PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **181**, 270–274 (1986)

Selective Expression of Hepatocellular Membrane Proteins in Mice Homozygous for a Lethal Chromosomal Deletion (42252)

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Abstract. Previous studies of mice homozygous for one of several overlapping radiation-induced deletions in chromosome 7 revealed reduced expression of a number of hepatocyte proteins. These proteins include the plasma membrane receptors for insulin, epidermal growth factor, and glucagon, all of which are reduced in number by over 70% with no change in apparent affinity constants. It is not known whether all or only select liver cell surface proteins are so affected in newborn deletion homozygotes. In the present study, we investigated expression of two additional, functionally distinct intrinsic hepatocellular membrane binding proteins: the hepatic binding protein (HBP: the receptor for asialoglycoproteins), and the organic anion binding protein (OABP) which may play a role in organic anion transport. Immunoblot analysis showed the content of OABP and HBP in neonatal mutants to be identical to that in controls. As compared to adults, neonates showed levels of only about 8% of HBP and 75% of OABP binding proteins. Assays of HBP binding activities confirmed these results. The normal levels of these hepatocyte binding proteins in the deletion homozygotes suggest that the DNA sequences deleted in these mutants do not regulate all genes encoding such proteins but only a selected number of them.

A series of overlapping radiation induced chromosomal deletions in chromosome 7 of the mouse has been shown to interfere specifically with the expression of liver specific enzymes and proteins as well as the differentiation of ultrastructure and components of hepatocyte cellular membranes in perinatally lethal deletion homozygotes (1, 2). The rough endoplasmic reticulum appears disrupted, dilated, and vesiculated, and focal loss of membrane bound ribosomes as well as disaggregation of polysomes are widespread. Furthermore, the nuclear membrane is dilated and the lamellae of the Golgi apparatus are swollen and vacuolated. These abnormalities of protein expression and ultrastructure are specific for hepatocytes; other cell types remain normal. While the ultrastructure of the hepatocyte plasma membrane of deletion homozygotes appears normal, receptors for insulin (3), epidermal growth factor, and glucagon (4) are reduced to approximately 25% of the number present in livers from control littermates. In the course of attempts to identify further the nature of these genetic defects, it appeared important to determine the state of additional hepatocyte specific surface proteins in deletion homozygotes. In particular, we chose two functionally distinct, hepatocellular membrane binding proteins: the hepatic binding protein (HBP) which binds asialoglycoproteins, resulting in their internalization by endocytosis (5, 6), and the organic anion binding protein (OABP) which binds organic anions such as bilirubin and sulfobromophthalein and may play a role in their uptake into the liver (7, 8). Electron microscopic immunocytochemical localization of HBP (9, 10) and OABP (9) in liver revealed that they were found exclusively in parenchymal cells predominantly at the sinusoidal aspect. OABP was identified in the lateral membrane of the cell as well (9). Earlier studies have also described a trace of HBP in the bile canaliculus. In contrast to OABP, HBP was not limited to the plasma membrane and was identified also in other hepatocyte subcellular organelle membranes (9, 10).

Since mice homozygous for the c^{14CoS} deletion die within the perinatal period (1), the quantities of HBP and OABP were determined in neonatal mutants and littermate controls. Earlier studies of the normal newborn mouse had shown that liver content of HBP was ap-

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proximately 10% of adult levels (11), but no data are available for the levels of OABP in neonatal or fetal livers.

Materials and Methods. *Mutant mice.* Mice carrying the lethal albino deletion c^{14CoS} were maintained and inbred as heterozygotes with c^{ch} (chinchilla) as the normal allele, and homozygous and normal littermate newborns were kindly provided by Dr. Salome Gluecksohn-Waelsch. Mutant homozygous newborns are recognized as albinos by their unpigmented eyes. Normal homozygous and heterozygous littermates, which are pigmented and indistinguishable from each other served as controls. Animals were sacrificed within a few hours after spontaneous birth or after dissection of the mother at the estimated term date. They were decapitated and their livers removed, frozen in liquid nitrogen, and stored at −80°C.

Immunoblot detection of HBP and OABP. Livers were homogenized on ice in 3 vol of 1 m*M* $NaHCO_3$, 0.5 m*M* $CaCl_2$, pH 7.4, containing 2 m*M* phenylmethysulfonyl fluoride and 5 m*M* EDTA, with a Brinkmann Polytron homogenizer. Protein concentration of homogenates was estimated by a Lowry method (12) using bovine serum albumin (BSA, fraction V, Sigma) as the standard. Aliquots of homogenate were diluted in 4 vol of SDS sample buffer containing 5% 2-mercaptoethanol and incubated at 100°C for 5 min before resolution of the proteins on 10% SDS–PAGE (13). Proteins were electrophoretically transferred to nitrocellulose paper from the SDS gel as described by Towbin (14). Following transfer, the nitrocellulose sheet was soaked for 1 hr in 300 ml of 0.35% BSA in 0.15 *M* NaCl containing 50 m*M* Tris, pH 7.6 (buffer A), followed by 1 hr in 10 ml of rabbit anti-rat OABP or rabbit anti-rat HBP serum (1:100 dilution in 3.5% BSA in buffer A). Prior to this we verified the cross-reaction of these antibodies to rat proteins with the corresponding mouse proteins. The sheet was next washed for two 30-min periods in 200 ml of 1% Tween-20 (Sigma) in buffer A. All procedures were performed at room temperature.

To detect protein-bound IgG on the nitrocellulose paper, we used protein A which had been radioiodinated by a chloramine-T procedure (5×10^6 cpm/μg) (15). The nitrocellulose sheet was incubated for 1 hr in 10^6 cpm of ^{125}I-protein A in 10 ml of 3.5% BSA in buffer A. The sheet was then washed for 30 min in 700 ml of 1% Tween 20 in buffer A followed by a second 30-min wash in 30 ml of buffer A. It was blotted with paper towels, air-dried, and exposed to Kodak XAR-5 X-ray film for 24 to 48 hr at −70°C. Radioactivity associated with the bands was also quantitated directly by excising them from the nitrocellulose paper using the autoradiogram as a template.

Determination of HBP binding activity. Asialoorosomucoid (ASOR) was purified from human serum as previously described (11). ^{125}I-ASOR (5×10^6 cpm/μg) was prepared with a solid phase lactoperoxidase, glucose oxidase coupled system purchased from Bio-Rad (Richmond, Calif.). To assay HBP activity, aliquots of liver homogenate, equivalent to 2 to 12 mg tissue, were incubated at 22°C for 10 min or at 4°C for 60 min with 1 μg of ^{125}I-ASOR in 0.5 ml of 0.05 *M* Tris–HCl buffer at pH 7.8, containing 0.05 *M* $CaCl_2$, 0.1 *M* NaCl, 1% bovine serum albumin, and 0.5% Triton X-100. Bound ASOR was determined following precipitation of the receptor-ligand complex with an equal volume of 20% (w/v) polyethylene glycol-6000 in the presence of 1 mg/ml of bovine IgG. The precipitate was recovered by filtration under reduced pressure through glass fiber filters (Whatman GF/A) followed by two 5.0-ml washes with 8% (w/v) polyethylene glycol-6000 in assay buffer without Triton X-100. Nonspecific binding was determined by omission of calcium or addition of 0.01 *M* EDTA to the assay mixture.

Results. Immunoblot analysis reveals that HBP is present as a 41,000 Da antigen and OABP as a 55,000 Da antigen in livers of newborn c^{14CoS} deletion homozygotes, control littermates and adults (Fig. 1). With this method, expression of OABP in newborns appears to be somewhat less than that in adults. In contrast, HBP content of newborn liver is considerably lower than that of the adult. The amounts of both proteins in livers of newborn mutant mice did not differ from those in livers of newborn controls.

For a better quantitative comparison, these proteins were excised from the nitrocellulose paper using the radioautogram as a template, and radioactivity due to bound ^{125}I-protein A was determined. While this method is not strictly quantitative, increasing the amount of

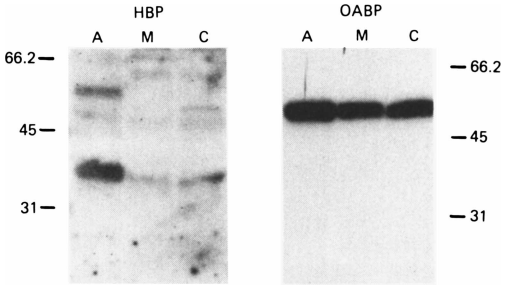

FIG. 1. Immunoblot analysis of HBP and OABP. Aliquots (10 μg) of liver homogenate proteins present in (A) adult c^{14CoS}/c^{ch} heterozygous and (M) c^{14CoS} homozygous deletion mutant or (C) control littermate neonates were applied to each lane. Numbers indicate mobility of molecular weight markers (in kDa).

homogenate applied to the SDS–PAGE resulted in a linear response within the range used. As suggested by the radioautogram (Fig. 1), no significant difference in radioactivity due to either protein could be detected in the c^{14CoS} homozygote when compared to control (Table I). HBP content in the newborn was only about 8% of the adult, while the concentration of OABP in newborns was within 75% of adult levels. This difference in the developmental expression of the two proteins was also evident in 18-day fetal livers (Table I).

The low level of HBP detected in newborn livers by the immunoblot method was confirmed by direct assay of asialoorosomucoid binding capacity. In livers from newborn homozygotes and control littermates, the amount of Ca^{+2}-dependent asialoorosomucoid bound was approximately 10% of the normal adult level (Table II). The low level of receptor activity in the newborn was evident when assayed at either 22 or 4°C indicating that there is no preferential degradation of receptor protein.

Discussion. The phenotype of mice homozygous for the c^{14CoS} deletion includes deficiencies of several liver-specific enzymes as well as a number of serum proteins which are synthesized by the liver. The tissue-specific expression of the mutant phenotype is further demonstrated by the exclusive reduction in liver cells of numbers of plasma membrane receptors for insulin (3), epidermal growth factor, and glucagon (4) with normal expression in other tissue. Based on these and other observations it has been suggested that the genes deleted in the c^{14CoS} mutants are instrumental in the normal regulation of certain aspects of hepatic parenchymal cell differentiation. In contrast to the previously cited 70% reduction of other cell-surface receptors, no difference in the hepatic concentration of HBP and OABP was detected. Furthermore, the molecular weights of the species present in liver homogenates from mutants were indistinguishable from those of controls. Since both proteins are normal constituents of the hepatocyte plasma membrane, the absence of quantitative or qualitative differences between mutants and controls indicates that the genes defined by the deletion do not regulate biosynthesis of all membrane proteins at this stage of hepatocellular differentiation.

TABLE I. QUANTITATION OF IMMUNOBLOT RADIOACTIVITY FOLLOWING EXCISION OF BANDS FROM THE NITROCELLULOSE SHEET

	OABP		HBP	
	cpm	Percentage of adult	cpm	Percentage of adult
Adult (3)[a]	4900 ± 230	100	797 ± 110	100
Neonate				
control (4)	3682 ± 210	74	63 ± 11	8
c^{14CoS} homozygote (3)	3661 ± 195	73	57 ± 12	7
18*d* Normal fetus (5)	3010 ± 120	60	47 ± 10	6

Note. Results are means ± SD.
[a] Number of animals is indicated in parentheses.

TABLE II. BINDING OF ^{125}I-ASIALOOROSOMUCOID TO MOUSE LIVER HOMOGENATES

	control	c^{14CoS} homozygote
	ng ASOR/mg protein	
Adult	92 (2)[a]	—
Newborn	8.8 ± 0.4 (5)	8.4 ± 0.6 (4)

Note. Results are the means ± SEM of triplicate determinations.

[a] Number of animals is indicated in parentheses.

The low level of HBP in the newborn and in the 18-day-old fetus detected by transblot analysis (Table I) confirms previous reports of the late development of this protein (11). The appearance of HBP in the perinatal period suggests that it may mediate specialized functions associated with extrauterine survival, although the nature of this role is unknown. A possible failure in the deletion homozygote of the expected increase of HBP expression during postnatal stages cannot be determined, because of the mutant's perinatal lethal period (1).

The data presented here also show that in contrast to the relatively low concentration of HBP, OABP is present at approximately 75% of the adult level in the newborn and at 60% in the 18-day-old fetus. The developmental increase of OABP parallels the cellular maturation of the liver during gestation. In the rat, hematopoietic cells occupy 35% of the liver volume up to the 18th day of gestation while at term the mass attributable to hepatocytes increases to 85% (16). Even though the function of OABP is not known, its significant expression in fetal liver suggests that it may be required for normal development.

The enzyme and protein deficiencies characterizing the c^{14CoS} deletion homozygotes do not result from the deletion of the respective structural genes. Support for this conclusion stems from results of somatic cell hybridization experiments showing normal activity of enzymes deficient in deletion homozygotes (17), demonstration of identical structure of the TAT gene in normal and mutant mice by Southern blotting (18), normal structure of reduced proteins (2, 3, 19) as well as identification of normal levels of mRNA sequences for deficient serum proteins (20). Another possible cause for the mutant phenotype, a reduction of hepatocyte numbers in the livers of deletion homozygotes, has been eliminated by the demonstration presented here of identical levels of the hepatocyte specific proteins HBP and OABP, in homozygous mutant and normal littermate livers. Our results, furthermore, exclude a general defect of plasma membrane biogenesis in the deletion mutants at least during pre- and perinatal stages of differentiation. The mechanisms responsible for the effects of the deletions on certain liver proteins to the exclusion of others remain to be determined.

This work was supported by NIH Grants AM-23026, AM-17702, AM-32419, and AM-32972 to A.W.W. and R.J.S., and GM-27250 and CD-38 (American Cancer Society) to Salome G. Waelsch. The authors acknowledge the encouragement and support of Dr. Salome G. Waelsch.

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Received July 8, 1985. P.S.E.B.M. 1986, Vol. 181.
Accepted October 15, 1985.