Developmentally regulated transcription of the four liver-specific genes for inter- α -inhibitor family in mouse

Jean-Philippe SALIER,*† Philippe CHAN,* Gilda RAGUENEZ,* Theresa ZWINGMAN‡ and Robert P. ERICKSON‡

*INSERM U-78 and Institute for Peptide Research, B.P. 73, 76233 Boisguillaume Cedex, France,

and ‡Dept of Pediatrics, University of Arizona Health Sciences Center, Tucson, AZ 85724, U.S.A.

The inter- α -inhibitor family is composed of the plasma-protease inhibitors inter- α -inhibitor, pre- α -inhibitor and bikunin. Inter- α -inhibitor and pre- α -inhibitor are distinct assemblies of bikunin with distinct sets from three heavy (H) chains designated H1, H2 and H3. These H chains are encoded by a set of three evolutionarily related *H* genes, and bikunin by an α -1-microglobulin/bikunin precursor gene (*AMBP*). This precursor is cleaved to yield bikunin, a member of the Kunitz-type protease-inhibitor superfamily, and α -1-microglobulin, which belongs to the lipocalin superfamily. Northern-blot experiments with RNAs obtained from various tissues in fetal and in adult mice indicated that the transcription of the four *AMBP* and *H* genes is liver-restricted, although there is expression of *H3* in brain. An analysis of the *H1*, *H2*, *H3* and *AMBP* transcripts, as well as of

transcripts for other control genes, in liver during development showed a progressive increase in the amounts of the *H1*, *H2*, *H3* and *AMBP* RNAs, which all peak transiently at day 5 after birth. This was shown by a nuclear run-on experiment to originate from a change in transcription rate. The transient and postnatal increase in transcription could be explained neither by the liver-restricted expression nor by a common origin of these four genes, nor by a perinatal requirement for many lipocalins or protease inhibitors. This suggests that all four genes are perinatally triggered at the level of similar elements in their transcriptional regulatory regions, a conclusion strengthened by the weak expression of the four genes that is seen in a mutant mouse strain (albino) that is deficient in some liver-specific transcription factors.

INTRODUCTION

The inter- α -inhibitor (*I α I*) family consists of a set of mammalian plasma-protease inhibitors, the major members of which are designated *I α I*, pre- α -inhibitor (*P α I*) and bikunin. These molecules are made up of a series of four distinct polypeptides, including three heavy (H) chains, H1, H2 and H3, and a light chain, also designated bikunin after its tandem arrangement of Kunitz-type protease-inhibitor domains. *I α I* corresponds to an H1 + H2 + bikunin assembly, whereas *P α I* is made of H3 + bikunin. Bikunin is also present as a free molecule in plasma (reviewed in [1,2]). The bikunin polypeptide originates from an α -1-microglobulin/bikunin precursor (*AMBP*). This precursor undergoes a post-translational processing that results in two mature polypeptides, α -1-microglobulin (*A1M*) and bikunin [3]. Unlike bikunin, *A1M* is not a component of *I α I* family members. Instead, *A1M* is found in plasma in a free state as well as partly complexed to other polypeptide chains, which consist mostly of immunoglobulin A heavy chains in humans [4] and the monomeric protease inhibitor α -1-inhibitor-3 (*A1I3*) in rodents [5]. In rat, this *A1I3*–*A1M* complex makes up 1–3 % of the total *A1I3* in plasma, and 60 % of the total *A1M* in plasma [5]. *A1M* is one of many lipocalins, a superfamily of proteins that are able to carry small, lipophilic molecules (reviewed in [4]). The *AMBP* gene therefore eventually gives rise to two mature lipocalin- or Kunitz-type-related polypeptides. Despite these features and the *in vitro* protease-inhibitory activities of *I α I* family members, very little is known about the functions of these molecules *in vivo*. *A1M* has been suspected of involvement in various immunoregulatory circuits [4,6,7], and one of the *I α I* family members may possibly

act as an endothelial-cell growth factor [8] or have a role in stabilizing the cumulus extracellular matrix of the oocyte at an early stage of ovulation [9].

I α I and bikunin have been found in the plasma of all mammalian species so far studied, from rodents to humans [10]. Using the mouse as a model, we are currently interested in determining the biological functions and regulation of the expression of *A1M*, bikunin and the H1, H2 and H3 chains. We demonstrated recently that four *AMBP*, *H1*, *H2* and *H3* genes are present in mouse as well as in man, with gene arrangements and chromosomal locations that are quite similar in the two species [11,12]. As nothing is known about the developmental regulation of expression of these four genes in mammals during ontogenesis, we have now analysed the tissue-specific expression of these genes from embryo to adulthood in mouse. We have observed that they display a transient and synchronous peak of expression in liver during development. As these four genes are located on three different chromosomes, this result indicates that similar transcriptional control elements are likely to be present in the *AMBP* and *H1*, *H2* and *H3* genes.

MATERIALS AND METHODS

Mouse strains and developmental stages

The mouse strains BALB/C, C3H/HeJ, C57BL/6J and SJL/J were obtained originally from the Jackson laboratory, and maintained by brother–sister mating. They were maintained on a 14 h light, 10 h dark cycle and provided with mouse chow and water *ad libitum*. The timing of fetal development depended on checking females for vaginal plugs; the day of finding the plug

Abbreviations used: *A1AC*, α -1-anti-chymotrypsin (contrapsin in mouse); *A1I3*, α -1-inhibitor-3; *A1M*, α -1-microglobulin; *A2HS*, α -2-HS glycoprotein; *AMBP*, α -1-microglobulin/bikunin precursor; *ASL*, argininosuccinate lyase; *FIX*, coagulation factor IX; *H*, heavy; *HNF*, hepatocyte nuclear factor; *I α I*, inter- α -inhibitor; *MUP*, major urinary protein; *P α I*, pre- α -inhibitor; *p.c.*, post coitus.

† To whom correspondence should be sent.

was considered to be day 0 post coitus (p.c.). The timing of post-natal development depended on daily inspection of cages for litters; the day the litter was born was considered to be day 0. Homozygous c^{14Cos}/c^{14Cos} lethal albino mice and control littermates were also used in this study (see below).

Human, rat and mouse cDNA probes

Partial or full-length AMBP, H1, H2 and H3 cDNAs obtained from a human liver cDNA library and subcloned in a pUC18 plasmid have been described previously [11,13]. Partial cDNAs for human α -1-anti-chymotrypsin (A1AC; 1.2 kb) and rat α -2-HS glycoprotein (A2HS; 1.05 kb) subcloned in pUC-based plasmids were kindly provided by Professor P. Arnaud (Charleston, SC, U.S.A.). Likewise, two mouse cDNAs for the oncogene protein 24p3 (830 bp) and for transferrin (1.5 kb), a rat cDNA for argininosuccinate lyase (ASL; 1.3 kb) and a human cDNA for coagulation factor IX (FIX; 1.4 kb) were generous gifts from Professors M. Kress (Villejuif, France), G. Kelsey (Institute of Cell and Tumour Biology, Heidelberg, Germany), M. Mori (Kumamoto, Japan) and K. Kurachi (Ann Arbor, MI, U.S.A.) respectively. A rat A113 cDNA pRLA113/23J (4.6 kb) in the pcDV1 plasmid [14] was purchased from ATCC (clone reference: 63100) and a *Bam*HI–*Bam*HI fragment (575 bp) of the insert was used as a probe. A mouse 28 S RNA probe (1.5 kb) in pUC18 was obtained from Professor J. Mallet (Gif/Yvette, France). All cDNAs were purified from the plasmids by restriction-enzyme digestion, submerged agarose-gel electrophoresis and electro-elution according to standard procedures [15].

Tissue isolation and purification of total RNAs

Young or adult mice were killed by decapitation or by cervical dislocation respectively. The organs of interest were quickly removed and immediately frozen in liquid nitrogen, and they were stored long-term at -70°C . Total RNAs were prepared by the guanidinium isothiocyanate procedure followed by centrifugation on to a CsCl cushion [15]. RNA integrity was controlled by visual inspection of the 18 S and 28 S ribosomal RNA bands after ethidium-bromide staining in agarose gels. RNA concentrations were determined from the absorbance at 260 nm. Total liver RNAs from homozygous c^{14Cos}/c^{14Cos} lethal albino mice and control littermates were kindly provided by G. Kelsey and G. Schutz (Institute of Cell and Tumour Biology, Heidelberg, Germany). Each batch of these RNAs was prepared from at least five mice killed 4 h after birth.

RNA electrophoresis and blot hybridization

Samples of total RNAs were denatured for 15 min at 65°C in $1\times$ Mops buffer ($10\times$ Mops contains 200 mM sodium 3-[*N*-morpholino]propanesulphonate, 50 mM sodium acetate, 10 mM Na_2EDTA pH 7.0, 2.2 M formaldehyde and 50% (v/v) deionized formamide, and then loaded on to a 0.8% (w/v) agarose gel in $1\times$ Mops buffer and 2.2 M formaldehyde, and separated by electrophoresis at 25–50 V for 14–20 h. Capillary blotting of RNAs on to nylon membranes (Hybond N⁺, Amersham) was for 3 h in 50 mM NaOH, following the manufacturer's instructions. The membrane was then neutralized briefly in $2\times$ SSPE ($20\times$ SSPE contains 3 M NaCl, 200 mM NaH_2PO_4 , 20 mM Na_2EDTA , pH 7.4) and air-dried. The cDNA probes were labelled by the random-oligo procedure with [α -

^{32}P]dCTP (3000 Ci/mmol, Amersham) at a specific radioactivity of $0.5\text{--}2\times 10^9$ c.p.m./ μg . Pre-hybridization (3–4 h), hybridization (16–20 h) and post-hybridization washings were done in a hybridization oven with rotating glass tubes, at high (rodent cDNA probes) or low (human cDNA probes) stringency, as described previously [11,16]. In many instances, successive hybridizations were made by dehybridization of the nylon filters in boiling 0.1% SDS (4 times for 5 min) and rehybridization with another probe. Autoradiographic exposures on to Hyperfilm-MP (Amersham) were done for 1–15 days at -80°C with intensifying screens. The autoradiographic signals were quantified by densitometric scanning with Biocom (Les Ulis, France) equipment which mainly consisted of a CCD camera (Canon) and 'Lecphor' software.

Isolation of hepatocyte nuclei and nuclear 'run-on' experiments

Nuclei were isolated from fresh hepatocytes at various stages of mouse development, essentially using the methods described in [17]. At least three livers were used for each preparation of nuclei. The final preparations of nuclei were suspended at 1.2×10^8 nuclei/ml in 15 mM Tris/HCl, pH 7.5, 0.34 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM 2-mercaptoethanol, 0.5 mM spermidine and 0.15 mM spermine, and aliquoted for storage at -70°C . Run-on experiments were carried out at 27°C for 30 min with $(1\text{--}2)\times 10^7$ nuclei ($100\ \mu\text{l}$), 200 μCi [α - ^{32}P]UTP (800 Ci/mmol; Dupont–NEN), 1 μl of RNAsin (Gibco–BRL) and 100 μl of a buffer containing 40 mM Tris/HCl, pH 8.0, 2.5 mM dithiothreitol, 5 mM MgCl_2 , 70 mM KCl, 0.1 mM Na_2EDTA , 0.5 mM EGTA, 32% (v/v) glycerol, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.6 mg/ml heparin and 0.5 mM spermidine, to which 2 units of nucleotide 5' diphosphate kinase (Sigma), 2 mM phosphocreatine (Sigma) and 7 μg of creatine phosphokinase (Sigma) was added just before use. The reaction was stopped by incubation for 15 min at 37°C with 2 μl of RNAase-free DNAase I (Gibco–BRL) at 2 mg/ml in 10 mM CaCl_2 , 2.5 μl of tRNA at 20 mg/ml in water and 2 μl of 100 mM CaCl_2 . The DNAase I was destroyed by incubation at 45°C for 30 min with 50 μl of proteinase K (Boehringer–Mannheim) at 5 mg/ml in 5% (w/v) SDS, 25 mM Na_2EDTA , 50 mM Tris buffer, pH 7.5. Then 6 μl of 5 M NaCl and 2.5 μl of 20 mg/ml tRNA were added to the mixture, and the mixture was phenol-extracted. The aqueous phase was precipitated with 5% (w/v) trichloroacetic acid and 50 mM sodium pyrophosphate (final concentrations) at 0°C . After centrifugation, the pellet was resuspended in 40 mM Tris buffer, pH 8.0, and ethanol-precipitated. The final pellet was resuspended in diethyl-pyrocyanate-treated water, heated at 65°C for 10 min and then added to a minimal volume (2 ml) of hybridization medium. For each sample from different developmental stages, the same number of incorporated c.p.m. was hybridized to the cDNA 'traps' that had been immobilized on to filters. The probes used to capture the newly synthesized RNAs were pUC-based plasmids containing one of a number of cDNAs (see the Results section). These plasmids were *Tag*I-restricted and immobilized on to a nitrocellulose/nylon membrane (BAS85, Schleicher and Schuell) in alkaline/denaturing conditions with a vacuum slot-blot apparatus (Millipore). Hybridizations and washes were done under the conditions detailed above (under blot hybridization), and then the membranes were incubated in 20 $\mu\text{g}/\text{ml}$ RNAase A in $2\times$ SSPE (37°C , 15 min) and then briefly washed in $2\times$ SSPE at room temperature. After autoradiography, the autoradiographic signals were quantified by densitometric scanning (see above). Control plasmids included pUCBM21 DNA without any insert cDNA as well as a pUC plasmid containing a mouse 28 S RNA

probe (see above). For a given gene, the transcription levels obtained with different batches of nuclei prepared at various times during mouse development were compared with the autoradiographic signal and retained on to the corresponding cDNA after the subtraction of background (pUCBM21) and normalization to the level of transcribed 28 S RNAs.

RESULTS

Tissue-specific expression of the *AMBP* and *H1*, *H2* and *H3* genes in adult or in fetal mouse

The tissue-specific expression of the four *AMBP*, *H1*, *H2* and *H3* genes was investigated in adult male mice with a (C57BL/6J $\times$ SJL/J)F2 genetic background by blot hybridization of total RNAs. The results, presented in Figure 1, clearly indicate that all four genes are transcribed in liver, whereas the corresponding RNAs are undetectable in brain, heart, kidney, lung and spleen with, however, the notable exception of a faint band that was detected in brain with the *H3* cDNA probe. This indicates that there is a low level of transcription of the corresponding *H3* gene in adult mouse brain, a feature that is also observed in the BALB/C mouse (results not shown). Since expression of the *AMBP* gene has been claimed to occur in rat kidney [18], we looked for similar expression in mice of both sexes with various genetic backgrounds. However, no expression of any *I α I*-family-related RNA could be observed in kidney from adult male or female mice, with either the BALB/C or C3H/HeJ genotype (results not shown). Finally, given the possible role of *I α I* or *P α I* in the stabilization of the cumulus extracellular matrix of the oocyte at an early stage of ovulation in mouse [9], we looked for transcripts of the four *I α I*-related genes in mouse ovaries obtained

14–16 h after stimulation with 5 units of human chorionic gonadotrophin. No transcripts were detectable under such conditions (results not shown).

To determine whether the tissue-specific pattern of expression of these four genes is developmentally regulated, a similar study was performed with organs from male and female fetuses at 18 days p.c. The results presented in Figure 2 again indicate that the four *AMBP*, *H1*, *H2* and *H3* genes are transcribed only in liver. These transcripts are identical in size to those found in liver RNAs from an adult mouse used as a control on the same blot.

Developmental expression of the *AMBP* and *H1*, *H2* and *H3* genes in mouse liver

We systematically studied the developmental expression of the four *I α I*-related genes in liver from (C57BL/6J $\times$ SJL/J)F2 mice. Northern blots for these four genes and for control genes are shown in Figure 3. Table 1(a) gives quantitative data obtained by the densitometric scanning of these blots. A low-level expression of the four *I α I*-related genes in liver is detectable as early as 15 days p.c. (results not shown). From 18 days p.c. until adulthood (Figure 3 and Table 1a), all four transcripts are present and exhibit a clear-cut, quantitative change between a minimal level, which is seen before birth and is similar in males and females, and a maximum level, which is reached at day 5 after birth, although the level for the *H3* transcript at birth is already close to this maximum (Table 1a). Finally, the transcript amounts in the adult decrease to a level that is either above (*AMBP* and *H1*) or below (*H2* and *H3*) the level that is seen before birth (Figure 3 and Table 1a). In conclusion, these results indicate a developmentally regulated expression of the four *AMBP*, *H1*, *H2* and *H3* genes in mouse liver, with a transient and synchronous maximum in RNA level at day 5 after birth for the four genes.

To assess whether another gene whose product can assemble

Figure 1 Liver-specific expression of the *AMBP*, *H1*, *H2* and *H3* genes in adult mouse

Total RNAs were extracted from various tissues of adult male mice. For each tissue, RNAs were prepared from pooled organs of three animals. The tissues tested were brain (B), heart (H), kidney (K), liver (Li), lung (Lu) and spleen (S), as indicated at the bottom. The RNAs (10 μ g/lane) were electrophoretically separated, blotted and successively hybridized with four human *AMBP*, *H1*, *H2* and *H3* cDNA probes, as indicated on the right. The 28 S lane is a control hybridization with a ribosomal 28 S RNA probe used to monitor the variations in RNA loading between lanes. The sizes of mouse *AMBP* and *H1* to *H3* RNAs, as published in [11], are on the left. For each probe, only the part of the blot that gave a positive hybridization signal is shown. Except with the 28 S probe, the autoradiograms shown have been overexposed to optimize the detection of weak transcripts: exposures for *AMBP*, *H1*, *H2*, and *H3* were 17 h, 20 days, 50 h and 7 days respectively. The anode is at the bottom.

Figure 2 Liver-specific expression of the *AMBP*, *H1*, *H2* and *H3* genes in mouse fetus

Total RNAs (10 μ g/lane) were extracted from various tissues of female (F) or of male (M) mouse embryos at 18 days p.c. For each tissue, RNAs were prepared from pooled organs from three animals. The heart was not studied in the female embryo. RNAs from an adult mouse liver (AL) are shown as a control. Except with the 28 S probe, the autoradiograms shown have been overexposed to optimize the detection of weak transcripts: exposures for *AMBP*, *H1*, *H2* and *H3* were 32 h, 20 days, 5 days and 15 days respectively. The faint signal seen in all lanes at the upper edge of the first four panels corresponds to a weak cross-hybridization of the probe with the 28 S RNAs. All other details and abbreviations used are as in the legend to Figure 1.

Figure 3 Developmental expression of the *AMBP*, *H1*, *H2* and *H3* and various control genes in mouse liver

Total RNAs (10 µg/lane) were extracted from the liver of mouse embryos at 18 days p.c. (female: F18; male: M18), male newborn (NB) or 1 day- (1 d), 3 day- (3 d), 5 day- (5 d), 1 week- (1 w), 2 week- (2 w), 4 week-old (4 w) male mice, or adult (A) male mice. For each time-course point RNAs were prepared from three pooled livers. The resulting blot was hybridized with the four human *AMBP*, *H1*, *H2* and *H3* cDNA probes, as well as six other probes as indicated on the right: *A1I3*, rat *A1I3* cDNA; *24p3*, mouse *24p3* cDNA; *A2HS*, rat α -2-HS glycoprotein cDNA; *FIX*, human *FIX* cDNA; *A1AC*, human *A1AC* cDNA; *ASL*, rat *ASL* cDNA. *A1I3*, *24p3*, *A2HS*, *FIX*, *A1AC* and *ASL* RNAs are 5.0, 1.0, 1.5, 3.0, 1.8 and 2.1 kb in size respectively. For each probe, the length of autoradiographic exposure was carefully chosen to avoid saturation of the film: exposures for *AMBP*, *H1*, *H2*, and *H3* were 12 h, 20 days, 32 h and 5 days respectively. The densitometric analyses of the hybridization signals are partly presented in Table 1(a).

with an *I α I* family gene product shows synchronous expression, we also investigated the developmental pattern of expression of the *A1I3* gene. This gene was tested because a fraction of *A1I3* molecules is bound to A1M in rodent plasma [5]. Our results (Figure 3, upper panel) indicate that *A1I3* gene expression occurs late in the postnatal period and reaches its maximum in adulthood. The *A1I3* pattern, therefore, does not correlate with the maximum of *AMBP* gene expression that is seen shortly after birth.

To assess whether the synchronous maximum in the expression of the *AMBP* and *H1*, *H2* and *H3* genes reflects a common, perinatal increase in the expression of many liver-specific genes or is roughly restricted to these *I α I* family genes, a series of other transcripts were investigated on the same blot. As exemplified by the hybridizations obtained from five cDNA probes (lower five

Table 1 Quantifications of changes in *I α I*-related mRNA levels in liver during mouse development: (a) levels of *I α I*-related mRNAs and 28 S RNAs during normal development (see Figure 3); and (b) levels of *I α I*-related mRNAs and 28 S RNAs in albino mice and controls (see Figure 4)

The values for a given RNA are from scanning densitometry of the autoradiographic bands that are shown in Figure 3 or 4. The values given are expressed as a percentage of the maximum value (100%) that was obtained for this RNA during development, after normalization to the 28 S RNA level.

(a)	
	RNA
Age	28 S H1 H2 H3 AMBP
18 days p.c.	100 12.1 27.7 41.1 53.7
Newborn	100 27.9 38.5 83.6 62.1
5 days	100 100 100 100 100
2 weeks	100 69.0 52.6 58.2 75.7
Adult	100 64.8 19.3 13.9 64.7

(b)	
	RNA
Animals	28 S H1 H2 H3 AMBP
Control mice	100 100 100 100 100
Albino newborns	100 43.5 47.2 47.2 68.5

panels in Figure 3), several distinct patterns of developmental expression emerged, including early expression (*24p3*), postnatal expression (*FIX*), essentially adulthood-restricted expression (*A1AC*), biphasic expression with early and late maxima (*ASL*), or finally an expression that mimicked that of *I α I* family genes (*A2HS*). Further cDNA probes for other control genes were also tested, such as the major urinary protein (*MUP*) gene, the product of which is a lipocalin. The *MUP* gene pattern (results not shown) was virtually identical to that of *A1AC* (Figure 3), indicating an adulthood-restricted expression of the *MUP* gene.

To establish whether the development-associated fluctuations of the four *AMBP*, *H1*, *H2* and *H3* mRNAs discussed above take place at the transcriptional level, nuclear run-on assays were performed with nuclei isolated from the livers of male mice at various stages in development. A representative experiment is detailed in Table 2 and shows that the transcriptional activities of the four *AMBP*, *H1*, *H2* and *H3* genes fluctuate during development so as to fit with the changes of the respective RNAs (see the correlations in Table 2). Notably, the *H3* transcriptional activity, but not that of the other three genes, is at a high level early in development. In adulthood, the transcriptional activities of the *H1* and of the *AMBP* genes plateau, whereas those of the *H2* and *H3* genes drop below their respective maximal levels that are seen earlier in development. In conclusion, our results indicate that the postnatal and transient increase in levels of the four *I α I*-related mRNAs, as described above, is at least partly accounted for by an increased transcription at, or shortly after, birth.

Expression of the *AMBP*, *H1*, *H2* and *H3* genes in liver from mice with the *c¹⁴CoS* lethal albino deletion

As a preliminary attempt to determine which transcription factor(s) are involved in the transient and synchronous maximum of transcription of the *I α I* family genes during development, we investigated whether these four genes display a similar level of expression in the liver of newborn mice that are homozygous for

Table 2 Nuclear run-on with nuclei from mouse liver at various stages during normal development

The radiolabelled RNAs newly synthesized by pooled livers obtained at a given age, as listed on the left, were hybridized to immobilized cDNA targets as listed on the top. The values for a given RNA are from scanning densitometry of the autoradiographic bands, after background subtraction and normalization to the 28 S RNA level; they are expressed as a percentage of the maximum value (100%) that was obtained during development. The *A2HS* and *MUP* genes are control genes of which the timing of the expression maximum in development is known (see text and Figure 3). The value of *r* is the correlation of run-on values with transcript levels at the corresponding ages (the values for I α I-related transcripts are given in Table 1a).

Age	RNA						
	28 S	H1	H2	H3	AMBP	A2HS*	MUP*
18 days p.c.	100	6.8	18.7	54.8	15.9	31.3	10.0
Newborn	100	20.1	100	54.7	54.6	41.9	10.3
5 days	100	60.5	82.8	100	100	100	20.4
2 weeks	100	100	97.6	40.0	96.0	87.6	17.3
Adult	100	84.9	48.2	8.8	85.7	50.7	100
<i>r</i>		0.74*	0.49	0.88†	0.78†	0.77†	0.99†

* $P < 0.05$.
† $P < 0.01$.

Figure 4 Developmental expression of the *AMBP*, *H1*, *H2* and *H3* genes in newborn mice with the *c^{14Cos}* albino deletion and in control littermates

Total RNAs (10 μ g/lane) were extracted from liver of *c^{14Cos}/c^{14Cos}* albino deletion mice (Alb) or from the liver of healthy, control littermates (C) 4 h after birth. RNAs were prepared from the pooled organs of five animals. Transferrin (TSF) cDNA identifies an RNA population that is not modified in level between Alb and C mice [19]. For each probe, the length of autoradiographic exposure was carefully chosen to avoid saturation of the film. All other details are as in the legends to Figures 1 and 3. The densitometric analyses of the hybridization signals are presented in Table 1(b).

the *c^{14Cos}* albino deletion. A homozygous genotype for this deletion, spanning the albino (*c*) locus, is responsible for the low level of transcription of many genes that are expressed in liver and/or kidney [19–22]. Our results (Figure 4, quantified in Table

1b) demonstrate that the expression of all four *AMBP*, *H1*, *H2* and *H3* genes is impaired to a moderate (*AMBP*) or to a severe (*H1*, *H2*, *H3*) extent in mouse newborns that are homozygous *c^{14Cos}/c^{14Cos}*. Controls included hybridization with a 28 S ribosomal cDNA probe and a transferrin cDNA probe (Figure 4). These two probes identified two RNA populations that are not quantitatively modified in *c^{14Cos}/c^{14Cos}* mice as against controls [19].

DISCUSSION

The tissue-specific expression of the I α I family-related *AMBP* or *H* genes has been documented previously in several species, but in all instances these studies used only one or two cDNA probes, namely H2 and *AMBP* cDNAs for the analysis of baboon tissues [13] and only *AMBP* cDNA for studies of rat [18,23] or of pig [24] tissues. Therefore, our present study of the four *AMBP*, *H1*, *H2* and *H3* gene transcripts in mouse tissues, whether before birth or in adulthood, is the first report of a complete pattern of tissue-specific expression for these four genes. In the mouse, the transcription of these genes occurs exclusively (*AMBP*, *H1*, *H2*) or mostly (*H3*) in liver, which is consistent with previous observations for the *H2* gene in baboon [13] and for the *AMBP* gene in baboon and pig [13,24]. Also our results in the mouse are consistent with the lack of *AMBP* gene transcription in the rat kidney which has been reported recently [23], despite an earlier claim that rat kidney expresses A1M [18]. Finally, our present observation of a weak transcription of the *H3* gene in mouse brain is consistent with an identical observation made in male and female rats (P. Chan and J.-P. Salier, unpublished work).

No change in the tissue-specific pattern of expression or in the size of the four I α I family mRNAs was observed in the mouse during development, as assessed from the analysis of various tissues in male and female fetuses at 18 days p.c. The only difference that was detected in the expression of these RNAs during development is in the level of expression. The low level of these four RNAs seen in the mouse embryo shortly before birth is reminiscent of a similarly low level of *AMBP* and *H2* RNAs previously described in liver from a 18 week-old human fetus [13]. Our present results show that the levels of all four *AMBP*, *H1*, *H2* and *H3* transcripts increase sharply during the first week after birth and decrease in adulthood. Although this part of our study was restricted to the male mouse, it is very likely that the same quantitative changes exist in the female mouse, since levels of the four corresponding RNAs in female are identical to those found in male, whether at 18 days p.c. (Figure 3) or in adulthood (results not shown). Control experiments with further cDNAs corresponding to genes expressed in liver were carried out first to verify that the development-associated changes seen on the Northern blots were reliable. Indeed, the various developmental patterns seen for the *A1I3*, *A2HS*, *FIX*, *A1AC*, *ASL* and *MUP* genes in this study agree perfectly with published analyses of these patterns in rat or mouse [19,25–28]. To our knowledge, no previous study has dealt with a developmental pattern of expression for the *24p3* gene. Furthermore, the control RNAs that are studied here corresponded to genes with an expression that is known either to be preferential for liver (*A1AC*, *A2HS* and *ASL*) [26,29,30] or to be strictly liver-restricted (*A1I3*, *FIX* and *MUP*) [25,31,32]. Some of these control genes code for plasma proteins (*A1I3*, *24p3*, *A2HS*, *FIX*, *A1AC*), also for protease inhibitors of the serpin (*A1AC*), cystatin (*A2HS*) or macroglobulin (*A1I3*) superfamilies [25,33,34], or for members of the lipocalin superfamily (*24p3*, *MUP*) [32,35]. Despite these similarities in the pattern of tissue-specific expression, and/or in the sequence or in the function of these control genes or of their

products compared with the I α I family genes and products, no analogy in the developmental expression of I α I family genes with any of these control genes could be found, except for the *A2HS* gene (Figure 3). From developmental studies carried out in rat embryos (but not extended after birth), three sets of plasma protein genes have been delineated by others [36]: (1) genes with high levels of mRNAs in the yolk sac compared with liver, coding for plasma proteins with a transport function, including some lipocalins; (2) genes whose highest expression is in liver during the fetal life, mainly protease inhibitors; and (3) genes weakly expressed in fetal liver compared with adult liver, without expression in yolk sac. Despite its obvious connections with lipocalins and with protease inhibitors, the rat *AMBP* gene has been included in the third group, which contains apparently unrelated proteins without any shared function [36]. The three rat H chain genes were not considered in that study [36]; however, the mouse *H3* gene is unlikely to fit into this third group, given its high level in fetus relative to that in the adult (Figure 3 and Table 1a) and none of the H chains has a known function that could help classify them into any group. The transient and synchronous peak of transcript levels that is seen for the three evolutionarily related *H1*, *H2* and *H3* genes is not the usual situation that is found in multi-gene families [37], and the existence of an *H* gene family cannot account for the synchronous peak of expression at day 5 for the *AMBP* gene, which is not evolutionarily related to the *H* genes. In conclusion, our observations indicate that in mouse during development the synchronous peak of expression of the four *AMBP*, *H1*, *H2* and *H3* genes cannot be accounted for on the basis of (1) the liver-restricted expression of many plasma protein genes, (2) the similar behaviour of a large set of gene products with similar functions, such as the transport of lipophilic molecules (lipocalins) or a protease-inhibitory activity, or (3) a shared evolutionary origin. Our results rather indicate that the overall similar patterns of developmental expression of these four genes reflects the requirement of the four corresponding gene products to be assembled into multichain molecules, such as I α I and P α I. In this respect, the high level of *H3* transcript at birth, which is close to the maximum seen at day 5 (Table 1a), suggests that the plasma level of P α I molecules, that is *H3* + bikunin, is close to a maximum immediately after birth. Also, the very low level of *A1I3* transcript that are observed in the young mouse (Figure 3) and in the young rat [25] contrasts with the propensity of *A1I3* and *A1M* to assemble as a circulating complex in rodent plasma [5], and indicates that such a complex exists mostly during adulthood when both *A1I3* and *AMBP* genes are transcribed at high rates.

Our observations point to a perinatal control mechanism that leads to a transiently high level of the four *AMBP* and *H1*, *H2* and *H3* transcripts despite the four corresponding genes mapping to three remote chromosomal locations [2,12]. A maximal RNA level seen at a given developmental stage may result from an increased mRNA stability or increased nucleocytoplasmic transport, and/or from an increased rate of gene transcription, as shown previously in rat hepatocyte for various mRNAs including those for some plasma proteins [37]. Our nuclear run-on experiments demonstrate that an increased rate of transcription is at least partly responsible for the observed changes in RNA levels in mouse liver, in particular the increased levels observed during the first week of life. This suggests that the four I α I family genes all have one or more regulatory elements that are activated by development-associated nuclear factors. Whether such factors are also involved in the liver-specific expression of these four genes is an open question. As a preliminary attempt to address this issue, we analysed the levels of the *AMBP*, *H1*, *H2* and *H3*

transcripts in newborn mice with the *c^{14CoS}* lethal albino deletion. These mutant mice lack the so-called *alf* or *hsdr-1* locus, the disruption of which prevents the normal transcription of many genes with a perinatal and liver-restricted expression [21,22]. These genes were first considered to represent a subset of dexamethasone- and cyclic AMP-inducible genes (reviewed in [19–22]). However, the *hsdr-1* locus has recently been tentatively identified as the *fumaryl acetoacetate hydrolase* locus [21]. In the homozygous *c^{14CoS}/c^{14CoS}* mouse, a deletion of this locus may be responsible for the concomitantly observed, up- or down-regulated expression of several transcription factors [19–22] that affect the liver-specific expression of many target genes, which may or may not be hormone-inducible [20,21,38]. Among the liver-enriched transcription factors (reviewed in [39]), the transcription of the genes for hepatocyte nuclear factor (HNF)-1 and HNF-4 is greatly impaired in *c^{14CoS}/c^{14CoS}* mice, whereas the transcription of the HNF-3 and C/EBP factors remains unaffected [19–21]. In human, the HNF-1 factor is essential for the strong and liver-specific transcription of the *AMBP* gene (P. Rouet, G. Raguenez and J.-P. Salier, unpublished work) [40], a feature that could account for the impaired expression of the *AMBP* gene in the *c^{14CoS}/c^{14CoS}* mouse (Figure 4). In contrast, a major enhancer in the *transferrin* gene is mainly driven by HNF-3 (C. Auge-Gouillou, I. Petropoulos and M. M. Zakin, personal communication) [41], which could explain the sustained transcription of this gene that is observed in the mutant mouse (Figure 4) [19], despite a secondary requirement for HNF-4 in the *transferrin* promoter (E. Schaeffer, F. Guillo, D. Part and M. M. Zakin, personal communication). Accordingly, and given the low levels of transcripts for the four *AMBP*, *H1*, *H2* and *H3* genes that are seen in the *c^{14CoS}/c^{14CoS}* mouse (Figure 4 and Table 1b), it is tempting to speculate that one or more of the *trans*-acting factors that are reduced in this mutant mouse could drive the liver-specific and developmentally regulated transcription of the four I α I-related genes. However, the actual importance of these factors for the transcription of the *H1*, *H2* and *H3* genes is unknown at present. Therefore, a functional analysis of the 5' flanking sequences in the *H1*, *H2* and *H3* genes will help to elucidate the factor(s) that are responsible for the transient and synchronous maximum in expression of the four I α I family genes seen in liver during development, a feature that ultimately raises the possibility of a co-ordinated expression of these genes.

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