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Identification of the Chick Neural Retina Cell Surface N-Acetylgalactosaminyltransferase Using Monoclonal Antibodies

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Intact embryonic chick neural retina cells have at their surface an N-acetylgalactosaminyltransferase which catalyzes the incorporation of N-acetylgalactosamine from UDP-N-acetylgalactosamine into endogenous macromolecular acceptors. The enzyme along with its endogenous acceptors can be isolated as a particulate complex following treatment of membrane-enriched fractions with Triton X-100. In this paper we report on two separate fusions generating monoclonal antibodies: one using as immunogen the particulate complex and the second using as immunogen a soluble N-acetylgalactosaminyltransferase found in tissue-culture-conditioned medium which lacks endogenous acceptor activity. Antibodies from both fusions recognize an antigen which is tightly associated with the particulate transferase/acceptor complex and a soluble antigen having N-acetylgalactosaminyltransferase activity toward exogenously added acceptors. The antibodies recognize a component of ca M_r 220,000, which shows N-acetylgalactosaminyltransferase activity after SDS-gel electrophoresis and transfer to nitrocellulose. This component comigrates on two-dimensional gel electrophoresis with an iodinatable cell surface component whose presence at the cell surface correlates with endogenous transferase activity. We conclude that the antibodies recognize the transferase enzyme itself. Immunohistochemical analysis shows that the enzyme is initially localized throughout the embryonic neural retina in a pattern indicative of a cell surface disposition but becomes restricted to the outer plexiform layer and to outer segments in the adult.

Abbreviations used: GalNac, N-acetylgalactosamine; UDP-GalNac, uridine diphosphate N-acetylgalactosamine; Hepes, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonylfluoride; PTA/TCA, 1% phosphotungstic acid in 6% trichloracetic acid; SDS, sodium dodecyl sulphate; Tris, tris(hydroximethyl aminomethane); RAM, rabbit antimouse; GAM, goat antimouse; FITC, fluorescein isothiocyanate; M_r, apparent molecular weight; pI, isoelectric point; Mab, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; staph A, fixed *Staphylococcus aureus* cells.

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Key words: N-acetylgalactosaminyltransferase, neural retina, monoclonal antibodies, enzyme, acceptor, cell surface complex

Intact embryonic chick neural retina cells catalyze the incorporation of GalNac from UDP-GalNac into endogenous acceptors. Approximately two thirds of the total GalNac transferase activity appears to be localized at the cell surface [1]. The enzyme in association with its endogenous acceptors can be isolated as stable complexes, following treatment of cells or tissues with Triton X-100. Three complexes, differing in buoyant density, have been identified. In agreement with the cellular distribution of enzyme activity, two of these complexes are cell surface forms, while the third is intracellular [2]. The isolated high-density cell surface complex, termed H particle, reflects in several ways the properties of the enzyme/acceptor complex when present at the cell surface. The transferase reaction among both intact cells and the isolated H particles is stimulated by Mn^{2+} [1,2] with an optimum at 2 mM. Following catalysis, the glycosylated endogenous acceptors are released from the cell surface or the particulate complex into the reaction medium. Freed of its associated endogenous acceptors, the enzyme derived from either the cell surface or the particulate complex will now recognize and glycosylate certain exogenously added acceptors [1,2].

It is our goal to identify the molecular nature of the cell surface N-acetylgalactosaminyltransferase and its associated endogenous acceptors, to understand their relationship and identify the physiological role that this complex plays. As one step in this direction, we have isolated monoclonal antibodies that recognize the cell surface enzyme. In this paper we present data identifying the molecular nature of the particulate cell surface enzyme. Our data further verify the intimate association of the cell surface enzyme with its endogenous acceptors and demonstrate immunochemically the cellular and tissue distribution of the enzyme.

MATERIALS AND METHODS

Preparation of the High-Density Transferase/Acceptor Complex (H Particles)

Neural retinas from 10-day-old chick embryos were homogenized in HSTI buffer (50 mM Hepes pH 7.2, 150 mM NaCl, 1% Triton X-100 and 50 μg/ml each of the protease inhibitors, antipain, leupeptin, and chymostatin [Sigma]) at a ratio of 5 retinas/ml. After 30 min on ice, the homogenates were centrifuged at 27,000g for 30 min at 4°C; the supernatant was collected and centrifuged at 50,000g for 30 min. The resulting supernatant was layered on 200 μl of 50% sucrose and 1 ml of 20% sucrose (both in HSTI) and centrifuged for 1 hr at 100,000g at 4°C. The 20/50% sucrose interface and the 50% sucrose cushion correspond almost exclusively to the high-density, membrane-associated transferase acceptor complex, termed "H," in prior publications [2]. Figure 1 illustrates the profile of transferase activity following sucrose density gradient centrifugation of crude tissue homogenates (Fig. 1a) and preparations enriched for H particles (Fig. 1b). Incorporation seen at the top of the gradient following centrifugation of crude homogenates was previously shown to be into material digestible by hyaluronidase while incorporation into each of the three peaks represents terminal addition to glycoprotein material.

Preparation of Soluble Transferase From Tissue-Culture-Conditioned Medium

Retina-tissue-culture-conditioned medium (TCM) was prepared, concentrated, and fractionated by gel filtration as previously described [3]. Column fractions

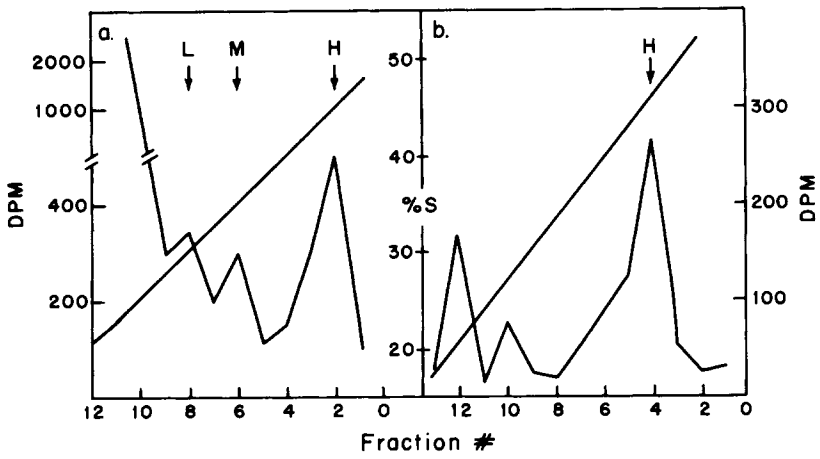

Fig. 1. Sucrose gradient profiles of GalNactransferase activity toward endogenous acceptors present in a total tissue homogenate and H particle-enriched fractions. Neural retinal homogenates prepared in HSTI buffer were centrifuged at 27,000 and the supernatant was further centrifuged at 50,000 and 100,000g as described. Aliquots of the initial 27,000g supernatant (a) and the 100,000g pellet (b) were layered on 15–50% sucrose gradients. After 18 hr of centrifugation at 100,000g, fractions were collected and assayed for endogenous GalNactransferase activity. L, M, and H refer respectively to "low," "medium," and "high" density particles (corresponding to ca 25%, 35%, and 42% sucrose [see 2]).

containing GalNactransferase activity toward endogenous and exogenous acceptors were pooled and concentrated by precipitation with ammonium sulfate (80% saturation). The ammonium-sulfate-precipitated protein was redissolved in about 5% of the original fraction volume and dialyzed against Hepes (10 mM)-buffered saline pH 7.0. Chelating Sepharose 6B (Pharmacia) was saturated with Co^{2+} ions by the procedure prescribed by the manufacturer. The cobalt-Sepharose conjugate was equilibrated in Hepes (10 mM)-buffered saline at pH 7.0, containing 1% NP-40. An equal volume of concentrated transferase fraction was added to the settled cobalt-Sepharose resin. After incubation for 1 hr on ice, the resin was washed with 3 volumes of the same buffer. GalNactransferase activity free of endogenous acceptors was eluted over a period of 1 hr on ice with the same buffer containing 1 M glycine at pH 8; the endogenous acceptors are eluted in the first washes without glycine. The fraction eluted with 1.0 M glycine at pH 8 was precipitated with 80% ethanol. This precipitate was used as immunogen. Assays for transferase activity were performed as previously described [1,2].

Preparation of Monoclonal Antibodies

The antibodies described in this paper were obtained from two independent fusions: one using H particles as immunogen and the second using the soluble transferase from TCM. In both cases, 4–6-wk-old BALB/c mice were injected intraperitoneally (IP) with 5 μg of antigen in 0.1 ml of complete Freund adjuvant. Over the next 6 wk, four more IP injections were given. Three days before the mice were to be killed, they were given an intravenous injection of antigen in physiological saline. Spleen cells from immunized mice were fused with the NS-1 (PS NS1 Ag4/1) myeloma cell line and cultured and hybrids were selected by using standard procedures [4].

Culture fluids from hybridomas obtained in either fusion were screened for their ability to bind transferase activity (see below). Positive wells were cloned by limiting dilution and the resulting clones were screened in a similar manner. Positive wells were expanded and ascities fluid was prepared by injection of cells into pristane-treated BALB/c mice. Purified IgG was obtained from the ascities fluid by using the Bio-Rad MAPS procedure, concentrated by ammonium sulfate precipitation, dialyzed against HBS containing 1 mM PMSF and 1 mM azide, and kept at −70°C at a concentration of approximately 1 mg/ml.

One clone resulting from the immunization using H particles was ultimately chosen for detailed analysis; this antibody is referred to as 6H5. Two clones resulting from the immunization with soluble transferase were chosen for analysis; these are referred to as 12E5 and 11D4. An unrelated monoclonal prepared against bovine capillary endothelium was used as a control. It was kindly supplied by Dr. Robert Auerbach; this antibody is referred to as BCE.

Screening of Culture Fluids and Analysis of Purified IgGs

Aliquots of 100 μl (approximately 100–200 μg of protein) of H particles in HSTI containing 40% sucrose were mixed with hybridoma culture fluid. After 1 hr on ice, 100 μl of a 20% suspension of staph A which had been previously incubated with rabbit antimouse Ig was added, the mixture was incubated for another hour on ice and the staph A was pelleted. The staph A pellet was washed 3 times with ice-cold HSTI and resuspended in 100 μl HSTI containing 2 mM $^{2+}$ and 1 μl UDP-[^{3}H]-GalNac (NEN; 10.7 Ci/mmol; 5.8 g/ml). The mixture was incubated for 1 hr at 37°C and the PTA/TCA- insoluble radioactivity was collected on glass fiber filters (Whatman GF/F) and counted as described [1,2]. Background binding was monitored by using an unrelated hybridoma supernatant. Identical procedures were used for analysis of purified IgGs. When analyzing for binding of soluble transferase, the reaction mixture contained 50 μg of asialo-agalactofetuin [1].

Immunoblot Analysis of the Antigens

For one-dimensional electrophoretic analysis, whole tissue homogenates in HSTI were mixed with an equal volume of 6% SDS, 10% β-mercaptoethanol, 0.25 M Tris-HCl pH 6.8, and 20% glycerol and run on slabs by using the discontinuous buffer system of Laemmli [5]. Two-dimensional electrophoretic analyses were performed as described previously [6].

Proteins in the gel were transferred to nitrocellulose according to Towbin et al [7]. The nitrocellulose blot was incubated overnight in "Blotto" [50% nonfat dry milk in 10 mM Tris, 150 mM NaCl, 10 mM azide, and 0.1% antifoam (Sigma)] [8] followed by incubation for 24 hr at 4°C with a 1:100 dilution of ascities fluid or 10 μg/ml purified IgG. The blots were washed in several changes of Blotto over a period of 2 hr and incubated with a solution of [^{125}I]-labeled rabbit IgG directed against mouse IgG (0.1 μCi/ml NEN) in Blotto for 2–4 hr. After several more washes in Blotto, the nitrocellulose sheets were washed in 10 mM Tris pH 7.2 buffer containing 0.1% SDS and 0.03% Tween-20 (Bio-Rad), rinsed extensively in distilled H_2O, dried, and autoradiographed using Kodak X-AR film and DuPont intensifying screens at −70°C.

Assay for Transferase Activity on Nitrocellulose Replicas of SDS-Page

Preparations of H particles were catalyzed with unlabeled UDP-GalNac; the 100,000g supernatant was collected, mixed with an equal volume of 2 × SDS sample

buffer (0.25 M Tris pH 6.8, 6% SDS, 10% β-mercaptoethanol, 20% glycerol), and heated at 100°C for 2 min. Approximately 500 μg protein in 1 ml was applied to a 4-cm-wide slot in the stacking gel. Two lanes of prestained molecular weight standards, one on each side of the sample, were used as markers. At the end of the run, the slabs were incubated at room temperature, under gentle agitation, in 0.01 M Tris pH 7.2 containing 25% isopropanol (v/v) for 30 min, with one change after 15 min, followed by another 30-min incubation in Tris buffer alone, with one change after 15 min [9]. After transfer to nitrocellulose, lengthwise strips 1.5 cm wide were cut into 1-cm sections. Each section was incubated for 1 hr at 37°C in a shaking water bath with 500 μl HSTI buffer containing 2 mM Mn^{2+}, 2 μl UDP-[^{3}H]GalNac, and 50 μg asialo-agalactofetuin. The reaction was stopped by transferring the reaction medium to tubes containing an equal volume of PTA/TCA. The precipitable radioactivity was determined as described previously.

Fractionation of Tissue-Culture-Conditioned Medium Transferase Activity by High-Pressure Liquid Chromatography (HPLC)

An aliquot of the partially purified tissue-culture-conditioned medium enzyme preparation was injected into a 0.75 × 60 cm TSK SW 2000 (Altex) column equilibrated in 20 mM Hepes, 150 mM NaCl, pH 7.2. The column was eluted at a flow rate of 1 ml/min and 0.5-ml fractions were collected. Each fraction was assayed for catalytic activity by incubation with 100 μg asialo-agalactofetuin 10 mM Mn^{2+}, 2 μl UDP-[^{3}H]GalNac for 2 hr at 37°C. Radioactivity incorporated into macromolecules was assayed as described before.

Transferase activity precipitated by 6H5 Mab was assayed by incubating the column fractions with 6H5 Mab for 1 hr on ice, followed by RAM Ig-coated staph A. The resuspended, washed pellet was incubated in 100 μl of 20 mM Hepes, 150 mM NaCl pH 7.2, containing 10 mM Mn^{2+}, 100 μg asialo-agalactofetuin, and 1 μl UDP-[^{3}H]GalNac for 1 hr at 37°C.

Iodination of Cells

Iodinated, repaired cells were prepared as described by Geller and Lilien [10]. H particles were prepared from iodinated, repaired cells as described above.

Binding of Monoclonal Antibody to Single Cells

Single cells were prepared by trypsinization in the presence or absence of Ca^{2+} as described previously [11] except that 10 mM azide was included in all solutions. The final cell suspension was resuspended in buffer containing 10 mM Hepes pH 7.4, 150 mM NaCl, 2 mM glucose, 3 mM KCl, 1 mM Ca^{2+}, and 2.5% normal rabbit serum at a concentration of 2×10^7 cells/ml; 100 μl of the cell suspension was added to 100 μl of buffer containing increasing concentrations of IgG. The mixture was incubated for 1.5 hr on ice, pelleted and washed 3 times in buffer and resuspended in 0.2 μCi of [^{125}I]-labeled rabbit IgG directed against mouse IgG (NEN) in the same buffer. Unlabeled RAM-IgG was included at a concentration that resulted in approximately 50% inhibition of binding of labeled antibody alone in order to ensure that the second antibody was not limiting. After incubation for 1 hr on ice the cells were washed 3 times in buffer and bound radioactivity was determined in a Gamma counter. Binding of antibody to intact, single cells was visualized by substituting

FITC-conjugated goat IgG directed against mouse IgG (Cappel) for the [^{125}I]-labeled IgG and viewing as detailed below.

Histochemical Procedures

The distribution of antigen in unfixed frozen sections of retina was determined by indirect immunofluorescence. Retinas were dissected in Dulbecco's modification of Eagle's minimal medium and incubated sequentially at 24°C in the same medium containing 10, 20, and 30% sucrose (w/v), 10 min for each solution. Tissues then were embedded in OCT compound (Miles Scientific) and frozen on dry ice. Sections 10 μm thick were cut on an IEC cryostat at −20°C and thaw-mounted on Teflon-grid printed histology slides with ten viewing areas (Roboz) that had been cleaned with ethanol. The mounted sections were dried in air for 30 min and stored overnight in a desiccator at 4°C. Sections were hydrated for 20 min in PBS (pH 7.4) with mM Ca^{2+} and Mg^{2+} and 10% normal goat serum, incubated with IgG diluted in PBS with mM Ca^{2+} and Mg^{2+} for 2 hr at 24°C, washed three times in the same buffer, and incubated with affinity purified, FITC-conjugated goat IgG directed against mouse IgG and IgM (Kierkegaard and Perry) for 2 hr at 24°C. The sections were washed three times with buffer and mounted in a solution containing 90% glycerol, 100 mM Tris-HCl, pH 8.0, and 5.5 mM p-phenylenediamine to retard fading of fluorescence during viewing [12]. Sections were examined and photographed on a Zeiss Universal microscope equipped with epifluorescence with a BP 485/20 nm excitation filter and a LP 520 nm barrier filter.

RESULTS

Immunization of mice with preparations enriched for H particles or with a semipurified preparation of GalNactransferase from retina-tissue-culture-conditioned medium resulted in the production of several hybridomas that precipitate endogenous transferase activity from H particle preparations. We have chosen three of those clones for analysis, one prepared by using the particulate transferase as immunogen (6H5) and two by using the soluble, tissue-conditioned medium transferase as immunogen (12E5, 11D4).

Immunoprecipitation of GalNactransferase and Its Endogenous Acceptors Present in H Particle Preparations

Mab 6H5 binding to H particles is dose dependent and as much as 100% of the total activity can be recovered in the immunoprecipitated pellet (Fig. 2a). Mabs 11D4 and 12E5 also precipitate H particle associated activity (Fig. 2b). All three forms of the enzyme/acceptor complex previously described [2] are immunoprecipitated by Mab 6H5 (not shown). These results indicate that the H particle enzyme is tightly associated with its acceptors and that the antigen recognized by Mabs 6H5, 12E5, and 11D4 is tightly associated with the enzyme/acceptor complex.

Immunoprecipitation of GalNactransferase Solubilized From H Particles

When H particles are centrifuged at 100,000g for 30 min, over 80% of the GalNactransferase activity is recovered in the pellet, reaffirming the stable association of the enzyme with the particulate complex (Table I, footnote b). It is likely that, in the intact particle, the enzyme is closely associated with its endogenous acceptors.

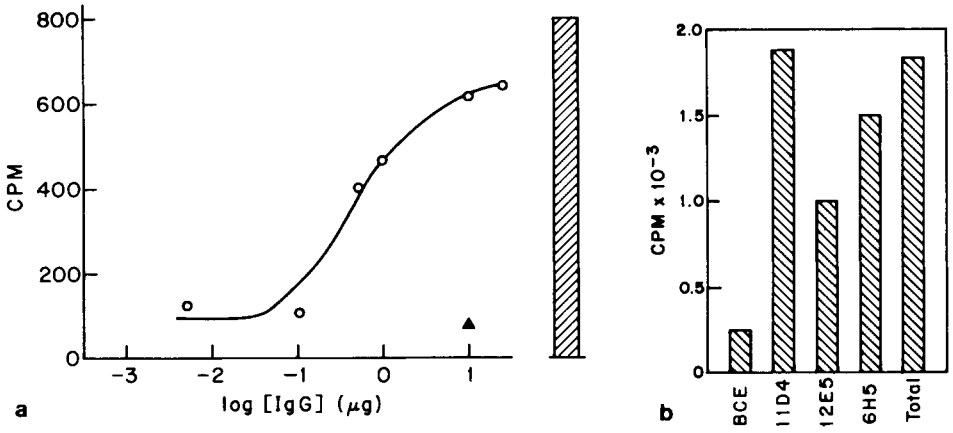

Fig. 2. Immunoprecipitation of particulate transferase activity. a) Aliquots of H particle preparation were incubated with increasing concentrations of 6H5 IgG and the antibody-bound components were precipitated with RAM-IgG-coated staph A. After washing, the pellets were resuspended in HSTI containing 2 mM Mn^{2+} and UDP-[^{3}H]GalNac and incubated for 1 hr at 37°C. Transferase activity was measured by determining the amount of radioactivity present in PTA/TCA-precipitable material. Total activity was determined on a similar aliquot of unprecipitated H particles, treated under similar conditions (bar graph). Mab 6H5, ○; Control Mab, ▲. b) Aliquots of an H particle preparation were incubated with 10 μg purified IgG and treated as in a. Results shown are from one experiment representative of several.

TABLE I. Release of Transferase From H Particles Upon Catalysis*

Treatment of H particle suspension	CPM incorporated into PTA/TCA-precipitable material			
	Endogenous acceptor		Exogenous acceptor	
	Supernatant	Pellet	Supernatant	Pellet
None[a]	830 ± 39		175 ± 15	
Centrifuged at 100,000g for 30 min[b]	110 ± 20	688 ± 42	78 ± 4	180 ± 7
Incubated with unlabeled UDP-GalNac prior to centrifugation at 100,000g for 30 min[c]	126 ± 12	156 ± 18	388 ± 25	187 ± 32

*A preparation of H particles was diluted to 20% sucrose with HSTI and divided into three aliquots of equal volume. All assays were performed in an equal volume with 2 μl UDP-[^{3}H]GalNac and 2 mM Mn^{2+} in the presence or absence of 50 μg asialo-agalactofetuin. Results are the average of duplicates.

[a]Total activity in aliquot.

[b]Following centrifugation, the pellet was resuspended in an equal volume of buffer and the supernatant and pellet were assayed separately.

[c]H particles were incubated with 20 μM UDP-GalNac prior to centrifugation and assay as in b.

The addition of a potential exogenous acceptor to such preparations results in inhibition of the endogenous activity with no glycosylation of the added acceptor (Table I, footnote b). After catalysis, however, the enzyme (or a fragment containing its active site) is released from the complex, remaining in the supernatant after centrifugation at 100,000g. This supernatant has little or no activity toward endogenous acceptors but is able to transfer [^{3}H]GalNac residues from UDP-[^{3}H]GalNac to an exogenous acceptor (asialo-agaletofetuin), while the residual pellet lacks activity (Table I, footnote c). This indicates that most of the particle-associated enzyme is solubilized on catalysis. There is no accurate way in which the amount of enzyme present in the particle preparation can be compared to that solubilized on catalysis as the acceptors themselves and their association with the enzyme differ. This solubilized form of the enzyme is recognized by all three monoclonal antibodies described in this paper. As shown in Figure 3, up to 100% of the total activity is recovered in the immunoprecipitates, while only background radioactivity is found associated with an unrelated antibody (BCE).

Immunoprecipitation of Soluble GalNactransferase Activity From Tissue Culture Medium

Intact neural retinas obtained from 10-day-old chicken embryos release GalNactransferase activity into culture medium over a period of 24 hr. This activity has been partially purified and obtained free of endogenous acceptor activity (see Materials and Methods). The partially purified enzyme is recognized by Mabs 6H5, 12E5, and 11D4 (data not shown). As shown in Figure 4, Mab 6H5 binds to the enzyme in a dose-dependent manner, removing essentially all activity from solution. The recognition of the tissue culture medium enzyme by this Mab, prepared by using H particles as antigen, further stresses the fact that both the particulate form and the soluble enzyme share common epitopes.

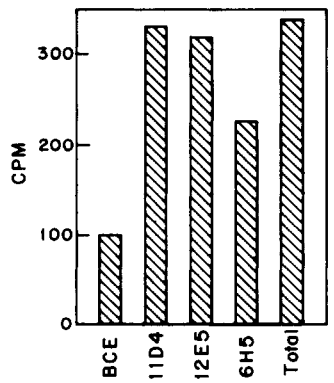

Fig. 3. Immunoprecipitation of transferase activity solubilized from H particles. Fractions enriched in H particles were incubated for 1 hr at 37°C with unlabeled UDP-GalNac and 2 mM Mn^{2+} and repelleted at 100,000g. Aliquots of the supernatant were reacted with purified IgG and the antibody-bound molecules were pelleted with RAM-IgG-coated staph A. After washing, the pellets were resuspended in HSTI containing Mn^{2+}, UDP-[^{3}H]GalNac, and asialo-agalactofetuin and incubated at 37°C for 1 hr. Radioactivity incorporated into macromolecules was determined as described. No incorporation was observed when the reaction was performed in the absence of asialo-agalactofetuin (not shown). Results shown are from one experiment representative of several.

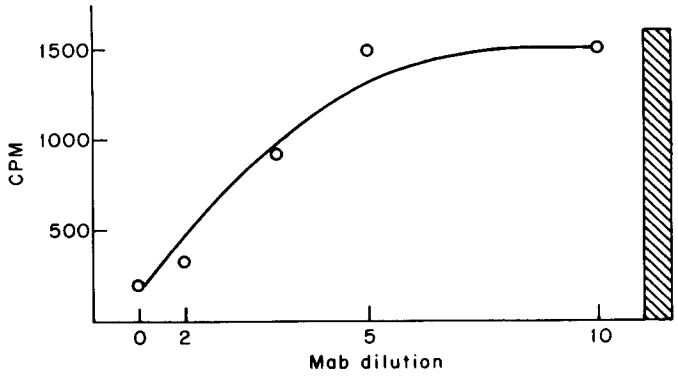

Fig. 4. Immunoprecipitation of transferase from tissue-culture-conditioned medium. Results are given as the activity remaining in the supernatant after pelleting the staph A-antibody-antigen complex. Total activity in a similar aliquot is shown in the bar graph. Results are from one experiment representative of several.

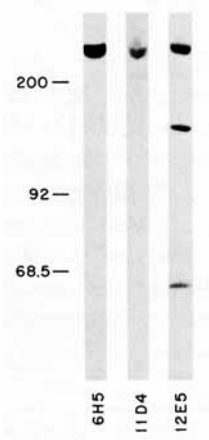

Fig. 5. Detection of antigens reacting with each Mab following Western blotting. SDS-PAGE of neural retina homogenates was transferred to nitrocellulose as described in the Materials and Methods section. Ascities fluid diluted 1:100 was used in each case, and bound Mab was detected with [^{125}I]RAM-IgG. Numbers to the left of the figure indicate the position of prestained molecular weight markers (200,000 myosin, 92,000 phosphorylase a, 68,500 bovine serum albumin).

Identification of the Target Molecules for 6H5, 12E5, and 11D4

All three monoclonal antibodies recognize a polypeptide with an apparent molecular weight (M_r) of 220,000 present in a crude membrane fraction prepared by centrifugation of retina homogenates at 27,000g (Fig. 5). A second species of ca M_r 130,000 is consistently recognized by 12E5 but not by 11D4 (Fig. 5). Mab 6H5 shows no reaction with the M_r 130,000 component on the autoradiograph shown in Figure 5, but has reacted in other experiments. The reason for this variability is unclear. A third species of M_r 60,000 is also recognized by Mab 12E5.

GalNactransferase Activity Comigrates With Mab Binding Sites on SDS-PAGE and HPLC

To demonstrate that the monoclonal antibodies recognize epitopes on the Gal-Nactransferase itself and not on a tightly associated component, we compared the

position of immunostained components with the distribution of catalytic activity following SDS-PAGE of the 100,000g supernatant from catalyzed H particles. As shown in a previous section (Table I) the 100,000g supernatant following catalysis of H particles has enzymatic activity toward exogenously added acceptors, but little or no detectable endogenous acceptors. Activity is lost after denaturation in SDS-gel sample buffer, but removal of SDS after electrophoresis by soaking the gel in isopropanol [9] and carrying out the electrophoretic transfer to nitrocellulose in the absence of detergent apparently results in partial renaturation. Three peaks of transferase activity are observed when asialo-agalactofetuin is used as an acceptor. These correspond to molecular weights of approximately 220,000, 130,000, and a broader area around 75,000. The 220,000 and 130,000 species comigrate with immunostained polypeptides (see Fig. 5). The broader peak of activity at ca M_r 75,000 may represent a fragment of the enzyme lacking the epitope recognized by the Mabs or it may represent a different enzymatic activity.

A similar analysis was done by using the transferase prepared from tissue-culture-conditioned medium. In this case, the enzyme preparation was fractionated by high-pressure liquid chromatography on a TSK SW 2000 column. The collected fractions were assayed for their ability to transfer [^{3}H]GalNac residues from UDP-[^{3}H]GalNac to asialo-agalactofetuin (Fig. 7a). The total activity recovered corresponds to approximately 100% of the activity applied to the column. Another aliquot of the same preparation was incubated with Mab 6H5 followed by RAM-Ig-coated staph A, and transferase activity was measured in the staph A pellet (Fig. 7b). In a third aliquot binding of Mab 6H5 to the antigen was measured directly by spotting aliquots of each column fraction on nitrocellulose, reacting with Mab 6H5 followed by incubation with [^{125}I]RAM-Ig, and measuring bound radioactivity (Fig. 7c). Comparison of Figure 7a–c reveals that immunoprecipitable activity and antibody binding are restricted to the fractions showing transferase activity at molecular masses of approximately 60 Kd and 30 Kd.

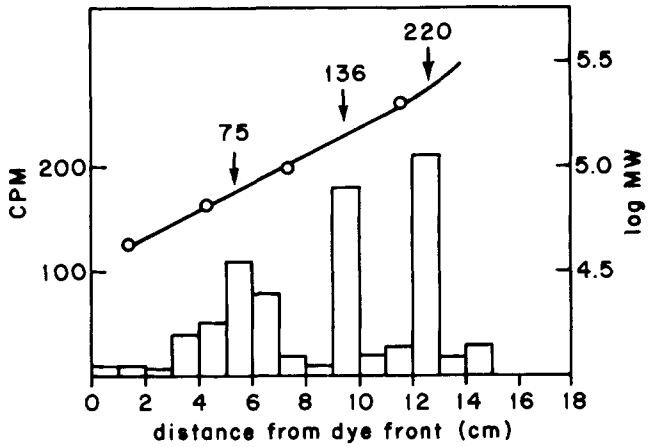

Fig. 6. Localization of particulate transferase activity on nitrocellulose replicas of SDS-polyacrylamide gels. The 100,000g supernatant from catalized H particles was submitted to SDS-PAGE, transferred to nitrocellulose, and assayed for GalNactransferase activity in the presence of asialo-agalactofetuin as described in Materials and Methods. The molecular masses corresponding to peaks of activity were calculated based on the migration of the prestained standards. Results are from one experiment representative of many.

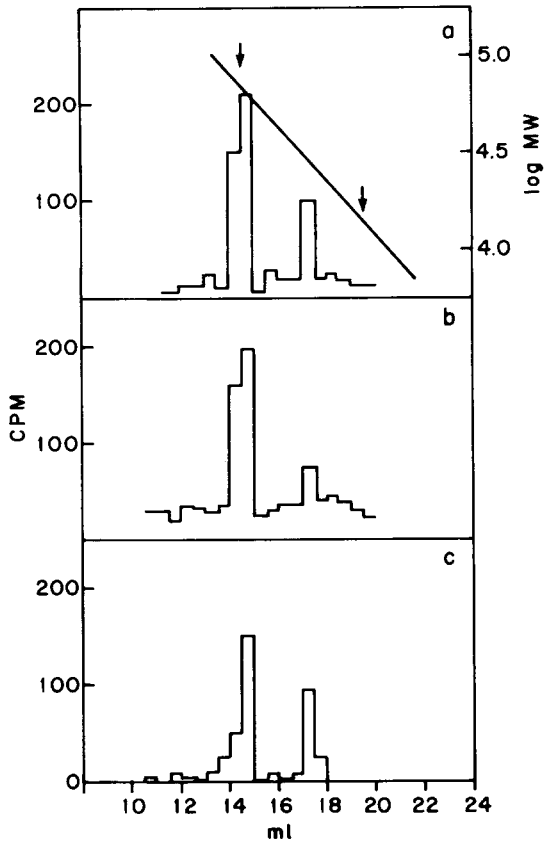

Fig. 7. Fractionation of soluble transferase by HPLC; 100-μl aliquots of semipurified enzyme from tissue-culture-conditioned medium were fractionated on a TSK SW 2000 column. a) Eluted fractions were assayed for transferase activity as described in Materials and Methods. The arrows represent the elution positions of BSA (68,400) and soybean trypsin inhibitor (14,000). b) Fractions were incubated with Mab 6H5 followed by RAM-Ig-coated staph A and the immunoprecipitated pellet was assayed for transferase activity as described. c) Fifty-microliter aliquots were applied to nitrocellulose and dried; the paper was blocked with Blotto, washed, and reacted with [^{125}I]RAM-Ig. After extensive washing, the radioactivity bound to each spot was determined on a γ-counter.

The Mabs Bind to the Cell Surface

Purified IgG from 6H5 and 12E5 bind in a dose-dependent manner to intact cells, as monitored by using [^{125}I]-labeled second antibody (Fig. 8). This is true whether the cells are prepared by trypsin treatment in the presence or absence of 1 mM Ca^{2+} [11]. The binding is saturable and the approximate number of target molecules was calculated to be on the order of 10^4/cell based on the radioactivity bound at plateau level and the specific activity of the secondary antibody.

Binding of the Mabs to the cell surface was also demonstrated by using FITC-labeled goat IgG directed against mouse IgG to detect bound primary antibody (Fig. 9).

The M_r 220,000 Component Recognized by the Mabs Comigrates With a Cell Surface Molecule in Neural Retina

To compare molecular species present at the cell surface with those recognized by the antibodies, we labeled single cells by lactoperoxidase-catalyzed iodination after

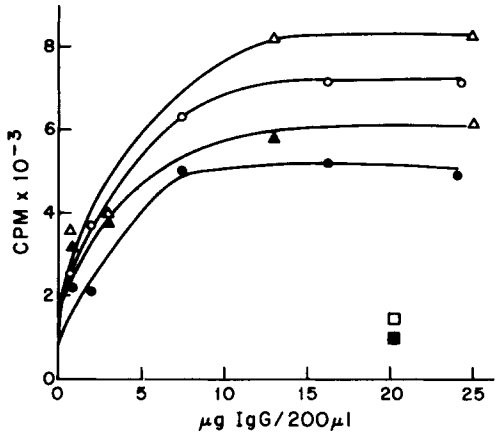

Fig. 8. Binding of Mabs to intact cells. Increasing concentrations of Mab IgG were reacted with 2 × 10^6 cells in a total of 200 μl of reaction medium. After incubation on ice, the cells were washed several times and incubated with [^{125}I]-labeled RAM-IgG. After washing, radioactivity bound to cells was determined by γ counting. Filled circles and triangles correspond to cells prepared by the standard trypsinization procedure; open circles and triangles correspond to cells prepared by trypsinization in the presence of 1 mM Ca^{2+}: Mab 6H5 (●, ○); Mab 12E5 (▲, Δ); Control Mab (□, ■).

5 hr of culture in serum-free medium. An autoradiograph of a 2D-PAGE of retina cells prepared in this way is shown in Figure 10A. A 2D-PAGE of unlabeled, repaired cells run simultaneously was blotted onto nitrocellulose and reacted with Mab 6H5 followed by [^{125}I]RAM-Ig. An autoradiogram of this gel is shown in Figure 10B. The iodinated component of M_r 220,000 (indicated by the arrowhead in Fig. 10A) comigrates with the major species recognized by the antibody in Figure 10B (also indicated by an arrowhead). This species is also present when H particles are prepared from iodinated repaired cells (Fig. 10C, arrowhead). It should be noted that two of the predominant iodinatable components present at the cell surface and in H particles are the Ca^{2+}-dependent adhesive components GP130 and GP70, previously described in this laboratory [13] (marked with an "X" in Fig. 10A,C).

The M_r 220,000 Component Is Present in Neural Retinas Throughout Development and in Different Tissues

The M_r 220,000 species recognized by the Mabs is present in neural retinas from all ages tested (7-, 10-, 12-, 16-, 18-day embryo, hatchling, and adult; results not shown). A similar molecule is also recognized by the Mabs in several embryonic neural tissues: retina, cerebral lobe, optic lobe, and two non-neural tissues examined: muscle and liver (Fig. 11).

Immunocytochemical Localization of Antigen 6H5 During Retinal Development

The distribution of transferase in sections of unfixed frozen retinas from embryonic and adult chickens was examined by indirect immunofluorescense by using Mab 6H5. Control antibodies (obtained from medium conditioned by P3×63 Ag8 myeloma cells which secrete an IgGl kappa of unknown binding specificity) show no binding to retina (Fig. 12A); however, Mab 6H5 binds strongly to sections of retinas obtained from all stages of development tested (Fig. 12B–H). At 6 days of embryonic

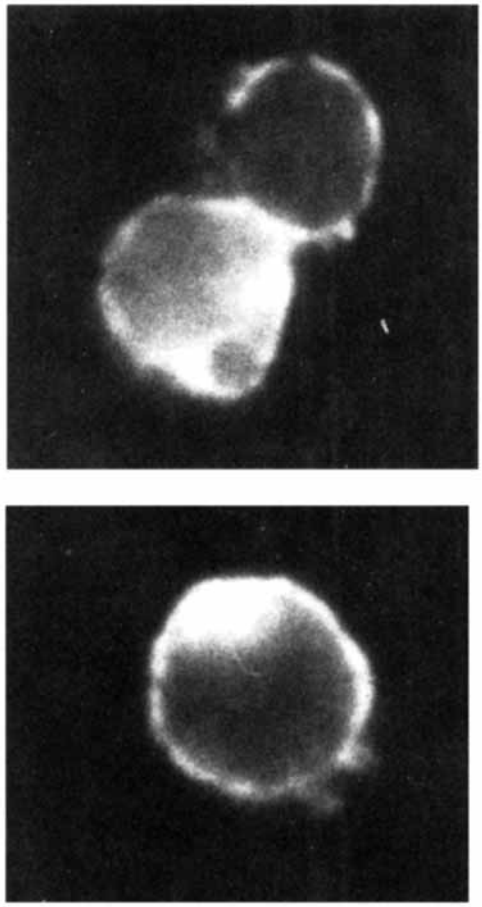

Fig. 9. Immunofluorescence detection of Mab 6H5 bound to single cells. Single cells prepared by trypsinization in the presence of Ca^{2+} were incubated on ice for 15 min with a 1:100 dilution of Mab 6H5 ascities fluid or control Mab, washed, and incubated with FITC-labeled GAM IgG. Controls are completely dark and are not shown (×3,000 approx.).

development, the earliest stage tested, all cells show ringlike fluorescence indicating that the antigen is localized at or near the cell surface (Fig. 12B). This cell-surface-like staining pattern persists at 10 days of development (Fig. 12C), by which time most retinal cells are postmitotic and have segregated into nuclear and plexiform layers. In occasional sections of 10-day embryo retinas, a more fibrillar pattern of antibody staining is found (Fig. 12D). The difference in appearance between panels C and D may be due in part to slight differences in the plane of section, but it is also possible that this represents a true transition in antigen distribution, since later stages of development uniformly exhibit a more fibrillar pattern (Fig. 12E–G, representing retinas from 16- and 18-day embryos and newly hatched chicks, respectively). In adult retinas, a further consolidation of antigen expression takes place, with most antibody binding limited to the outer plexiform layer and among the photoreceptor outer segments (Fig. 12H).

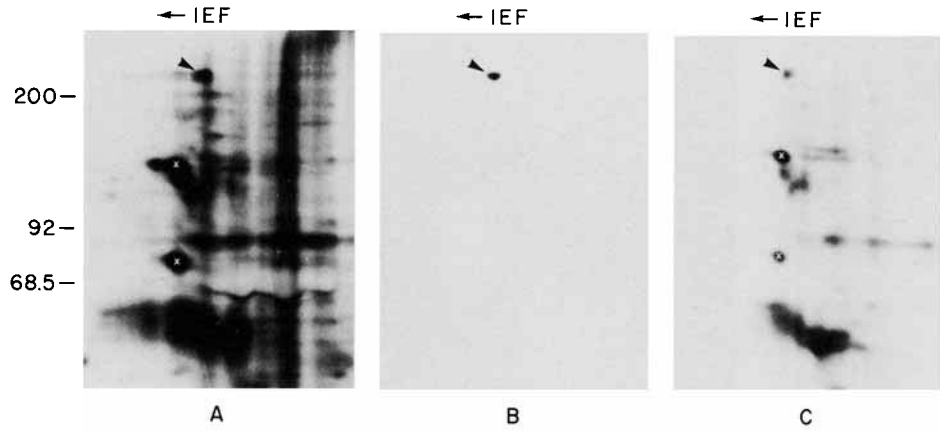

Fig. 10. Detection of antigens reacting with Mab 6H5 on Western blots of 2-D gels. Neural retina cells were prepared by the standard trypsinization procedure and allowed to "repair" in culture for 5 hr. A fraction of the resulting cell suspensions was labeled by lactoperoxidase-catalyzed iodination. Labeled and unlabeled cell homogenates were analyzed by 2D SDS-PAGE: A) Autoradiogram of labeled cells; B) autoradiogram of unlabeled cell homogenates transferred to nitrocellulose and reacted with Mab 6H5 followed by reaction with [^{125}I]RAM-IgG; C) autoradiogram of H particles prepared from iodinated cells. This gel is from a separate run and differs slightly from the other two in the isoelectric focusing (IEF) dimension. Numbers to the left of the figure indicate the position of prestained molecular weight markers (200,000 myosin, 92,000 phosphorylase a, 68,500 bovine serum albumin). Arrowheads indicate the component immunoblotted by Mab 6H5, and "X" identifies GP130 and GP70, previously described by this laboratory [see 6,10].

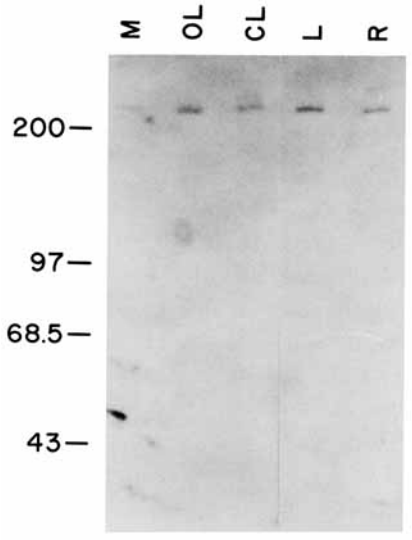

Fig. 11. Detection of antigens reacting with Mab 11D4 in various tissues. SDS-PAGE of whole tissue homogenates was transferred to nitrocellulose reacted with Mab 11D4 IgG followed by [^{125}I]-labeled RAM-IgG and autoradiographed. Approximately equal quantities of protein were run in each lane: M, muscle; OL, optic lobe; CL, cerebral lobe; L, liver; R, retina. Numbers to the left of the figure indicate the position of prestained molecular weight markers (200,000 myosin, 97,000 phosphorylase b, 68,500 bovine serum albumin).

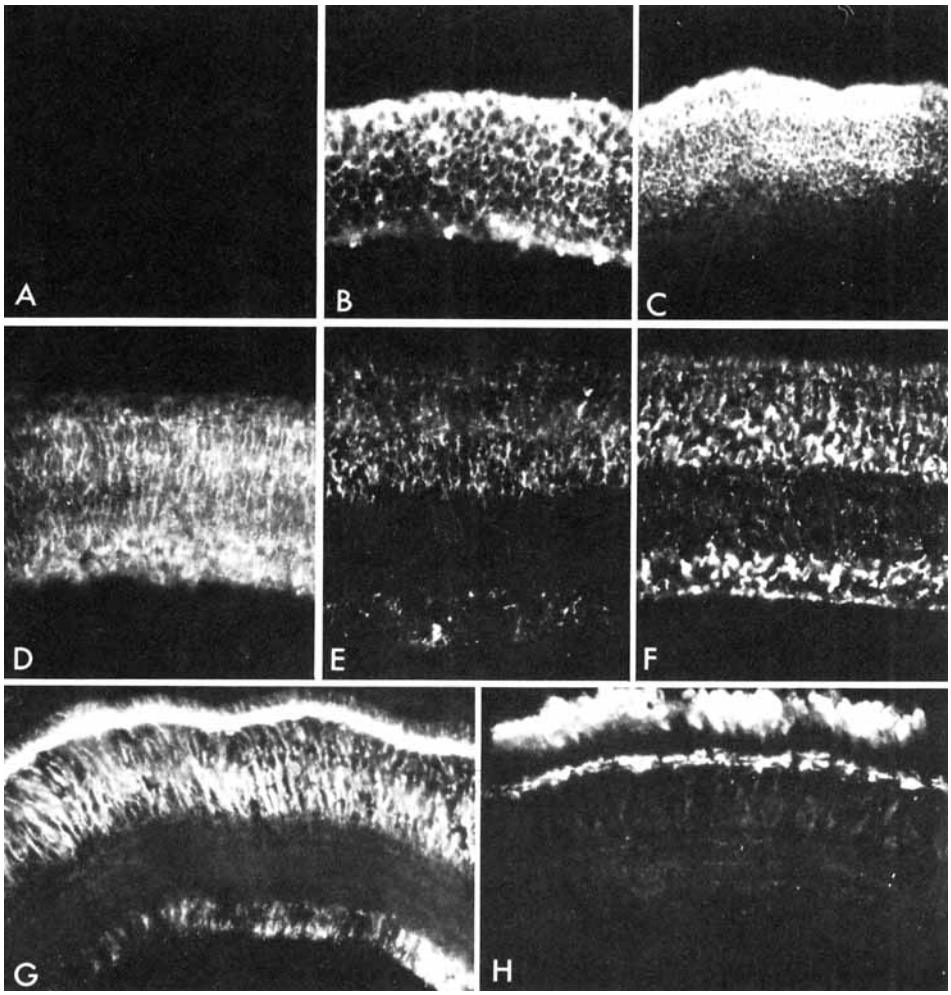

Fig. 12. Immunocytochemical localization of antigen 6H5 during retinal development. Fresh-frozen sections of retinas from embryonic and adult chicks were reacted with antibody 6H5 as in Materials and Methods. Panel A is a retina from a 6-day embryo reacted with a control myeloma antibody. Panels B–H are retinas from embryos of 6 (B), 10 (C, D), 16 (E), or 18 (F) days incubation, from newly hatched chicks (G), or from adult chickens (H). All panels are to the same scale (×300).

DISCUSSION

Monoclonal antibodies originating from two independent fusions bind to the cell surface of intact embryonic chick neural retina cells and recognize polypeptides with GalNactransferase activity. The antigens used as immunogens to generate the monoclonal antibodies were a cell surface GalNactransferase/acceptor particulate complex from chick neural retina and a partially purified, soluble form of the enzyme found in tissue-culture-conditioned medium. Antibodies from both fusions recognize the enzyme associated with the particulate complex and the soluble enzyme present in tissue culture medium. Both forms of the enzyme thus share a common or immunologically cross-reactive epitope.

A direct comparison of polypeptides with transferase activity with those showing Mab binding was possible as the particulate enzyme retains its catalytic activity after SDS-PAGE and transfer to nitrocellulose. The nitrocellulose replica shows three areas of GalNactransferase activity; two of these comigrate with components immunostained by the monoclonal antibodies, corresponding to molecular masses of approximately 220,000 and 130,000 daltons. The third area of activity may represent a distinct activity or a fragment of the enzyme which has lost the epitope recognized by the antibodies. The same approach was not feasible with the soluble form of the transferase found in tissue-culture-conditioned medium as it loses activity after SDS-PAGE and transfer to nitrocellulose. However, we were able to compare the distribution of activity and of components recognized by Mab following fractionation by molecular sieve chromatography on HPLC. Two peaks of activity are seen which correspond to the position of immunoprecipitable activity and to the distribution of the antigen. These fractions correspond to molecular masses of ca 60 Kd and 30 Kd. While a direct comparison of molecular weights between SDS-PAGE and HPLC cannot be made with assurance, Mab 12E5 does recognize a molecular species of ca 60 Kd in immunoblots of tissue homogenates (Fig. 5) which may correspond to this soluble form of the enzyme.The 30-Kd form of the enzyme may represent a degradation product generated following turnover of the transferase into the culture medium.

The 220,000 component recognized by the Mabs is identical in molecular mass and pI to a component previously identified by radioiodination studies to be at the surface of cultured single cells [10]. This component is absent from the cell surface following standard trypsinization procedures, but appears after 3 hr of repair in culture, even in the presence of cycloheximide. Colchicine, however, does block its reappearance. The molecule is thus present in an intracellular pool, which is mobilized and exposed at the cell surface in a process triggered by trypsinization. This behavior is consistent with GalNactransferase activity toward endogenous acceptors on intact cells [1]. Cells prepared by standard trypsinization procedures lack their cell-surface-associated complement of GalNactransferase activity toward endogenous acceptors; however, activity is restored with time in culture in a process which is independent of protein synthesis but which is inhibited by colchicine. Thus, the presence at the cell surface of the M_r 220,000 component correlates with transferase activity toward endogenous acceptors. The antibodies react with a similar M_r 220,000 antigen in all tissues analyzed. It will be of interest to determine if all these polypeptides show the same substrate specificity.

Under most conditions, the enzyme remains tightly associated with its endogenous acceptor whether at the cell surface or in the particulate complex. However, there are two conditions under which the enzyme is separated from its endogenous acceptor. Following catalysis of the transfer reaction in the high-density particle, the enzyme is liberated from the particle and available to bind Mab independently of endogenous acceptors. A second set of conditions where a catalytically active enzyme is freed of endogenous acceptor is at the cell surface following trypsinization. Freshly trypsinized cells lack endogenous transferase activity at the cell surface but are able to glycosylate exogenously added acceptors [1]. This is consistent with the observation that the Mabs bind to freshly trypsinized cells. We believe this to be a fragment of the enzyme since the M_r 220,000 component is absent from the surface of fresh, trypsin-treated cells [11].

The M_r 220,000 component recognized by the antibody is present in the retina throughout several stages of embryonic development from 6 days through hatching

and in the adult. However, there is a clear shift in its distribution. The enzyme is initially present throughout the retina, whereas in the adult the enzyme antigen is restricted to the outer plexiform layer and outer segments. This shift may represent a change in the functional role of the enzyme during development and in the adult.

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