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Inflammation of the dam has different effects on the binding of maternal and fetal rat liver nucleoproteins to the rat haptoglobin gene promoter

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Abstract. Transcriptional regulation of binding interactions between nucleoproteins and the hormone response element (RE) of the rat haptoglobin (Hp) gene was investigated in adult and fetal livers of rats exposed to inflammation on day 19 of pregnancy. Nuclear extracts from the embryonal liver displayed a barely detectable binding-affinity for hormone RE, but in extracts from the adult liver it was noticeable. The acute phase reaction of the mother promoted an increase of Hp gene expression in both adult and fetal livers, relying on stage-specific changes in hormone RE binding activities of nucleoplasmic proteins. The results indicated that the elevation of Hp gene expression in fetal liver to the steady basal level found in adults required the induction of new *trans*-acting proteins, whereas an overexpression of this gene in adult acute phase liver relied essentially on an increase in the binding-affinity of the preexisting hormone RE binding proteins.
Key words. Haptoglobin; transcription; *trans*-acting factors; acute-phase; development.

Haptoglobin (Hp) is a plasma acute phase glycoprotein of heterotetrameric structure. Its functions are pleotropic, and mostly related to the binding and clearance of hemoglobin[1,2]. Birth[3,4] and acute phase injury[5] have been identified as events that promote the induction of the Hp gene in the liver and a consequent increase in the rates of Hp synthesis and secretion. Though it is classified as a gene expressed only after birth[3], the level of Hp gene expression in fetal liver was observed to increase with the time of gestation from a few percent to 40% of the level in adult liver[4]. The transition of fetuses to an extrauterine environment has been shown to promote an increase in the expression of the Hp gene, which is followed by a gradual decrease of Hp mRNA to the steady basal level[4]. Another increase in the level of Hp gene expression has been observed during the acute phase reaction of adults, when complex changes in the functioning of several systems lead to a release of cytokines and hormones mediating the transcriptional activation of acute phase protein (APP) genes in the liver[5–7]. Inflammation of the dam was found to induce the APP genes in fetal liver as well, the magnitude of increase in the level of the Hp mRNA being similar to that observed for maternal liver[4]. The Hp mRNA concentration in acute phase fetal liver thus reached the basal level of the adult liver, whereas that in maternal liver exceeded this value 2- to 3-fold.

The observed persistance of a gradient difference between the levels of the Hp gene expression in adult and fetal livers suggested a distinctive feature of the underlying molecular events. Considering that the mechanisms regulating the rate of gene transcription are believed to rely on *cis-trans* interactions of the gene promoter with nuclear proteins, it was tempting to assume that *trans*-acting nucleoproteins from fetal and maternal livers binding to the hormone response element (RE) of the Hp gene were not identical. In this work, this presumption was tested on the model of the pregnant rat exposed to inflammation on day 19 of gestation. The proteins in the nuclear extracts from adult and fetal livers were found to differ in composition, and the binding affinity of the Hp gene for hormone RE also differed. The acute phase-induced activation of the Hp gene in adult liver promoted a selective increase in the hormone RE binding potential of several nucleoproteins, with no significant changes in their composition. The acute phase reaction of the mother, however, elicited remarkable alterations in both the composition and the hormone RE binding patterns of fetal nucleoproteins.

Materials and methods

Animals. Male and female 75–90-day-old albino rats of the Wistar strain weighing 220–250 g were used. Fetuses were isolated from dams on day 19 of gestation. Livers were removed from males, females, dams and fetuses. Fetal livers were pooled to comprise a single sample. Inflammation of the rats was induced by subcutaneous injection of turpentine oil (1 µl per g b.wt) in the lumbar region and animals were sacrificed 12 h later.

Probe. Plasmids with a DNA sequence complementary to rat Hp (pIRL-25), as well as the promoter region of the Hp gene, were kindly donated by Dr Heinz Baumann (Roswell Park Cancer Institute, Buffalo, USA). A fragment of the Hp gene spanning from −170 to −56 was inserted into the Hinc II site of pUC_{13} and isolated according to the standard procedure described by Sam-

brook et al.[8]. A DNA probe was (α^{32}P) dCTP-labeled by the random priming technique[9] for Southwestern blot analyses. For the retardation assay the Hp fragment was end-labeled with (γ^{32}P)ATP.

Preparation of rat liver nuclear extracts. Nuclear extracts were prepared from control and acute phase adult and fetal rat livers following the procedure of Gorski et al.[10]. Tissues were homogenized in 10 mM Hepes pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM Na-EDTA, 1 mM PMSF, 2 M sucrose and 10% glycerol. The nuclei were collected by centrifugation through a cushion of the same buffer at 24,000 rpm for 30 min at 4 °C. The nuclei were resuspended in lysis buffer (10 mM Hepes pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM Na-EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) and chromatin was pelleted by centrifugation of the lysate at 35,000 rpm for 60 min at 4 °C. Solid $(NH_4)_2SO_4$ was added to the supernatant to give a final concentration of 0.3 g/ml, and the precipitated proteins were sedimented at 35,000 rpm for 30 min at 4 °C. Nuclear extracts were dialyzed against 25 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM Na-EDTA, 1 mM DTT and 10% glycerol and resuspended in dialysis buffer. Samples of nuclear extracts were frozen in small aliquots at −80 °C.

Polyacrylamide gel electrophoresis. Nuclear extracts from control and acute phase adult and fetal rat livers were applied to 11% separation gels according to Laemmli[11]. Proteins were visualized by staining with Coomassie brilliant blue R-250.

Gel retardation assay. 10 µg of the nuclear extract of adult or fetal rat livers was incubated with poly dI-dC and salmon sperm DNA in a buffer containing 25 mM Hepes pH 7.6, 60 mM KCl, 7.5% glycerol, 0.1 mM Na-EDTA, 0.75 mM DTT and 5 mM $MgCl_2$ at room temperature[12]. After 10 min, 5 ng (10,000–20,000 cpm) of the end-labeled Hp fragment was added and the incubation continued for 20 min. Samples were resolved on a 5% polyacrylamide gel in 0.5x TBE (45 mM Tris, 45 mM boric acid and 2 mM EDTA). The gels were dried and autoradiographed for 3 to 5 days.

Determination of mRNA concentrations in adult and fetal livers. Total liver RNA from fetal and/or adult rats was isolated by the procedure based on the extraction of the RNA with guanidine-HCl, as described by Harding et al.[13]. Samples of RNA (2.5, 5, 10 µg/spot) were dot-blotted onto Schleicher-Schuell nitrocellulose filters and hybridized with a nick-translated plasmid for Hp cDNA[14]. Relative changes in mRNA concentrations were determined by scanning the autoradiograms (Beckman Microzone Densitometer, model 110) and expressed as a percentage of the control (100%). The estimates were expressed relative to the corresponding values for untreated dams and their fetuses.

Nuclear transcription assay. The assay was performed as described by Vannice et al.[15]. Rat liver nuclei were isolated by homogenization in 0.25 M sucrose, 50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT and purified by ultracentrifugation in the same buffer containing 2.2 M sucrose[16]. The nuclei were counted in a Burker-Turk counting chamber. The isolated nuclei were incubated with 18.5 MBq/ml of α[^{32}P]UTP (9.25 MBq/ mmol) in a mixture containing 0.5 M Hepes pH 8.0, 0.9 M NH_4Cl, 50 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM EDTA), 10% glycerol, 10 µg/ml BSA, 2 mM DTT and 0.4 mM each of unlabeled ATP, CTP and GTP. The ^{32}P-labeled RNA was hybridized to a nitrocellulose filter containing 3 µg of linearized denatured plasmid DNA containing inserts complementary to rat Hp mRNA and pBR_{322}. The filters were hybridized with all of the in vitro labeled RNA isolated from 10^8 nuclei of adult and/or fetal livers. The conditions of prehybridization, hybridization and washing were as described by Vannice et al.[15]. The transcriptional activity was quantified by scanning the autoradiograms and expressed as the percentage increase with respect to the control (100%).

Southwestern blot analysis. Southwestern blot analysis was performed according to the modified method of Bowen et al.[17]. Nuclear extracts from adult or fetal rat livers were electrophoresed through an 11% polyacrylamide gel. The gel was electroblotted onto a Hybond TM-C membrane. Filters were kept in binding buffer (1 mM Na-EDTA, 10 mM Tris-HCl pH 7.0, 0.02% BSA, 0.02% Ficoll, 0.02% polyvinilpyrrolidone, 50 mM NaCl) for 1 h and then incubated in the same buffer containing 10^6 cpm of α^{32}P-labeled DNA per pattern for the next 1 h. For removal of nonspecifically bound radioactivity the filters were washed with three changes of the binding buffer containing 200 mM NaCl and then exposed to X-ray film for 1 to 4 days.

Immunoblot analysis. In order to prepare polyclonal rabbit anti-HMG antibodies, HMG proteins were isolated according to the procedure of Sanders[18]. Identical volumes of protein solution (2 mg/0.5 ml) and Freund's complete adjuvant were mixed and subcutaneously injected into a rabbit. Over a period of 4 weeks 3–5 more injections with incomplete adjuvant were given. Antisera were prepared one week after the last immunization. Nucleoproteins from adult or fetal rat livers were separated through a 10% polyacrylamide gel and transferred to nitrocellulose filters. After incubation with blocking solution (0.05% Tween 20, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2% non-fat condensed [Carnation] milk) the filters were incubated with polyclonal rabbit anti-HMG antibodies (dilution 1:50) for 3 h at room temperature. Following rinsing the blots were incubated with ^{125}I-labeled anti-rabbit antibodies (dilution 1:1000) for 1 h. After washing, the filters were exposed to X-ray film.

Results

The complexes obtained with nuclear extracts from the liver of 19-day fetuses from an untreated dam with hormone RE of the Hp gene formed a few diffuse, fast-moving and barely detectable bands in the gel shift assay (fig. 1, fetus, lane C). When nuclear extracts from untreated adult rats (males, females and dams) were probed with the same sequence, two sets of complexes designated in figure 1 by numerals 1 to 3 and 4 to 6, and a fast-moving complex F, were obtained (figure 1, adult, lane C). These results suggested that birth-related activation of the Hp gene relied on a remarkable increase in the ability of nucleoproteins to bind the hormone RE of this gene.

The acute phase reaction of the mother promoted to a 2- to 3-fold increase in the level of Hp gene expression in both the fetal and maternal livers (ref. 4; fig. 3A). Thus Hp gene activity in the embryo liver reached the same level as the basal level of its expression in adult liver, and in maternal liver it exceeded this value threefold.

The results of gel shift analyses showed that this differential activation of the Hp gene in the two livers is accompanied by discernible changes in the hormone RE binding activity of the proteins present in the maternal and fetal nucleoplasms (fig. 1, lanes T). In fetuses, the changes were characterized by a striking increase in abundance of the fast-moving set of complexes and the appearance of several slower-moving bands, which rendered the pattern comparable to that of the control adult liver. In marked contrast to the fetal polypeptides, nucleoproteins extracted from the adult acute phase liver displayed no dramatic changes in the ability to bind hormone RE. The corresponding gel shift pattern revealed an increase in the relative amounts of complexes 3, 5 and 6 at the expense of 1, 2 and 4, as well as the appearance of a novel, slowest-moving band S (fig. 1, adult, lane T).

The results of gel shift experiments suggested that hormone RE binding proteins in nuclear extracts from fetal and adult livers were not identical. Possible differences were investigated by Southwestern blot analyses of nuclear extracts from the two livers. The patterns obtained after Coomassie blue staining of proteins separated in the SDS-PAGE system pinpointed differences in the composition of polypeptides in the nuclear extracts from the two livers (fig. 2). Those extracted from the livers of fetuses from an untreated dam formed several prominent bands, and numerous diffuse faint ones, most of which migrated at rates different from those of proteins extracted from the livers of untreated adult rats (fig. 2, lanes C). In addition, nuclear extracts from the fetal livers were depleted of the 45 kD proteins which were abundantly present in nuclear extracts from adult rats. Inflammation of the mother promoted remarkable changes in the composition of fetal nucleoproteins, whereas the pattern of maternal proteins remained virtually unaltered (fig. 2, lanes T). Nuclear extracts from the liver of fetuses from an inflamed dam were enriched with several proteins, the most abundant

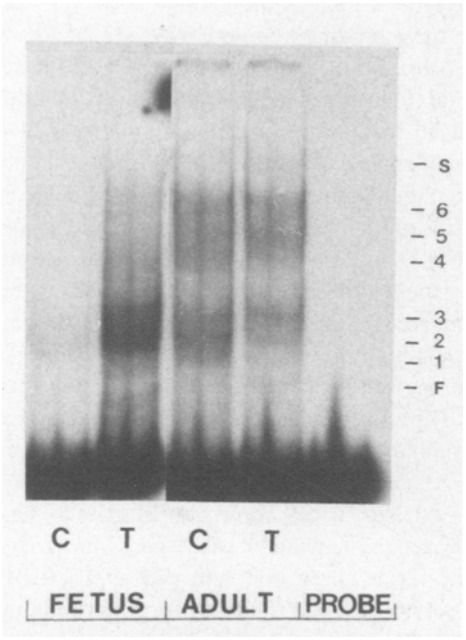

Figure 1. Gel mobility shift patterns of complexes obtained using hormone RE of the Hp gene with nucleoplasmic proteins from control (lanes C) and 12 h-acute phase (lanes T) livers of fetal and adult rats; lane 'probe', no extract. Numbers and letters indicate the different bands formed.

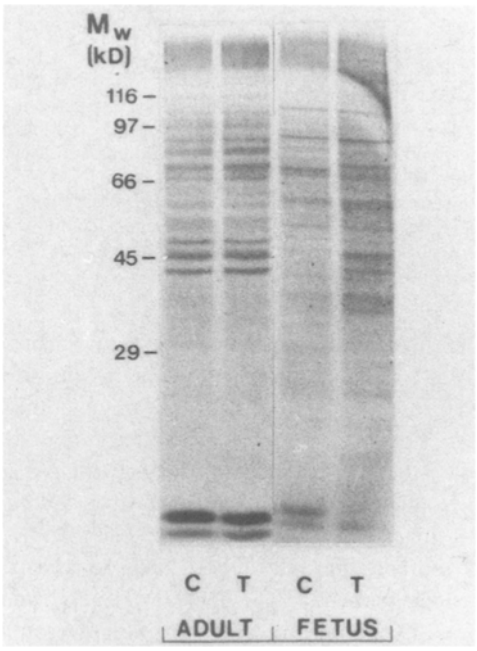

Figure 2. Coomassie brilliant blue stained SDS-PAGE patterns of nucleoplasmic proteins from control (lanes C) and 12 h-acute phase (lanes T) livers of adult and fetal rats.

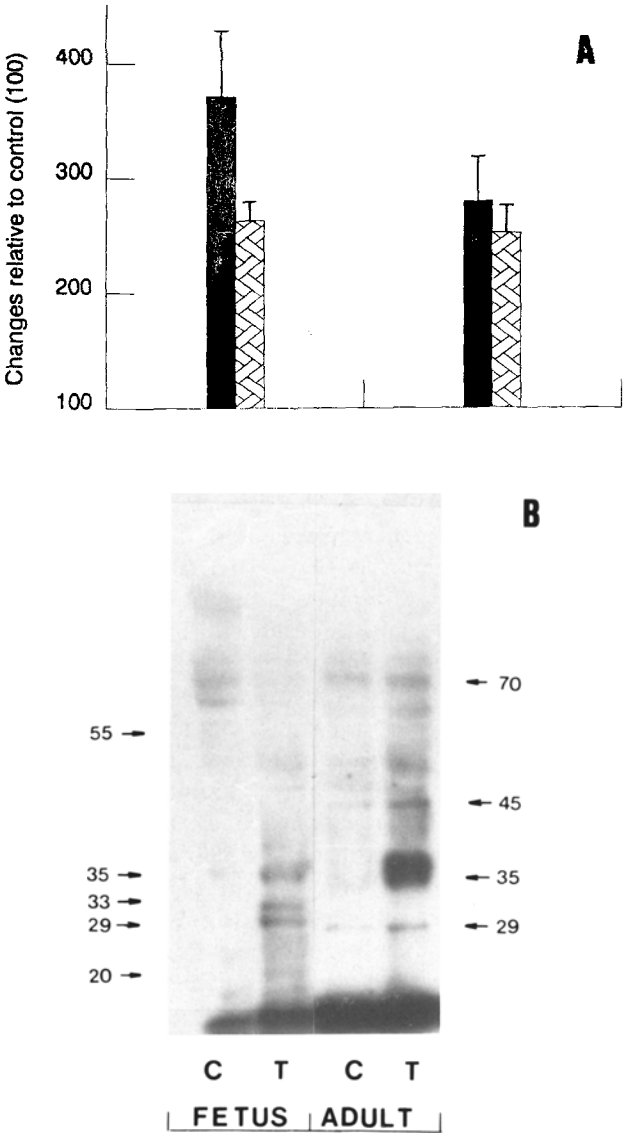

Figure 3. *A*. The relationships between the increases in the nuclear transcriptional activity for Hp gene and concentrations of the corresponding mRNA of the inflamed dams and their fetuses; □ mRNA, ■ transcriptional activity.
B. Southwestern blot patterns of complexes obtained with the hormone RE of the Hp gene and size-separated nucleoproteins from control (lanes C) and 12 h-acute phase (lanes T) livers of fetal and adult rats.

of which had molecular masses ranging from 20 to 45 kD (fig. 2, fetus, lane T). A high affinity of the induced proteins, particularly those at positions of 35, 33 and 29 kD for hormone RE resulted in a Southwestern blot pattern of nuclear extracts from the liver of fetuses from an inflamed dam which was qualitatively different from that observed for fetuses from an untreated mother (fig. 3B, fetus, lanes C and T). The observed enrichment of the nuclear extracts from acute phase fetal liver with low molecular weight proteins also provided an explanation for the appearance of fast-migrating complexes in gel shift experiments (fig. 1, fetus, lane T).

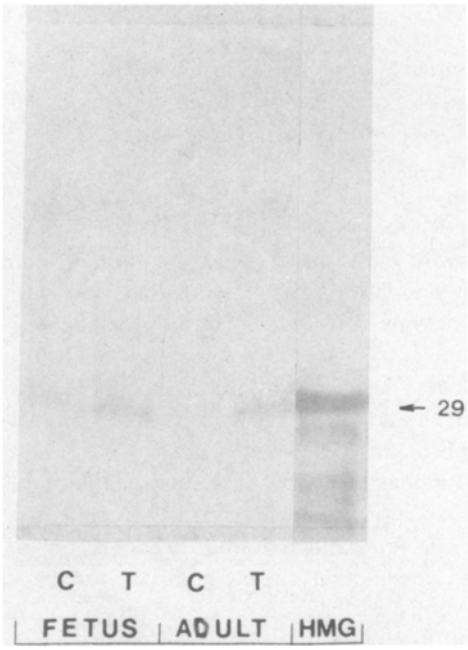

Figure 4. Western blot analysis of nucleoplasmic proteins from control (lanes C) and 12 h-acute phase (lanes T) livers of fetal and adult rats. Lane 'HMG' pattern of rat liver HMG proteins; arrow, p29 kD.

As reported previously[4], and confirmed in the present work, transcriptional activity of liver nuclei from fetuses from an inflamed dam for the Hp gene was 2.5-fold higher than that of control fetal nuclei (fig. 3A, fetus). Although a concurrent appearance of novel hormone RE binding proteins does not prove that acceleration of the Hp gene transcription in fetal liver was achieved through these binding interactions, it argues strongly for such a possibility. The Southwestern blot patterns of nuclear extracts from acute phase liver of adult rats revealed an increase in the binding-affinities of several preexisting proteins, especially of p29, p35, p45 and p70, for hormone RE (fig. 3B, adult, lane T), which coincided with a 2.5-fold enhancement of transcriptional rate (fig. 3A, adult). These results indicated that an elevation of the Hp gene transcription rate in fetal liver to the basal level observed in the adult liver required induction of new *trans*-acting proteins, whereas a further acceleration of this process in the acute phase liver of adult rats relied essentially on the modification of the preexisting protein species, enhancing their ability to bind hormone RE.
Among several hormone RE binding proteins in the nuclear extracts from adult rat liver, p70 has been characterized as a protein with the same size, charge and epitopes as lamin A[19], and p35 as a C/EBPβ isoform. Protein p29 was found to form an immune complex with HMG1 antibody (manuscript submitted). When the nuclear extracts from fetal liver were processed for Western blot analyses, the antibodies raised against rat liver HMG interacted with nucleoplasmic proteins at the 29 kD position (fig. 4, fetus). With

HMG antigens and HMG antibodies several immune complexes were obtained, the most prominent of which was formed with the 29 kD antigen, corresponding to HMG1 (lane HMG). Nuclear extracts from the adult liver contained a barely detectable amount of the HMG1 antigen (lane C), and that from fetal livers a noticeable amount, whereas those from acute phase livers were enriched with it (lane T). The C/EBPβ antibody, which has been shown to recognize the p35 residing in the nuclear extracts from the adult livers (manuscript submitted) failed to form a detectable amount of immune complex with nuclear extracts from the fetal livers (not shown).

Discussion

Nuclear extracts from rat livers of 19-day-old fetuses were found to contain proteins whose composition and binding-affinity for hormone RE of the Hp gene were markedly different from those observed for adult liver. According to the already-established timing for the induction of liver stage-specific proteins, late gestation represents one of at least three stages in development during which the modification of gene expression takes place[20,21]. The phase of changes aimed at preparing the liver for extrauterine functioning is succeeded by the period of postnatal liver maturation-related modification of gene expression, which ends with the attainment of an adult pattern of differential expression of specific genes. When assessed by one-dimensional SDS-PAGE, the composition of proteins residing in nuclear extracts from terminally differentiated rat liver was not found to depend on sex or to be affected by pregnancy. A largely different composition of nucleoproteins extracted from maternal and fetal livers on day 19 of gestation could be related, therefore, to a distinct pattern of differential gene expression in the two livers.

Based on the low concentration of Hp in fetal blood[22] and Hp mRNA in fetal liver[23], as well as on the 47 times lower Hp gene transcription rate in fetal than adult liver[3], the Hp gene has been classified as one of the group of genes expressed after birth. Nevertheless, its expression has been observed to increase during gestation to a level which, by day 19, was only a few times lower than that in adult liver[4]. The results presented in this work showed that liver nucleoplasm of 19-day-old embryos was depleted of protein species displaying an affinity for hormone RE of the Hp gene, whereas nuclear extracts from adult liver contained several proteins recognizing this sequence. This indicated that the Hp gene switch after birth correlated with an increase in the ability of nucleoproteins to bind hormone RE.

The acute phase reaction has been shown to promote a temporal modification of the final pattern of differential expression of a specific gene in adult liver, through induction of Hp and other acute phase protein genes, mediated by hormonal stimuli[24]. The principal mediators of a 3- to 6-fold increase in the Hp transcriptional rate, mRNA concentration, Hp protein synthesis and plasma concentration[25–27] have been identified as IL-6 and glucocorticoids in human cell systems[28–30] and IL-1, IL-6 and glucocorticoids in rat cells[31,32]. The hormone RE used in the present work has been characterized as a promoter region containing three *cis*-acting sequences, termed A, B and C, through which IL-6 and glucocorticoids control the activity of the Hp gene[33]. The gel shift and Southwestern blot patterns of complexes obtained using this fragment with nuclear extracts from acute phase livers of adult rats were, however, quantitatively rather than qualitatively different from those observed for nuclear extracts from uninduced adult liver. The main feature of the acute phase-dependent changes was an increase in the amounts and/or affinities of hormone RE-binding nucleoproteins migrating with molecular weights of 29, 35, 45 and 70 kD in the SDS-PAGE system. As shown previously and confirmed now, p29 has been recognized by anti HMG1 antibodies, p35 was identified as C/EBPβ isoform (manuscript submitted) and p70 was found to share size, charge and epitopes with lamin A[19]. Based on the results of a study addressing the contribution of each of the three *cis*-acting sequences to the protein binding property of the whole hormone RE, the relation of preexisting hormone RE binding proteins to those forming the acute phase inducible complex S in the gel shift assay has been proposed to be that between monomers and their dimeric combinations (manuscript submitted).

In response to inflammation of the mother, the Hp gene activity in fetal liver increased to the basal level of its expression in uninduced adult liver (ref. 4; fig. 3A). Induction of the Hp gene coincided with the appearance of novel binding activities in fetal liver, making the gel shift and Southwestern patterns of nuclear extracts from induced fetal and uninduced adult livers comparable. However, binding activity migrating with a molecular mass of 33 kD was present in the fetal liver only, whereas the ability of 29 and 35 kD proteins to bind hormone RE was much greater for the acute phase fetal than the uninduced adult liver. In addition, p35 and p20 residing in nuclear extracts from adult liver were recognized by anti-C/EBPβ antibodies, whereas those extracted from fetal liver were not. In contrast to the C/EBPβ isoforms, a p29 protein sharing epitopes with rat liver HMG1 was present in both liver extracts. An active role of HMG1 in DNA synthesis[34] and RNA transcription[35], as well as its structural homology with acidic transcription factors[36], have indicated that HMG1 may function as a transcription activator. Based on the results showing that Lex A-HMG fusion protein constructs failed to elevate the level of β-galactosidase activity in transfected yeast cells, HMG1 as well as HMG14 and HMG17 have

been proposed to function as quasi transcription factors[37]. Although proteins sharing molecular sizes with HMG14 and HMG17 did appear in nuclear extracts from the acute phase fetal liver, they were not recognized by rat liver HMG antibodies. Thus all the acute phase inducible activities in fetal liver, except for p29, remain to be characterized.

The fact that binding activities in fetal and adult livers remained distinguishable regardless of whether the Hp gene in them was expressed to a different or similar level, argued for a stage- and perhaps stimuli-specific character of the regulatory mechanisms. Those operating in the developing liver relied on the induction of new *trans*-acting proteins, whereas in terminally differentiated cells the mechanisms act through modulation of the hormone RE binding property of the preexisting protein species.

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