharose and AsAgAGP-Sepharose columns were pooled and applied to the AsOSM-Sepharose column. About 70% of the (1→3)-transferase activity and 9% of the (1→4)-transferase activity is retained. About 28% of the bound (1→3)-transferase activity is eluted with 0.5M-KSCN, although the (1→4)-transferase activity in this eluate represents about 20% of the (1→3)-transferase activity (Table 3). Taking into account the specific activity of the (1→4)-transferase, contamination by this enzyme is less than 10%.

Overall, as presented in Table 2, both enzymes are purified about 5000–7000-fold.

Fig. 5 shows SDS/polyacrylamide-gel-electrophoretic patterns after each purification step. For both the (1→4)- and the (1→3)-galactosyltransferases, silver staining shows the relative increase in the density of a protein band that migrates with an apparent M_r of 68000. There are more than ten

Fig. 3. DEAE-Sepharose CL-6B chromatography of galactosyltransferase activities in detergent extracts from 2g of liver. The concentrated extract was applied to a column equilibrated with 0.1 M-NaCl. Free glucose is eluted at fraction 15, as indicated. ●, (1→4)-Transferase activity; ○, (1→3)-transferase activity. A 10 μ l portion of each fraction (2 ml) was used to assay each enzyme. The protein values (▲) represent one-tenth the total protein per fraction. For full details see the text.

Fig. 4. Effect of α -lactalbumin on galactosyltransferase activities

Galactosyltransferase activities towards GlcNAc (○), AsAgAGP (●) and AsOSM (△) were measured in the presence of increasing concentrations of α -lactalbumin. For full details see the text.

bands in the UDP-Sepharose-bound fraction, and more than six bands in the lactalbumin-Sepharose-bound fraction. Finally, only a single band

appears for each transferase activity. Because of the similarity in M_r values between bovine serum albumin and both purified proteins, no bovine serum albumin was ever introduced to the purification steps.

Activity stains after cellulose acetate electrophoresis

Activity stains were performed to determine if the visible protein bands coincide with the appropriate galactosyltransferase activities, and if both activities co-migrate during cellulose acetate electrophoresis as they do during SDS/polyacrylamide-gel electrophoresis. Lactalbumin-Sepharose pass-through and retained fractions were subjected to cellulose acetate electrophoresis. The electrophoretograms were stained with the coupled-enzyme assay as described in the Experimental section, and with silver. Fig. 6 shows the correspondence between the (1→4)- and (1→3)-transferase activities when both are subject to cellulose acetate electrophoresis. The NADH fluorescence indicates galactosyltransferase activity, and for both enzymes is absolutely dependent on the presence of UDP-galactose and a galactose acceptor. In parallel experiments, silver staining shows a major protein band that coincides with the NADH fluorescence, and a minor band at the

Table 2. *Purification of two galactosyltransferases from chick-embryo livers*

Enzyme activities at each purification step were determined as described in the Experimental section, with AsAgAGP and AsOSM as acceptors within the linear time ranges of the assays. Endogenous activities, which are appreciable in the homogenate and the detergent extract, were determined without the addition of acceptor, and these values were subtracted in each case. —, Not determined.

Purification step	(1→4)-Transferase			(1→3)-Transferase		
	Activity (nmol of galactose transferred/ 60 min per mg of protein)	Purification (fold)	Recovery (%)	Activity (nmol of galactose transferred/ 60 min per mg of protein)	Purification (fold)	Recovery (%)
Homogenate	0.56	1	100	0.06	1	100
Detergent extract	5.10	9	76	0.44	7	80
DEAE-Sepharose	32.7	58	58	3.55	59	55
UDP-Sepharose	106	189	43	15.9	265	38
Lactalbumin-Sepharose	215	384	34	34.3	571	30
GlcNAc-Sepharose	427	762	16	—	—	—
AsAgAGP-Sepharose	3934*	7025	4	—	—	—
AsOSM-Sepharose	—	—	—	257*	4280	5

* Protein values are estimated from SDS/polyacrylamide-gel-electrophoresis band intensities stained with silver and compared with dilutions of bovine serum albumin.

Table 3. *Relative galactosyltransferase activities toward five different acceptors in the detergent extract compared with the purified (1→4) and (1→3)-transferases*

Transferase activities were determined as described in the Experimental section. Endogenous activities (with no added acceptor) were measured and subtracted from each of the values. For the AsAgAGP-Sepharose-bound fractions that were eluted with GlcNAc, endogenous values were calculated in accordance with the competition analyses presented in Table 1. Abbreviation: N.D., not detectable.

Acceptor	Activity of detergent extract (pmol of galactose transferred/60 min per μ g of protein)	Activity of AsAgAGP-bound fractions (pmol of galactose transferred/60 min per 10 μ l of 0.8 ml fractions)	Activity of AsOSM-bound fractions (pmol of galactose transferred/60 min per 10 μ l of 0.8 ml fractions)
GlcNAc	2.47	42.3	3.3
AsAgAGP	5.70	204	4.7
GalNAc	1.69	12.9	N.D.
AsOSM	0.47	3.2	21.0
Ganglioside GM ₂	1.07	0.6	2.3

origin that shows no fluorescence (results not shown).

SDS/polyacrylamide-gel electrophoresis of iodinated galactosyltransferases

To obtain iodinated enzymes for Cleveland digest analysis, the proteins that were purified by using all six steps were iodinated as described in the Experimental section. Both labelled protein fractions were separated from the iodine on a Sephadex G-25 column. The peak fractions for each of the transferases contained less than 1 μ g of protein, as judged by the intensity of silver staining on SDS/polyacrylamide-gel electrophoresis compared with dilutions of bovine serum albumin standards that were treated identically. At the

same time, the total peak fractions for the (1→4)-transferase contained 1.2×10^9 c.p.m. and those for the (1→3)-transferase contained 2.7×10^8 c.p.m. When each radioiodinated transferase was applied to SDS/polyacrylamide-gel electrophoresis and the electrophoretograms were analysed radioautographically, each transferase showed a single labelled band with a mobility identical with that of bovine albumin (results not shown). When subjected to limited proteolysis with *S. aureus* V8 proteinase, four of the five labelled peptides from each enzyme migrated identically, as depicted in Fig. 7. Both transferases show bands b, c, d and e. Band a appears in (1→3)-transferase peptides but not in grossly over-exposed radioautograms of (1→4)-transferase peptides. Band f appears in digests of

Fig. 5. SDS/polyacrylamide-gel electrophoretograms, stained with silver, of the proteins present after each purification step. The gels contain: in lane 1, chick-embryo liver homogenate; lane 2, detergent extract; lane 3, fractions 13–23 from DEAE-Sepharose; lane 4, bound proteins from UDP-Sepharose; lanes 5 and 6, M_r standards; lane 7, bovine serum albumin; lane 8, α -lactalbumin-Sepharose-bound proteins; lane 9, pass-through fractions from GlcNAc-Sepharose; lane 10, bound fraction from GlcNAc-Sepharose; lane 11, bound fraction from AsAgAGP-Sepharose; lane 12, bound fraction from AsOSM-Sepharose; lane 13, M_r standards. The M_r standards are, in order of decreasing size, phosphorylase *b* (M_r 94 000) (A), bovine serum albumin (M_r 68 000) (B), ovalbumin (M_r 43 000) (C), carbonic anhydrase (M_r 30 000) (D), soya-bean trypsin inhibitor (M_r 21 000) (E) and lysozyme (M_r 14 300) (F). For full details see the text.

Fig. 6. Galactosyltransferase activity staining of partly purified transferases after cellulose acetate electrophoresis.

The bound (A, C and E) and pass-through (B, D and F) fractions from the lactalbumin-Sepharose column were subjected to cellulose acetate electrophoresis in cacodylate buffer as described in the Experimental section. After electrophoresis, NADH fluorescence was detected under u.v. light, also as described in the Experimental section. Sheets A and B were developed with the coupled-

the (1→4)-transferase but not in those of the (1→3)-transferase.

Discussion

Two galactosyltransferases from chick-embryo livers have been highly purified. One of the enzymes transfers galactose to AsAgAGP and to free GlcNAc with a β -(1→4)-linkage. The other transfers galactose to AsOSM with a β -(1→3)-linkage. The two enzymes co-purify when subjected to many conventional separation methods, but complete separation could be achieved only by affinity columns of their respective immobilized large- M_r acceptors.

Poor recoveries from affinity columns might be due to contamination of the subsequent transferase assays with the inhibitors (e.g. UMP and KSCN) used to wash the columns and elute the bound enzymes. The extremely low protein concentrations towards the end of the purification procedure might also contribute to enzyme inactivation and serve to lower, still further, the recovery values.

Two tentative conclusions are possible from the similarities between these galactosyltransferases. First, they behave identically on Bio-Gel A-1.5m,

enzyme mixture containing GlcNAc, sheets C and D with AsOSM, and sheets E and F served as controls with no acceptor.

Fig. 7. *Cleveland peptide mapping after limited proteolysis* For full details see the text. Radioiodinated protein bands were excised from SDS/polyacrylamide-gel electrophoretograms and transferred to new wells containing *S. aureus* V8 proteinase, in accordance with the method of Cleveland *et al.* (1977). After re-electrophoresis, radioautograms were obtained: A, 1h exposure; B and C, 30h exposure; D, 48h exposure. A and B are from the AsAgAGP-Sephacel-bound fraction. C and D are from the AsOSM-Sephacel-bound fraction.

DEAE-Sephacel, affinity chromatography on UDP-hexanolamine-Sephacel and α -lactalbumin-Sephacel, cellulose acetate electrophoresis and SDS/polyacrylamide-gel electrophoresis. Also, they behave very similarly on flat-bed isoelectric focusing. It is therefore possible that glycosyltransferases previously purified by using protocols without acceptor affinity columns might be contaminated with other transferases.

Secondly, the galactosyltransferases may make up a family of enzymes with significant sequence homologies. Such a possibility is made likely by the similarities in M_r values shown by the (1-4)- and (1-3)-transferases, as well as by their almost identical behaviour in a very wide variety of separation methods. This possibility is suggested by the results of the limited proteolysis of the purified iodinated enzymes. When incubated with *S. aureus* V8 proteinase, both the (1-4)- and the (1-3)-transferases show five labelled polypeptides of differing M_r values. Four of these five are shared. Peptide and, ultimately, sequence analyses will be required to address the possibility of structural homology in an unequivocal fashion. The extent to which all glycosyltransferases share common structures, and the extent to which this primitive and highly polymorphic class of enzymes is related to other proteins [e.g. those produced by the major histocompatibility complex, as suggested by McKenzie *et al.* (1977)], must await primary structure information.

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