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Two-dimensional Electrophoretic Analysis of Hepatitis-associated Polypeptides in Liver of LEC Rats Developing Spontaneous Hepatitis

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High-resolution two-dimensional polyacrylamide gel electrophoresis in combination with silver staining was used to analyze between 800 and 1000 cytosolic and particulate polypeptides from age-matched livers of normal male Long-Evans rat with Agouti coat color (LEA) and Long-Evans rat with Cinnamon-like coat color (LEC) rats with hereditary trait of hepatitis at ages long before, immediately prior to, and just after the onset of hepatitis. Although the electrophoretic patterns of polypeptide expression were very similar with respect to the overall spot patterns, a number of polypeptides which differed either qualitatively or quantitatively were noted. Two constitutively expressed cytosolic polypeptides, P29.5 (M_r 29.5 kDa/pI 6.73) and P30 (30 kDa/6.70), were not detected in livers of LEC animals at any age. In the normal LEA rats both P29.5 and P30 were detected as early as one day after birth and both were expressed at similar concentrations at all ages. In the LEC rats P30-C (30 kDa/6.68) was constitutively expressed in close proximity to the expected position of P30, and P30-C was not detected in the LEA rats. By means of non-equilibrium pH gradient electrophoresis two relatively basic polypeptides were detected in the LEC rats. P18ne was detected immediately prior to and P27ne immediately after the clinical manifestation of hepatitis. Experiments in F_1 backcross ([LEA × LEC] × LEC) animals, however, failed to demonstrate any genetic link between either the expression or lack of expression of P29.5, P30, P30-C, or P18ne and hepatitis development. P27ne was detected in all backcross animals exhibiting hepatitis, but was never observed in LEC rats prior to the onset of hepatitis. Although we were unable to identify any unique loss of expression of polypeptides which are genetically linked to hepatitis susceptibility in LEC rats, specific subsets of quantitatively modulated polypeptides were detected.

Key words: Hepatitis — LEC rat — Two-demensional polyacrylamide gel electrophoresis — Polypeptides

We have previously described a mutant rat strain, Long-Evans rat with Cinnamon-like coat color (LEC),[6] that suffers spontaneous hepatitis about four months after birth.[1,2] The clinical signs exhibited in some of the LEC rats resemble those found in human fulminant hepatitis and are characterized by hyperbilirubininemia, subcutaneous bleeding, loss of body weight, increased levels of serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) and alkaline phosphatase, and single cell and spotty necrosis of hepatocytes without inflammatory cell response. Within one week after the onset of hepatitis approximately 20–30% of the rats die from submassive necrosis of the liver. Among the long-surviving animals more than 90% of the animals develop preneoplastic lesions such as enzyme-altered foci and nodules, and in rats of 8–12 months and older, hepatocellular carcinomas develop.[2,3] Since the incidence of spontaneous tumors in the livers of rats is relatively rare[4–6] the LEC rat offers a valuable animal model for studying the relationship between the pathogenesis of hepatitis and hepatocellular carcinogenesis. Electron microscopic analysis failed to reveal any viral particles in the affected liver and intraperitoneal injections of cell-free liver homogenates of severely jaundiced LEC rats failed to induce hepatitis in neonatal LEA rats, a sibling line of LEC rats.[2] Pulse labeling with bromodeoxyuridine indicated the presence of a higher number of proliferating hepatocytes in LEC rats after the age of two weeks as compared to LEA, although no cell death was detected by histological analysis until week 14.[7] Flow cytometric analysis of the DNA content of jaundiced rats, in which enlarged hepatocytes with huge nuclei were found, showed very high polyploids (32 and 64n), and bi-, tri-, and tetranucleated hepatocytes with

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[6] Abbreviations: LEC, Long-Evans rat with Cinnamon-like coat color; LEA, Long-Evans rat with Agouti coat color; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; IEF, equilibrium pH gradient isoelectric focusing; NEPHGEL, nonequilibrium pH gradient electrophoresis.

variable-sized nuclei were observed.[7] At present, the molecular mechanism(s) for the development of hepatitis and carcinogenesis in the LEC rat is not known although genetic analysis has demonstrated that a single autosomal recessive gene, tentatively designated *hts* (hepatitis), is responsible for the hepatitis.[8]

A sensitive and quantitative approach to identify alterations in gene expression which may be essential in the spontaneous development of hepatitis and hepatocarcinogenesis in the LEC rat is analysis of polypeptides of the liver using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).[9] This approach has been successfully used to identify several polypeptides associated with cellular proliferation and transformation.[10–14] In this report we describe the 2D-PAGE analysis of cytosolic and particulate polypeptides from LEC and LEA rat liver at ages before and after the development of hepatitis. The LEA rat was used as a control animal since this is an established strain derived from the same parents as Long-Evans rat and does not develop hepatitis.[2] Comparative analysis of 800–1000 silver-stained cytosolic and particulate polypeptides from age-matched male LEC and LEA livers revealed five qualitative polypeptide differences between LEC and LEA animals. The relationship between these polypeptides and the *hts* gene product and manifestation of hepatitis is discussed.

MATERIALS AND METHODS

Animals The LEC strain was established from noninbred Long-Evans rats maintained under normal conditions at the Center for Experimental Plants and Animals of Hokkaido University. Details of the origin, maintenance, and breeding history of the LEC rats have been given previously.[1, 2]

Tissue preparation Animals were killed and livers were quickly frozen in liquid nitrogen. Liver (200–400 mg) were homogenized in ice-cold 10 m*M* Tris-HCl, pH 7.2 containing 0.2 m*M* phenylmethylsulfonyl fluoride as a protease inhibitor (100 mg of tissue per 600 μl of buffer). All subsequent manipulations were performed at 4°C. Homogenates were centrifuged at 200,000*g* for 30 min at 4°C in a Beckman TL-100 ultracentrifuge and separated into cytosolic and particulate fractions. An aliquot of the cytosolic fraction was carefully removed and adjusted to a final solution of O'Farrell's lysis buffer A[9] which contains 2% NP-40, 5% 2-mercaptoethanol, 9.5 *M* urea, and 2% ampholytes (1.6% pH range 5–7 and 0.4% pH range, 3.5–10.0). For the preparation of the particulate polypeptide fraction, after centrifugation the surface of each pellet was gently washed with 10 m*M* Tris buffer and then solubilized in O'Farrell's lysis buffer A. Cytosolic and particulate polypeptide fractions were centrifuged at 300,000*g* for 20 min at 10°C and then stored at −70°C. These were thawed just prior to use for electrophoresis. Protein concentrations of solublized fractions were determined by the method of Bradford using bovine serum albumin as a standard.[15]

2D-PAGE 2D-PAGE analysis of cytosolic and particulate polypeptides from LEC and LEA rat livers was performed as described by Wirth *et al.*[14, 16, 17] Equilibrium pH gradient isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGEL) was used in the first dimension and SDS-PAGE in the second dimension. In IEF ampholytes (LKB Instruments, Rockville, MD) were used at concentrations of 1.6% (pH range 5–7) and 0.4% (pH range 3.5–10.0). Two hundred micrograms of protein dissolved in buffer A was added at the basic end of the first-dimensional isoelectric focusing tube gel (inside diameter, 2.0×160 mm). Samples were electrophoresed at 750 V for 16 h at room temperature and finally at 1000 V for 1 h (13,000 V-h total). In NEPHGEL ampholytes were at a concentration of 1.6% (pH range 7–9) and 0.4% (pH range 3.5–10.0). Samples were loaded at the anode (acidic end) and focused toward the cathode at 375 V for 16 h (6000 V-h total). SDS-PAGE was performed at constant current (20 mA/gel) using 1.0 mm thick 10% and 15% polyacrylamide gels. Following electrophoresis, gels were silver-stained using the procedure of Hochstrasser *et al.*[18] with minor modifications and replicate images of the stained gels were made using Kodak X-Omat duplicating film.[16, 17]

RESULTS

Separation of particulate and cytosolic polypeptides from LEA and LEC rat liver Fig. 1 illustrates the IEF 2D-PAGE separation of silver-stained particulate (Fig. 1A, B) and cytosolic polypeptides (Fig. 1C, D) of livers from 15 wk LEA and 16 wk LEC rats suffering from hepatitis. Aproximately 800–1000 individual polypeptides over the pH range of 4.8–7.0 and M_r 24–100 kDa were readily visible on each electrophoretogram. The electrophoretic patterns of both particulate (Fig. 1A, B) and cytosolic (Fig. 1C, D) polypeptides were very similar between normal LEA and hepatitis LEC rat livers with respect to both the total number of polypeptides detected and the overall protein patterns. It should be noted, however, that some of the less abundantly expressed polypeptides, e.g. those expressed at less than 10 ng/mm^2, might not be visible on the composite photographs.[14]

Comparison of particulate polypeptides in livers of LEC (Fig. 1B) and LEA (Fig. 1A) revealed no qualitative polypeptide differences. However, comparison of cytosolic polypeptides (Fig. 1C, D) revealed the apparent loss of expression, or expression at greatly reduced con-

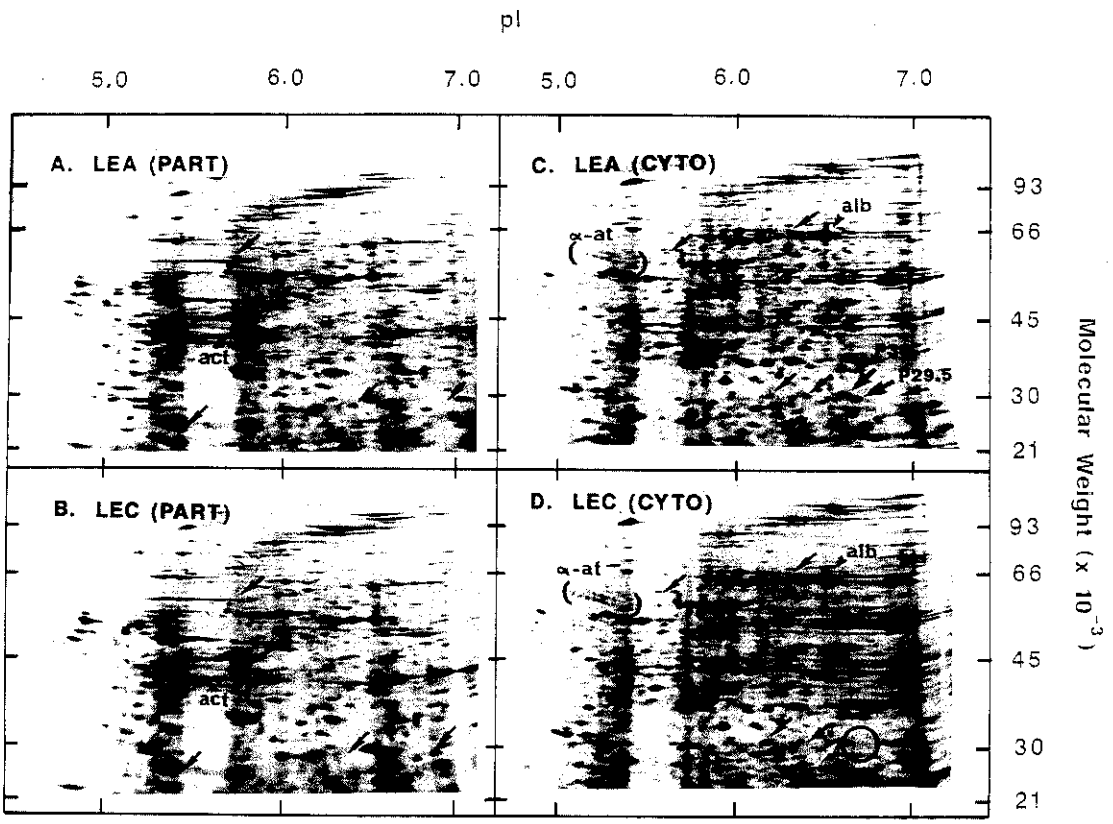

Fig. 1. IEF 2D-PAGE separation of polypeptides from 15 wk LEA and 16 wk LEC rat livers. Polypeptides were separated in the first dimension by IEF between pH 4.2 (left) and 7.4 (right) and in the second dimension between M_r 20 and 100 kDa. A, particulates of LEA; B, particulates of LEC; C, cytosol of LEA; D, cytosol of LEC. For reference, the particulate-associated polypeptide actin (act) and the cytosol-associated polypeptides albumin (alb) and α-antitrypsin (α-at) are indicated on the respective gels. Large arrows indicate the positions of P29.5 and P30 in the LEA rat liver; these were not detected in LEC cytosol. The open circle has been positioned at the expected M_r and pI values for P29.5 and P30 in the LEC rat liver. The white arrow indicates the position of P30-C in the LEC rat liver; it was not detected in the LEA cytosol fraction. Small arrows indicate significant quantitative differences.

centrations (below the limits of detection), of two polypeptides in hepatitis LEC rat liver (Fig. 1D). These polypeptides, P29.5 (29.5 kDa/6.73) and P30 (30 kDa/6.70), are indicated in Fig. 1C with the numbered arrows. Open circles have been positioned at the expected M_r and pI values for P29.5 and P30 on Fig. 1D, illustrating the apparent lack of expression of these polypeptides in the LEC rat liver. In the LEC rats one cytosolic polypeptide, P30-C (30 kDa/6.68), which was not detected in LEA, was expressed close to the expected position of P30 and it is indicated in Fig. 1D with the white arrow. Although qualitative differences were noted in only three peptides between LEA and hepatitis LEC rats, quantitative differences were observed in numerous polypeptides and these are illustrated with the small arrows. No apparent differences were noted in the particulate polypeptide, actin, or the cytosolic polypeptides, albumin and α-antitrypsin, in LEC and LEA rat livers as illustrated in Fig. 1.

Age-dependent expression of P29.5 and P30 in LEA and LEC rat liver In order to examine whether age-dependent expression of P29.5, P30 and P30-C existed in LEA and LEC rat livers, animals were sacrificed at various times after birth and their liver cytosolic polypeptides were compared. Fig. 2 is a composite representation of mosaic images from areas of IEF 2D-PAGE gels illustrating the expression of P29.5, P30 and P30-C in LEA and LEC rat livers 1 day (Fig. 2A, G), 2 wk (Fig. 2B, H), 8 wk (Fig. 2C, I), 12 wk (Fig. 2D, J), 16 wk (Fig. 2E, K), and 24 wk (Fig. 2F, L) after birth. Expression of both P29.5 and P30 was detected as early as 1 day after birth in the LEA rat liver (Fig. 2A).

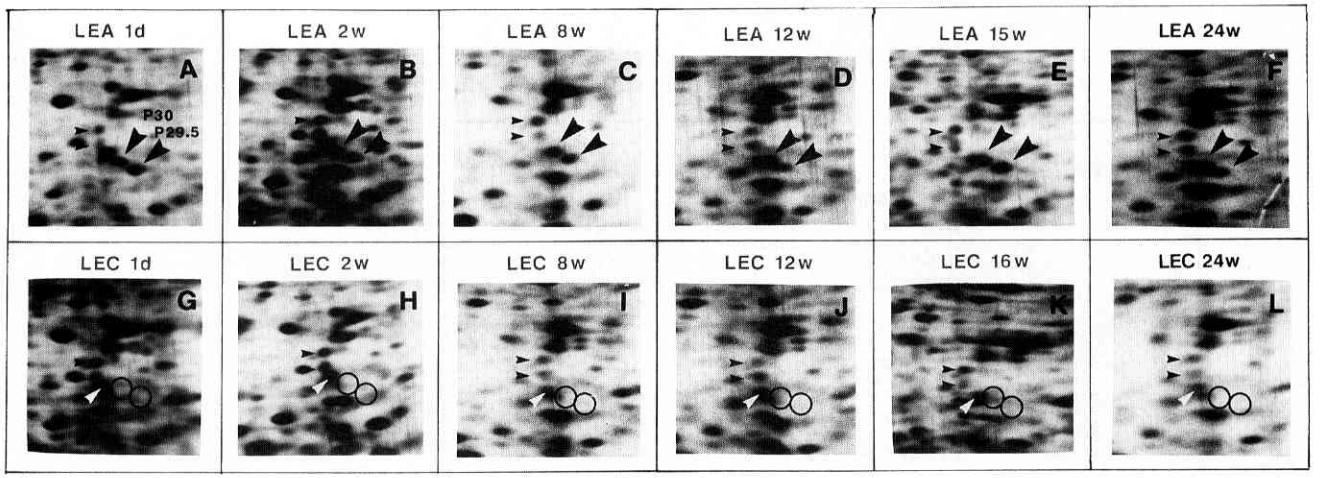

Fig. 2. Age-dependent expression of P29.5, P30 and P30-C. Mosaic composites of areas of IEF 2D-PAGE gels from the cytosolic fraction of LEA (A–F) and LEC (G–L) illustrating the expression of P29.5 and P30 at 1 day (A, G), 2 wk (B, H), 8 wk (C, I), 12 wk (D, J), 16 wk (E, K) and 24 wk (F, L). Open circles have been placed at the expected M_r and pI regions for missing polypeptides. White arrowheads indicate the expression of P30-C. To aid in orientation and spot comparison, two constitutively expressed polypeptides found in both LEA and LEC rat livers are illustrated with the small closed arrowheads.

Neither P29.5 nor P30 was detected in the LEC rat liver at any age (Fig. 2G–L). Expression of P30-C was observed in LEC of all ages as shown in Fig. 2G–L with white arrowheads, but not in LEA at any age (Fig. 2A–F).

No other qualitative polypeptide differences were noted between LEA and LEC rat livers although numerous quantitative differences were detected among the individual polypeptides at various ages (gels not shown).

P29.5 and P30 were also detected in cytosolic polypeptides of kidney preparations from the LEA rat but not from the LEC rat. Both these polypeptides were also detected as small spots in liver preparations from normal F344 rat but neither P29.5 nor P30 was detected in the regenerating 14-week-old LEC rat liver 20 h after two-thirds partial hepatectomy (gels not shown).

NEPHGEL analysis of particulate and cytosolic polypeptides in LEA and LEC rat livers In addition to P29.5, P30 and P30-C two relatively basic polypeptides were detected in the liver of LEC rat but not in the LEA. P18ne was detected in 12 wk LEC rat livers immediately prior to the onset of hepatitis as illustrated in Fig. 3. P27ne was detected in 16 wk hepatitis LEC rat liver but not in the 15 wk LEA as illustrated in Fig. 4.

Expression of P29.5, P30, P30-C, P18ne and P27ne in backcross animals In order to determine whether any

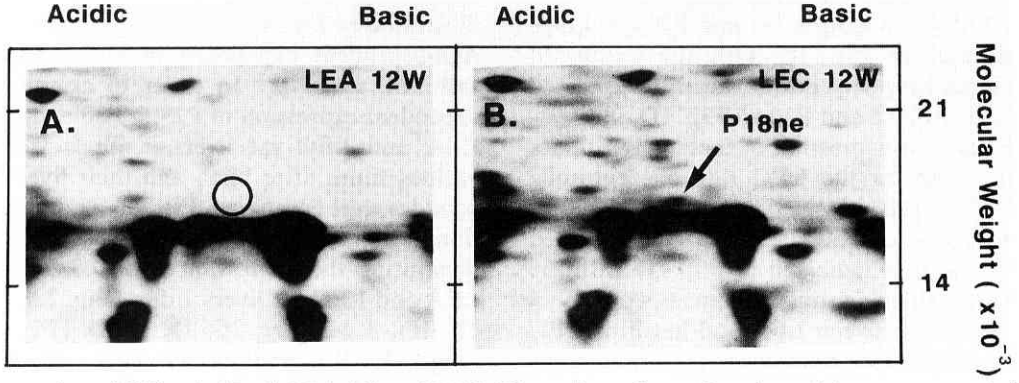

Fig. 3. Expression of P18ne in 12 wk LEA (A) and LEC (B) rat liver. Cytosolic polypeptides were separated in the first dimension under NEPHGEL conditions as described in "Materials and Methods."

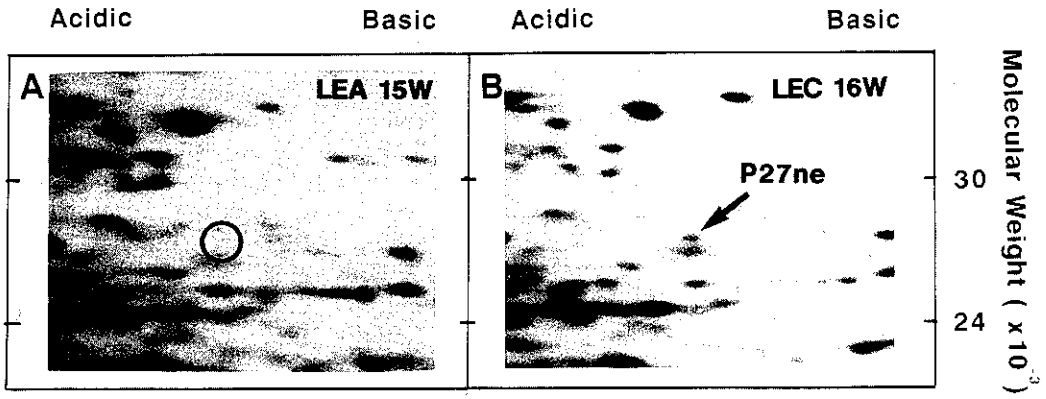

Fig. 4. Expression of P27ne in 15 wk LEA (A) and 16 wk LEC rat suffering from hepatitis. Cytosolic polypeptides were separated in the first dimension under NEPHGEL conditions as described in "Materials and Methods."

of these polypeptides was genetically linked to hepatitis susceptibility in the LEC rat 2D-PAGE analysis was performed in F_1 backcross ([LEA × LEC] × LEC) animals. In 23 wk backcross animals 50% (3/6) of the animals showed various degrees of hepatitis as determined from histological evaluation. One of the animals showed severe signs while the remaining two exhibited mild signs of hepatitis. However, no genetic link could be found between the expression of either P29.5, P30, P30-C or P18ne and hepatitis development. In one animal exhibiting relatively mild hepatitis both P29.5 and P30 were detected and P30-C was not, while P18ne was observed in one of the hepatitis-negative animals.

P27ne was detected in all three of the backcross animals exhibiting hepatitis, but was not observed in 14 wk LEC rat liver prior to the onset of hepatitis.

DISCUSSION

2D-PAGE analyses revealed that in the LEA rat polypeptides, P29.5 and P30 are expressed as early as one day after birth (Fig. 2A). However, neither P29.5 nor P30 was detected in the LEC rat liver at any age (Fig. 2G–L). In the LEA rat liver P29.5 and P30 were expressed at approximately equal and constant concentrations at all ages of development (1 day to 24 wk). P30-C was expressed constitutively in the LEC rats, but not in the LEA rats and migrated in close proximity to the expected position of P30. It is not known whether P30-C is related to P30 or not. In addition, one relatively basic polypeptide, P18ne, was detected in 12 wk LEC rat livers immediately prior to the onset of hepatitis but not in LEA rat livers at the same age (Fig. 3). Experiments in F_1 backcross ([LEA×LEC]×LEC) animals, however, failed to show any genetic link between either the expression or lack of expression of P29.5, P30, P30-C or P18ne and hepatitis development. An additional basic cytosolic polypeptide, P27ne was detected in all of hepatitis rats containing backcross animals, but was never observed in the rats prior to the onset of hepatitis. In both acute and chronic phases, this polypeptide was present at similar concentrations. It is possible that P27ne was a consequence rather than a cause of hepatitis. Sugiyama *et al.*[19] have found that certain isoenzymes of cytochrome P-450, probably P-450$_b$ and P-450$_e$, were selectively depressed, and γ-GTP activity was, in contrast, significantly increased in the LEC rat liver, and they suggested that these alterations may predispose the LEC rat to hepatitis and hepatoma development.[19] No linkage analysis to hepatitis susceptibility, however, has been done. If these alterations have a linkage to *hts* gene, certain critical biochemical alterations may occur in the livers of LEC rats at the beginning, or immediately after, birth and hepatitis may occur after the progressive accumulation of these alterations over a toxic threshold.[20]

The present study, which was limited to the analysis of approximately 1000 of the most abundantly expressed polypeptides, indicates that gene expression at the protein level is not significantly altered in the LEC rat liver as compared to the normal LEA rat. There is a possibility, however, that the maintenance of the normal phenotype in the LEA rat liver is under the control of polypeptide(s) expressed not abundantly. Since many of the proteins with DNA-binding and gene-regulatory activity are nuclear proteins and are expressed in very low concentrations, the existence of such proteins in LEA and LEC rat liver may be obscured by the more abundantly expressed proteins. Another possibility is that *hts* mutation in the LEC rat may involve a single amino acid substitution which may not change the apparent M_r/pI of the mutated gene product but yet completely destroys the enzymic or gene regulatory activity of the protein. LEC

rats have also been found to show remarkable hypogammaglobulinemia.[21] However, no genetic linkage has been found to exist between *hts* and immunological abnormality by the analysis of F_1 backcross animals (A. Hattori, personal communication). These data suggest that in addition to the *hts* gene mutation, at least one more mutation exists in the LEC rat strain. Establishment of a congenic rat strain which has an *hts* but none of the other abnormalities present in LEC rats is necessary to approach directly the cause of hepatitis.

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