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Developmental Regulation and Tissue Distribution of the Liver Transcription Factor LFB1 (HNF1) in *Xenopus laevis*

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The transcription factor LFB1 (HNF1) was initially identified as a regulator of liver-specific gene expression in mammals. It interacts with the promoter element HP1, which is functionally conserved between mammals and amphibians, suggesting that a homologous factor, XLFB1, also exists in *Xenopus laevis*. To study the role of LFB1 in early development, we isolated two groups of cDNAs coding for this factor from a *Xenopus* liver cDNA library by using a rat LFB1 cDNA probe. A comparison of the primary structures of the *Xenopus* and mammalian proteins shows that the myosin-like dimerization helix, the POU-A-related domain, the homeodomain-related region, and the serine/threonine-rich activation domain are conserved between *X. laevis* and mammals, suggesting that all these features typical for LFB1 are essential for function. Using monoclonal antibodies, we demonstrate that XLFB1 is present not only in the liver but also in the stomach, intestine, colon, and kidney. In an analysis of the expression of XLFB1 in the developing *Xenopus* embryo, XLFB1 transcripts appear at the gastrula stage. The XLFB1 protein can be identified in regions of the embryo in which the liver diverticulum, stomach, gut, and pronephros are localized. The early appearance of XLFB1 expression during embryogenesis suggests that the tissue-specific transcription factor XLFB1 is involved in the determination and/or differentiation of specific cell types during organogenesis.

In recent years, a growing number of transcription factors that coordinate the activity of target genes and are involved in the determination of a particular cellular fate or phenotype have been defined. In some cases, the importance of these regulatory proteins and their corresponding target sequences in the DNA is supported by the observation that the *trans*-acting factors and their *cis* elements have been conserved throughout evolution. Concerning liver-specific gene expression, four different types of regulatory elements and transacting factors have been identified. The prototypes of these factors involved in liver-specific transcription are C/EBP, LFB1 (HNF1), HNF3, and HNF4 (reviewed in references 17 and 27).

By analyzing the liver-specific expression of the *Xenopus laevis* albumin genes, we have identified the hepatocyte-specific promoter element HP1, which has been conserved throughout evolution, as it is functional in mammalian cells (41). HP1 not only constitutes one of the most important regulatory elements for liver-specific expression of mammalian albumin genes (15, 24, 29, 39) but also is involved in the expression of a group of genes specifically expressed in hepatocytes, such as the genes encoding α-fetoprotein, α_1-antitrypsin, and α- and β-fibrinogens (9, 14, 24). HP1 is recognized by the liver transcription factor LFB1 (14), also referred to as HNF1 or HNF1α (9, 31). The cDNA encoding LFB1 has been cloned from rats (4, 7, 12), mice (26), and humans (2). The deduced amino acid sequences for these three mammalian proteins are highly conserved and revealed that LFB1 is the most diverged homeoprotein so far identified (7, 11, 32). In addition, weak homology with the POU-A box of POU proteins can be found upstream of the homeodomain (4, 7, 12). Another typical feature of LFB1 is a myosin-like dimerization domain at the N terminus that has not been found so far in other groups of transcription factors (32).

It is generally assumed that tissue-specific transcription factors appear at some stage during the development and differentiation of an organism. As the target genes of LFB1 are activated in mammals early in development before birth (6, 46 and references therein), we decided to investigate the temporal and spatial expression of LFB1 during embryogenesis. Since early stages of development can more easily be studied in amphibian systems than in mammals (reviewed in references 8 and 30) and our previous data suggest that LFB1 has been conserved throughout vertebrate evolution (41), we used *X. laevis* for these investigations.

MATERIALS AND METHODS

Isolation and sequencing of cDNA clones. A randomly primed cDNA library of *Xenopus* liver was constructed from poly(A)-containing RNA by use of cDNA synthesis and λ-gt10 cloning kits from Amersham. The hybridization probe for screening of the cDNA library was a 530-bp *Eco*RI fragment of a clone previously isolated from a genomic *Xenopus* DNA library (unpublished data) by use of the 774-bp *Sma*I fragment of a rat cDNA (generous gift of R. Cortese) containing the POU-A-related and the homeodomain-related sequences (12).

Filters were prehybridized in 4× SET (1× SET is 0.15 M NaCl, 5 mM EDTA, and 50 mM Tris [pH 8])–5× Denhardt's solution–50% formamide–0.1% sodium PP_i–250 μg of yeast RNA per ml–100 μg of denatured calf thymus DNA per ml for 2 h at 37°C. Hybridization was performed at 37°C overnight in the same buffer containing 10^7 cpm of probe per ml, labeled by random priming to a specific activity of 6 ×

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10^8 cpm/μg. Washing of the filters was carried out in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) twice for 20 min each time at 65°C and once for 60 min at 37°C.

The DNA from positive clones was purified from liquid lysates, and the inserts were subcloned into the pBluescript II SK$^+$ vector (Stratagene). Sequencing was performed with a Pharmacia sequenase kit by use of a double-stranded plasmid as a template. Computer analysis of the sequence data was carried out with the Beckman Microgenie program.

Construction of expression vectors. Bal 31 digestion (20) was used to truncate the 5′-flanking region of the a1 cDNA clone up to position −13 in front of the open reading frame. Using a *Hin*dIII linker and the *Xba*I site 3′ of the open reading frame of the cloned sequence (Fig. 1), we inserted XLFB1a between the *Hin*dIII and *Xba*I sites of expression vector Rc/CMV (Invitrogen). Similarly, a *Hin*dIII site was introduced at −7 in the b1 cDNA clone, and this segment was ligated to the b2 cDNA clone by use of an *Esp*I site in the 70-bp overlap (see Fig. 1). An *Xba*I site present in the pBluescript II SK$^+$ vector was used with the *Hin*dIII linker to clone XLFB1b into Rc/CMV. The *Sna*B1 site present in the intron of the b2 cDNA (see Fig. 1) was used to delete the C-terminal portion of XLFB1b to generate b del. To delete the C-terminal activation domain of XLFB1a, we used the unique *Eco*RI site of the a1 cDNA.

Transient transfection and CAT assays. The dedifferentiated rat hepatoma cell line C2, grown in a 1:1 mixture of Dulbecco minimal essential medium (DMEM) and Ham F12 medium with 100 U each of penicillin and streptomycin per ml and 10% fetal calf serum, was transfected with 4 μg of reporter plasmid and 50 ng to 1 μg of the expression vector by the calcium phosphate precipitation method (41). Depending on the experiment, 100 to 200 μg of protein was used for the chloramphenicol acetyltransferase (CAT) assays (20).

Embryos and dissections. The stages of embryos obtained by in vitro fertilization (1) were determined as described previously (34). For early developmental stages, embryos were dejellied in 2% cysteine hydrochloride (pH 8) and cultured in 10% normal amphibian medium (42).

RNase protection analysis. One hundred embryos at a given developmental stage were homogenized in 2× SET–1 mg of proteinase K per ml–2% SDS. After 30 min of incubation at 25°C, phenol-chloroform extraction was carried out and the RNA was precipitated with ethanol.

An antisense probe covering the 5′ 206 nucleotides (nt) of the a1 clone (including the ATG) and 119 nt of polylinker sequences was prepared by transcribing the corresponding linearized pBluescript II SK$^+$ vector with T3 RNA polymerase at 10°C (22). RNase protection was carried out as described previously (40). The probe yielded a protected fragment of 206 nt.

Production of monoclonal antibodies. The 3′-terminal *Eco*RI fragment of the a1 cDNA clone, encoding the activation domain of XLFB1a spanning from the *Eco*RI site at position 1058 (see Fig. 1) to the *Eco*RI site in the cloning adaptor, was ligated into the *Eco*RI site of prokaryotic expression vector pGEX-3X (Pharmacia). The fusion protein was purified from isopropyl-β-D-thiogalactopyranoside (IPTG)-induced bacteria on a glutathione-Sepharose 4B column (Pharmacia) as described by the manufacturer.

BALB/c mice were immunized with 50 μg of the fusion protein (first immunization and first booster injection) and with 150 μg of the fusion protein (second booster injection) by use of the ABM-2 Adjuvans system (Sebak, Aidenbach, Germany). Spleen cells from an immunized mouse were fused 3 days after the last booster injection with cells of murine myeloma cell line P3-X63-Ag8.653 (19) by use of polyethylene glycol as a fusion reagent. Cultures of hybrid cells were grown in selective hypoxanthine-aminopterin-thymidine medium in 96-well tissue culture plates. Positive hybridomas initially identified by an enzyme-linked immunosorbent assay were subsequently characterized by the addition of an aliquot of the culture medium to band shift assay mixtures with labeled HP1 and *Xenopus* or rat liver extracts.

Extract preparation. Extracts from whole embryos, *Xenopus* liver, and dissections of stage-35 embryos were made as described previously (44) with benzamidine (0.5 mM), pepstatin A (25 μg/ml), and leupeptin (25 μg/ml) as protease inhibitors and 150 μl of homogenization buffer per 30 embryos. For embryo dissections, 100 μl of homogenization buffer was used per 30 pieces.

Extracts from adult *Xenopus* tissues were made by homogenization of frozen tissue samples in 5 volumes of the loading buffer of the SDS gel (40), boiling for 4 min, and clearing by centrifugation for 10 min at 10°C and 100,000 × *g*. The extract from rat liver nuclei was prepared as described previously (13).

Band shift assays. The conditions used for the band shift assays were defined previously (23). For upshift experiments, the incubation mixture contained 0.1 μl of the medium from either a control cell culture or the corresponding hybridoma culture. For analysis of extracts derived from *Xenopus* liver, the protease inhibitors used for extract preparation were included, and the incubations were done on ice.

In general, the complex containing LFB1 and HP1 not only was upshifted by the antibody but also was more intense, probably because of some stabilization by the added antibody. All upshifted complexes could specifically be inhibited by competition with unlabeled HP1.

In vitro transcription and translation. Capped sense RNA was synthesized in vitro by use of 100 U of T7 RNA polymerase and 3 μg of *Xho*I-linearized DNA template: the Rc/CMV construct containing the coding region either for XLFB1 protein a (amino acids [aa] 1 to 605) or for truncated XLFB1 protein b (aa 1 to 410). Incubation was done for 1 h at 37°C and was followed by a 10-min digestion of the DNA template with RNase-free DNase I, phenol-chloroform extraction, and ethanol precipitation (33). A 0.5-μg quantity of the synthesized RNA or of the control Brome mosaic virus RNA was translated in vitro for 1 h at 30°C with a rabbit reticulocyte lysate (Promega). For the band shift assays, 2 or 1 μl of the lysate containing the a or b translation product was used, respectively.

Western blot (immunoblot) analysis. Aliquots of the extracts were separated on an SDS gel and electrotransferred to a nitrocellulose membrane (40). The blot was incubated overnight with LFB1-specific monoclonal antibody XAD5 as a primary antibody, and horseradish peroxidase-conjugated rabbit antibodies to mouse immunoglobulin G were used to visualize bound XAD5 with the ECL-system (Amersham).

Nucleotide sequence accession number. The DNA sequences of the cDNA clones (see Fig. 1) have been submitted to the EMBL data bank. The accession numbers for the a1 and b1 XLFB1 cDNA clones are X64759 and X64760, respectively. The sequences for the XLFB1 b2 cDNA clone are deposited as X64761 and X64762 for the portions 5′ and 3′ of the presumed intron, respectively.

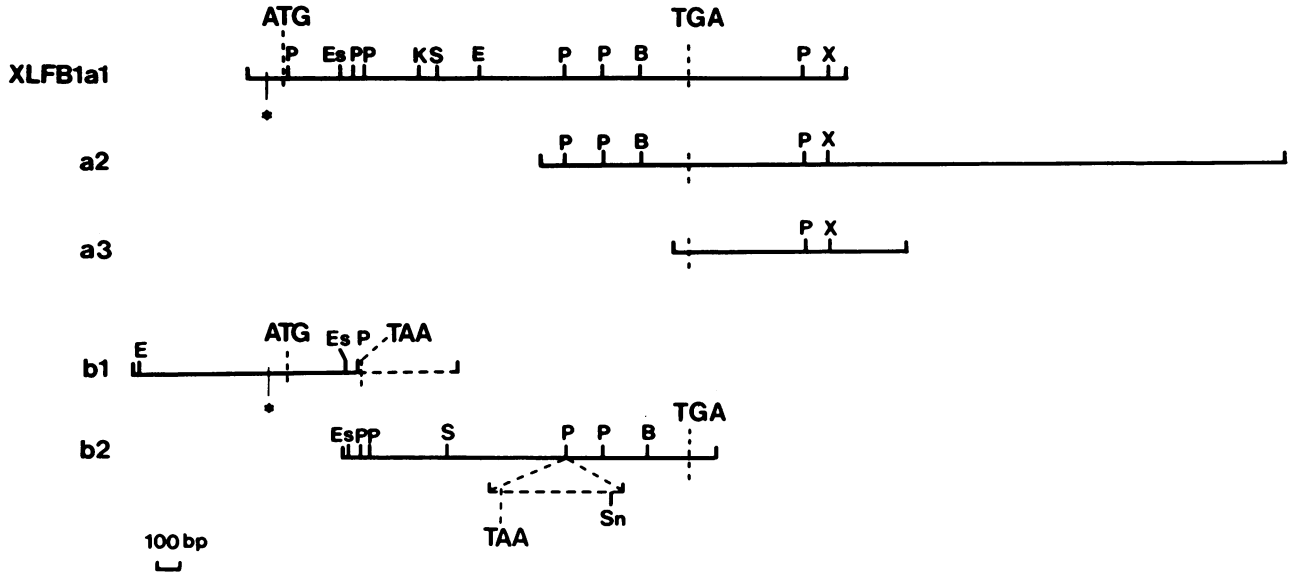

FIG. 1. Groups a and b for the five isolated overlapping *Xenopus* LFB1 cDNA clones. Start (ATG) and stop (TGA or TAA) codons of translation are indicated, and the putative unspliced introns in b1 and b2 are represented by broken lines. The restriction sites for *Bam*HI (B), *Eco*RI (E), *Esp*I (Es), *Kpn*I (K), *Pst*I (P), *Sna*B1 (Sn), *Spe*I (S), and *Xba*I (X) are given. The 8-bp consensus sequence TTTGGGGG also present in the rat and mouse 5'-untranslated region is marked by an asterisk.

RESULTS

Identification of cDNAs encoding the *Xenopus* transcription factor LFB1. From a Northern (RNA) blot analysis of *Xenopus* liver RNA, we observed that the potential *Xenopus* LFB1 mRNA is about 6.8 kb in size (data not shown). This value represents about twice the size found in the rat (12). Therefore, we used a randomly primed *Xenopus* liver cDNA library to allow efficient representation of the entire protein coding sequence in the cDNA clones. We isolated from this library five overlapping XLFB1 cDNA clones. Restriction analysis and partial sequencing revealed that the cDNA clones represent two distinct groups, a and b (Fig. 1).

Sequencing revealed that clone a1 of group a contains a large open reading frame of 1,815 bp and coding for 605 aa and some 5'- and 3'-flanking sequences. Clone a2 elongates clone a1 in the 3' direction for another 2.0 kb but includes neither a poly(A) tail nor a polyadenylation signal. Clone a3 contains the 3' end of the protein coding region and about 1 kb of a 3'-flanking sequence present in clones a1 and a2.

The cDNA clones of group b contain open reading frames much smaller than the one identified in the a1 clone. It is clear that clone b1 contains a large stretch homologous to the 5' portion of the clone a1 sequence, including the 180 bp preceding the open reading frame as well as the first 105 aa of the open reading frame. However, this clone extends another 340 bp in the 5' direction. In addition, clone b1 contains in its 3' portion a sequence completely unrelated to the one found in clone a1 and with an in-frame TAA translational stop codon (broken line in Fig. 1). Similarly, clone b2 has two stretches of homology to clone a1 interrupted by a completely unrelated sequence (broken line in Fig. 1). As the clones of group b contain an overlap of 70 bp that is completely identical but differs at 3 nt positions from the corresponding a1 sequence, we assume that clones b1 and b2 are derived from the same gene and are distinct from the cloned transcripts of group a. Since this perfect 70-bp overlap is followed by the splice donor site CA ↓ GTA, we speculate that the unrelated sequence of clone b1 represents an unspliced intron. This assumption is consistent with the exon-intron structure of a genomic clone containing the sequences of clone b1 (data not shown). In clone b2, the 70-bp overlap and the adjacent 3' region are highly homologous to the open reading frame found in the group a sequence; therefore, this clone most likely represents a spliced transcript at this position. Clone b2 seems to carry another intron that contains a presumed in-frame translational stop codon, since the sequence unrelated to the group a clone sequence is bordered by the potential splice donor site TG ↓ GTA and the splice acceptor site TTCAG ↓. Splicing at this position would generate continuous high homology to the a1 sequence.

In conclusion, the two group b cDNA clones contain a sequence that is homologous to the entire open reading frame found in clone a1. The homologies between groups a and b in the 5'-untranslated region, the protein coding region, and the 3'-untranslated region are 96, 92, and 82%, respectively.

Taken together, these results indicate that it is most likely that the a and b cDNAs originate from two different LFB1 genes. This suggestion is in agreement with our recent identification of two distinct families of genomic clones coding for the group a and b cDNAs (unpublished data).

Primary structure of *Xenopus* LFB1 compared with those of the mammalian proteins. The open reading frame of XLFB1 cDNA clone a1 encodes a protein of 605 aa, a size that is somewhat smaller than the one reported for the mammalian LFB1 proteins (631 aa for humans [2]; 628 aa for rats [7, 12] and mice [26]). A comparison of the deduced amino acid sequence of the *Xenopus* protein with the sequence reported for the rat reveals as the most conserved portion the DNA binding region, which includes the POU-A-related and the homeodomain-related sequences, with homologies of 83 and 96%, respectively (Fig. 2A). The main difference in the POU-A-related sequence is an insertion of the 5 aa LFTFT in XLFB1a (Fig. 2B). Most notable in the homeodomain-related sequence is a stretch of an extra 21 aa between helix

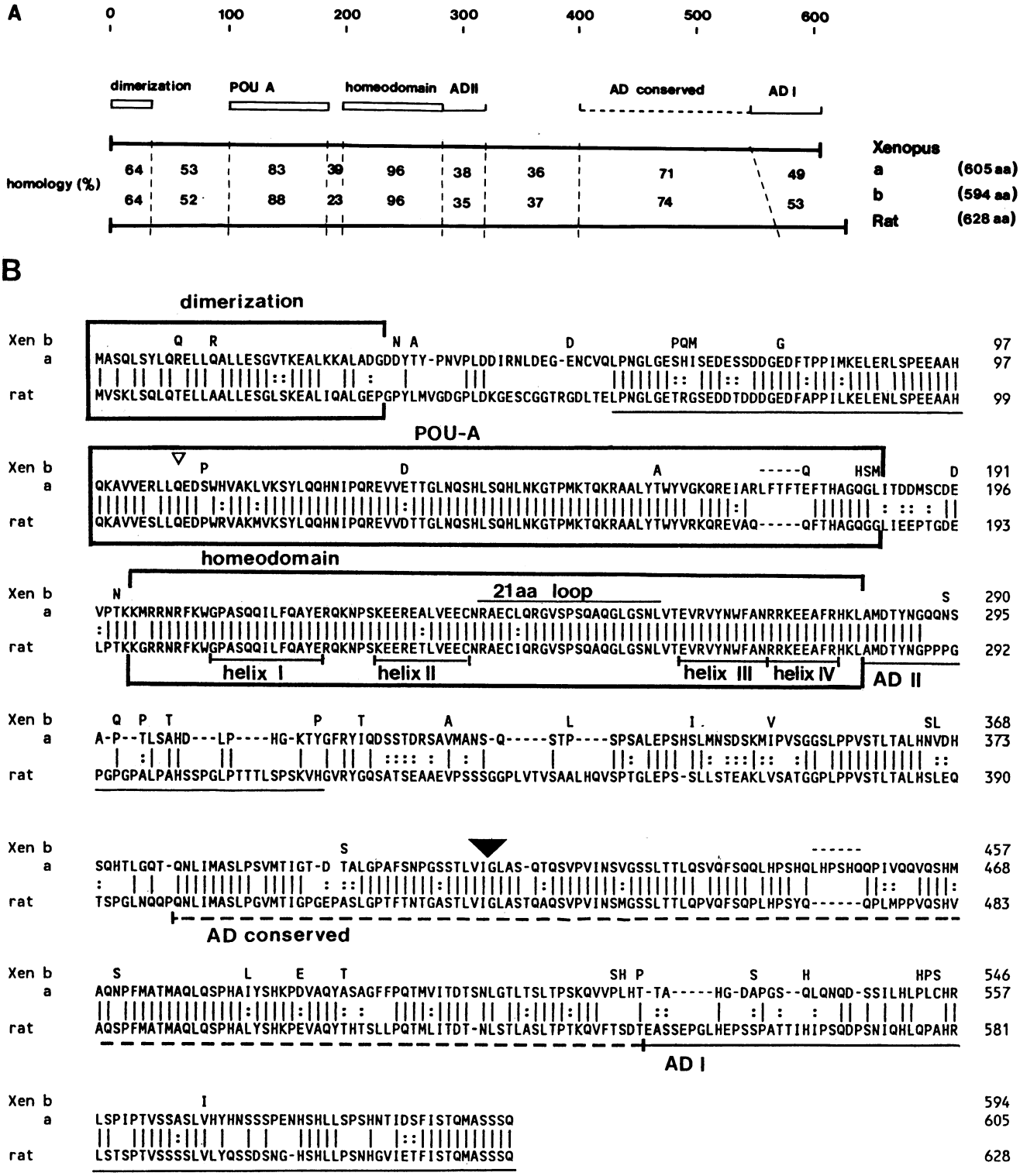

FIG. 2. Sequence homologies between the deduced amino acid sequences of XLFB1 and rat LFB1. The highly conserved regions corresponding to the dimerization domain (aa 1 to 33), the POU-A-related domain (aa 100 to 184), and the homeodomain (aa 198 to 281), as defined previously (32), are indicated in panel A and boxed in panel B. (A) Schematic representation of the homologous regions between XLFB1 and rat LFB1. The homologies, including conservative changes, between particular regions of the *Xenopus* (Xen) a or b sequence and the rat sequence are given as percentages. (B) Alignment of the deduced amino acid sequences of XLFB1a, XLFB1b, and rat LFB1. Gaps were added to optimize the alignment. Vertical bars indicate identical amino acids between XLFB1a and rat LFB1, whereas a colon is placed between amino acids representing conservative exchanges. For the amino acid sequence of XLFB1b, only those amino acids that are different from the sequence of XLFB1a are given. Dashes indicate missing amino acids. In the homeodomain-related sequence, the presumed helices are indicated, as defined previously (37), and the 21-aa loop is marked (11, 12). The positions of the presumed introns found in the b1 and b2 cDNA clones are marked by open and closed triangles, respectively. The high homology preceding the POU-A-related domain is underlined. The activation domains (AD) I and II, as defined previously (32), as well as the conserved activation domain (AD conserved), as indicated by our data, are underlined.

II and helix III that distinguishes the homeodomain of the LFB1 proteins from the classical homeodomains (11, 32) and is identical between the *Xenopus* and *rat* sequences, with the exception of one conservative change of Ile to Leu (Fig. 2B). This high evolutionary conservation between amphibia and mammals strengthens the notion that this unique structural feature has an essential function.

The amino-terminal dimerization domain (aa 1 to 33) is less well conserved (64% homology), whereas a region rich in acidic amino acids (aa 58 to 97; underlined in Fig. 2B) preceding the POU-A-related domain is highly conserved (78% homology).

The C-terminal portion of the LFB1 protein, which confers transcriptional activation (see below), is characterized by richness in serine and threonine in both *Xenopus* and rat sequences but shows only an overall homology of 54%. The smaller size of the *Xenopus* LFB1 protein than of the mammalian LFB1 proteins reflects the absence of several amino acids in this C-terminal portion. A proline-rich region (AD II in Fig. 2) which is just downstream of the homeodomain and which has been proposed to function as a hinge structure (7) and to play an important role in transactivation by LFB1 in in vitro transcription systems (32) is absent in the *Xenopus* LFB1 protein. Other stretches of the C-terminal portion are well conserved, especially the region from aa 382 to 531, which shows high homology (71%) and is therefore defined as the conserved activation domain (AD conserved in Fig. 2).

Analysis of the cDNA clones (see above) suggested the presence of a protein related to LFB1a, which we refer to as LFB1b. The amino acid sequence deduced from the combined open reading frames of the b1 and b2 cDNA clones shows an overall homology of 90% to the amino acid sequence of the a protein. Therefore, the homologies to the various domains of rat LFB1 are similar for the *Xenopus* a and b proteins (Fig. 2A). Interestingly, the amino acid sequence deduced from the group b clones matches the rat LFB1 protein sequence better (Fig. 2A) because of the absence of the extra 5 aa LFTFT that are in the POU-A-related domain of XLFB1a (Fig. 2B) and the absence of 6 aa (LHPSHQ) in the activation domain of XLFB1b.

A comparison of the *Xenopus* cDNA sequences with those reported for the mammalian LFB1 cDNA clones reveals homologies mainly restricted to the protein coding sequences. However, it is noteworthy that the sequence TT TGGGGG is found within a 100- to 130-bp sequence in front of the *Xenopus* translation initiation codon (Fig. 1) and those in rats (12) and mice (26). Interestingly, a recent footprint analysis of the 5′ region of the mouse LFB1 (HNF1) gene revealed exactly at this position (A site in reference 25) a sequence encoding a bound protein whose identity and function are not known. As the sequence of this binding site has been conserved during evolution, we assume that it plays an essential role in transcriptional regulation.

XLFB1a and XLFB1b act as transcriptional activators. To prove that the cloned cDNAs code for transcription factors that mediate transcriptional activation dependent on LFB1 binding site HP1, we cotransfected expression vector XLFB1a/CMV, encoding *Xenopus* LFB1a, and reporter construct $(HP1)_3$-TATA-CAT, containing three LFB1 binding sites (41), into dedifferentiated hepatoma cell line C2 (Fig. 3A). The expression of XLFB1a clearly increased CAT activity about fivefold, whereas the activity of a reporter construct lacking HP1 was unaffected (data not shown). As a deletion clone retaining just the DNA binding domain (aa 1 to 293) was unable to transactivate the $(HP1)_3$-TATA-CAT reporter construct (Fig. 3B), we conclude that the C-terminal portion of the protein contains the activation domain.

To verify that the potential protein encoded by the cDNA b clones can also transactivate an HP1-containing reporter construct, we fused the b1 and b2 cDNAs in their overlapping region and inserted this construct into expression vector Rc/CMV. Thus, we generated an open reading frame encoding the dimerization, DNA binding, and potential activation domains interrupted by the intron region. It is clear that XLFB1b was capable of conferring transactivation to the $(HP1)_3$-TATA-CAT reporter construct (Fig. 3C), as was XLFB1a (Fig. 3A). As it was not clear whether the intron present in the activation domain was spliced out upon transfection and thus whether the protein coding sequence downstream of the intron was translated, we transfected a construct lacking the sequence downstream of the intron. As shown in the CAT assay of Fig. 3D, this truncated clone was fully functional, establishing that the amino-terminal portion of the activation domain in XLFB1b (aa 280 to 410; Fig. 2B) is sufficient for transcriptional activation.

Characterization of monoclonal antibodies against XLFB1. Although LFB1 originally was thought to be a liver-specific factor, recent data indicate that in mammals, LFB1 transcripts are also present in other tissues, such as kidney, small intestine, and stomach (4, 26, 28, 49). Since the occurrence of LFB1 mRNA does not prove the presence of the transcription factor that mediates the function, we decided to look directly for the XLFB1 protein in different *Xenopus* tissues. Transcription factor LFB1 can be identified on the basis of its sequence-specific binding to HP1 (18, 24). However, this assay is not specific enough, as LFB3 (3, 10, 31, 37) and some other less well characterized proteins also bind to HP1 (10). Therefore, we prepared monoclonal antibodies against a fusion protein containing XLFB1 linked to the C terminus of the glutathione-*S*-transferase. Because in mammals the part of LFB1 that is most distinct from the related factor LFB3 is the activation domain, we used the fragment from aa 293 to 605 of XLFB1a in the fusion protein (Fig. 2B).

By using gel retardation assays with labeled HP1, i.e., the LFB1 binding site, as a probe, we selected six monoclonal antibodies for further analysis (Table 1). Five monoclonal antibodies, XAD1 to XAD5, were able to upshift the LFB1-HP1 complex generated by the *Xenopus* liver extract but failed to react with the corresponding complex formed by rat liver nuclear proteins (Fig. 4A, lanes 3 and 6 for XAD1; data for other monoclonal antibodies are not shown). In contrast, monoclonal antibody RAD1 was able to upshift the LFB1-HP1 complex generated by the *Xenopus* liver extract as well as by rat liver nuclear proteins (Fig. 4A, lanes 2 and 5), thus recognizing an epitope that has been conserved during evolution between *X. laevis* and the rat. To prove further the specificity of the monoclonal antibodies, we analyzed translation products made in reticulocyte lysates in vitro. Figure 4C illustrates that an HP1-containing complex was only detected when the translation system was programmed with XLFB1 mRNA a (compare lanes 1 and 9). The LFB1-HP1 complex could be upshifted by monoclonal antibody XAD1 (Fig. 4C, lane 2). Sequence-specific binding was proven by adding a 100-fold excess of unlabeled HP1, which inhibited the radioactive complex efficiently by competition (Fig. 4C, lane 3), whereas the same amount of an unrelated oligonucleotide did not interfere with binding (lane 4). Similar results were obtained for all other monoclonal antibodies (Table 1). In addition, we analyzed the in vitro translation product made by XLFB1 mRNA b. Since this RNA encodes

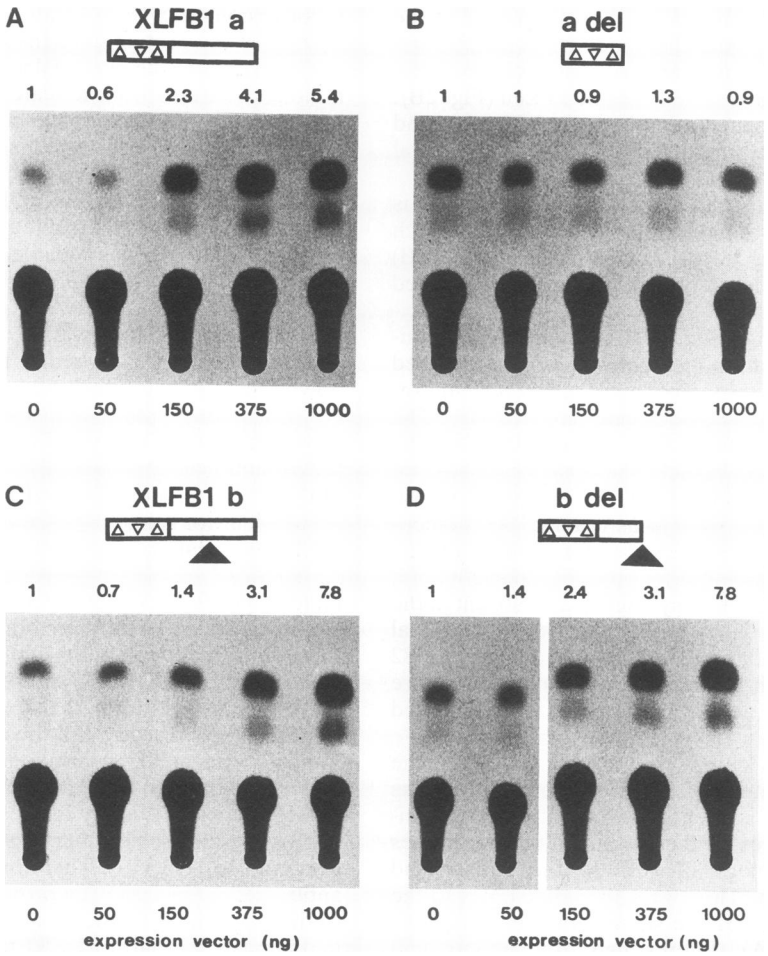

FIG. 3. Transcriptional activation by XLFB1a and XLFB1b in C2 cells. CAT assays were performed with extracts from C2 cells transfected with increasing amounts of expression vector Rc/CMV containing sequences from either XLFB1a or XLFB1b cDNA. The amount of DNA transfected was maintained constant by the addition of Rc/CMV. The reporter construct $(HP1)_3$-TATA-CAT, containing three copies of LFB1 binding site HP1, was used (41). The amounts of expression vector containing XLFB1 are given in nanograms, and above each panel is indicated the fold induction compared with that obtained by transfection with expression vector Rc/CMV alone. Schematic representations illustrate the different constructs. The DNA binding region is marked by open triangles, and the intron region in the activation domain of the b cDNA clones is marked by a closed triangle. The different expression vector constructs were as follows: A, the whole sequence of XLFB1a cDNA cloned into the Rc/CMV expression vector; B, a deletion of the C-terminal portion of the XLFB1a cDNA sequence (the remaining sequence contains aa 1 to 293); C, the XLFB1b cDNA derived from the fusion of the b1 and b2 cDNA clones and containing the intron region in the activation domain; and D, a deletion of the C-terminal portion of the XLFB1b cDNA sequence downstream of the intron (the remaining sequence contains aa 1 to 410).

only aa 1 to 410 of the open reading frame of the b protein (Fig. 2B), the complex detected in the gel retardation assay had a higher mobility (Fig. 4C, lane 5). The complex was upshifted by monoclonal antibody XAD1, and the complex showed sequence-specific binding (Fig. 4C, lanes 6 to 8). Using the other monoclonal antibodies, we observed that, except for XAD2, they failed to upshift the expression of the b protein (Table 1). We conclude that the epitopes recognized by XAD1 and XAD2 are amino terminal of aa 410 of the b protein. Since the a and b proteins are very similar (Fig. 2), it is unlikely that some of the antibodies are specific for the a protein. We rather assume that the antibodies that do not react with the b protein recognize epitopes carboxy terminal of aa 410. As the available cDNA does contain an intron at this position, a full-length b protein cannot be made easily in vitro to prove this assumption. We analyzed whether monoclonal antibody RAD1 cross-reacts with LFB1-related LFB3 (3, 10, 31, 37) in the dedifferentiated C2

TABLE 1. Properties of monoclonal antibodies raised against XLFB1

Antibody	Reactivity with LFB1[a]			
	In vivo[b]		In vitro[c]	
	X. laevis	Rat	a (aa 1 to 605)	b (aa 1 to 410)
XAD1	+	−	+	+
XAD2	+	−	+	+
XAD3	+	−	+	−
XAD4	+	−	+	−
XAD5	+	−	+	−
RAD1	+	+	+	−

[a] Reported as the presence (+) or the absence (−) of an upshift in the gel retardation assay.
[b] *Xenopus* liver extract or rat liver nuclear proteins used in gel retardation assays as described in the legend to Fig. 4A.
[c] Translation products made from mRNA a or mRNA b encoding aa 1 to 605 or aa 1 to 410 of the corresponding proteins, respectively.

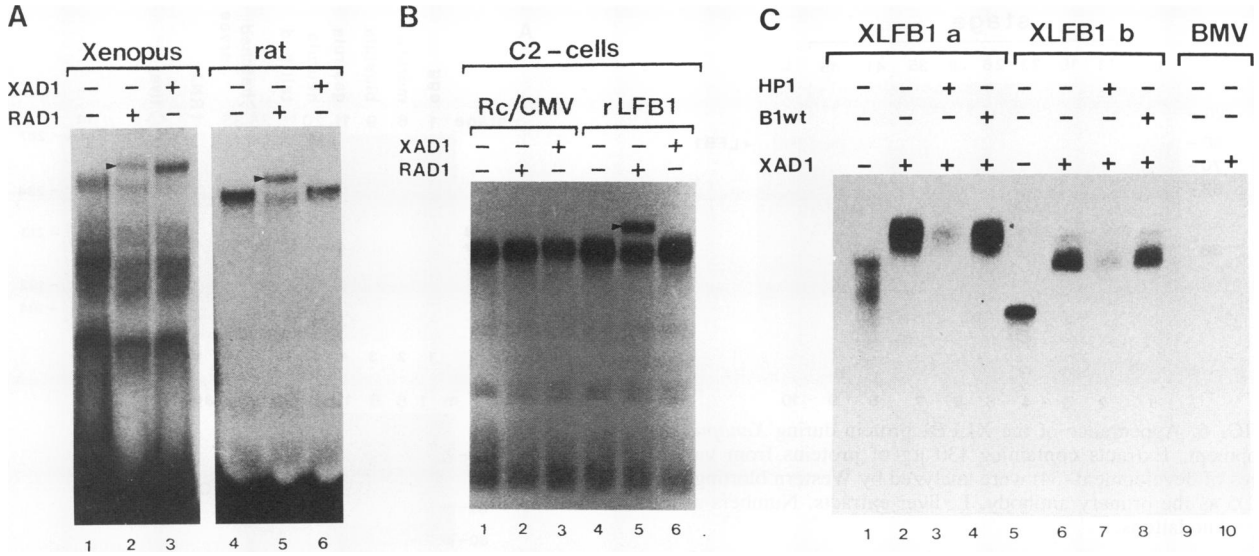

FIG. 4. Characterization of monoclonal antibodies specific for XLFB1. (A) Total *Xenopus* liver extract (lanes 1 to 3) or rat liver proteins from purified nuclei (lanes 4 to 6) were analyzed by gel retardation assays with labeled HP1 (18, 41). Monoclonal antibodies were added as indicated, and the upshifted complexes are marked by arrowheads. (B) Nuclear extracts from the dedifferentiated hepatoma cell line C2 transfected with either expression vector Rc/CMV without an insert (lanes 1 to 3) or an expression vector encoding rat LFB1 (45) (lanes 4 to 6) were analyzed by gel retardation assays with the indicated monoclonal antibodies. The free labeled HP1 oligonucleotide has left the bottom of the gel. (C) Reticulocyte lysates containing the XLFB1 a protein (lanes 1 to 4), the corresponding b protein (lanes 5 to 8), or the translation products encoded by Brome mosaic virus RNA (lanes 9 and 10) were incubated with labeled HP1. Monoclonal antibody XAD1 and an excess of unlabeled HP1 or B1wt (23) were included as indicated. The free labeled HP1 probe has left the bottom of the gel.

rat hepatoma cell line, which contains LFB3 but not LFB1 (31). It is clear that the LFB3 complex formed with HP1 was not upshifted by the antibodies (Fig. 4B, lanes 1 and 2). However, in extracts derived from C2 cells transfected with an expression vector encoding rat LFB1, monoclonal antibody RAD1 upshifted the expression of the HP1-LFB1 complex (Fig. 4B, lane 5), which comigrated with the HP1-LFB3 complex in the absence of the antibody (lane 4). As expected, the *Xenopus*-specific antibody XAD1 did not react with any of these rat proteins (Fig. 4B, lanes 3 and 6). This control experiment further documented the specificity of monoclonal antibody RAD1, as an upshift of the expression of the HP1-containing complex was absolutely dependent on the addition of LFB1. Similar experiments could not be done with *Xenopus*-specific antibodies XAD1 to XAD5, since we do not know whether a *Xenopus* LFB3 homolog exists.

Tissue-specific expression of XLFB1. To analyze the tissue distribution of XLFB1, we used the characterized monoclonal antibodies for the analysis of extracts derived from various adult *Xenopus* tissues. As illustrated in Fig. 5, a protein of 85 kDa could be detected on Western blots with monoclonal antibody XAD5 in liver, stomach, small intestine, colon, and kidney samples, whereas all the extracts derived from the other tissues analyzed, i.e., spleen, lung, blood, heart muscle, skeletal muscle, testis, and brain, yielded no signal. Thus, we conclude that the XLFB1 protein is not restricted to the liver but is also present in the stomach, small intestine, colon, and kidney. The same conclusion was drawn from band shift assays with HP1 as a DNA probe and the monoclonal antibodies (data not shown).

Temporal and spatial expression of XLFB1 during *Xenopus* embryogenesis. Assuming that the tissue-specific transcription factor XLFB1 might be involved in the differentiation of specific cell types, we wondered at what time and in what part of the *Xenopus* embryo XLFB1 can be found. We prepared extracts from various stages of developing embryos and analyzed them in a Western blot with monoclonal antibody XAD5, specific for XLFB1. Figure 6 demonstrates that a protein of 85 kDa could be detected with monoclonal antibody XAD5 at stage 35 (2-day-old hatched larvae; lane 7) and that the abundance of this protein increased to stage 45, on the fifth day of development (lane 9). The major band was identical in size to the one found in liver extracts (lanes 1 and 10). These data prove the developmental appearance of XLFB1 in 2-day-old hatched larvae. To localize XLFB1 in

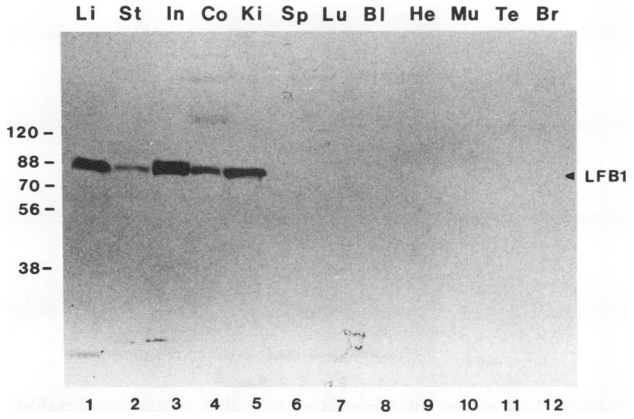

FIG. 5. Western blotting to detect the XLFB1 protein in different *Xenopus* tissues. Aliquots (25 μl) from cleared extracts of liver (Li), stomach (St), small intestine (In), colon (Co), kidney (Ki), spleen (Sp), lung (Lu), blood (Bl), heart muscle (He), skeletal muscle (Mu), testis (Te), and brain (Br) were separated on an SDS gel, and the Western blot was probed with XAD5 as the primary antibody. Numbers at left are in kilodaltons.

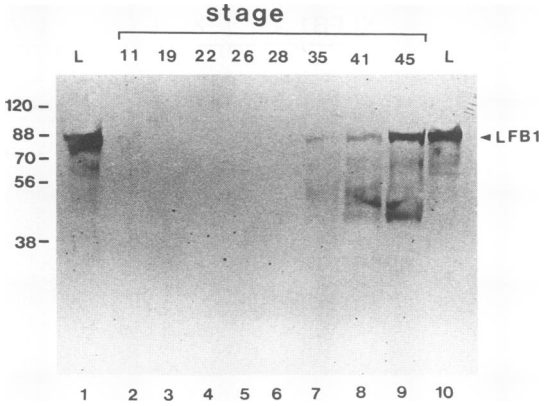

FIG. 6. Appearance of the XLFB1 protein during *Xenopus* development. Extracts containing 130 μg of proteins from various stages of development (34) were analyzed by Western blotting with XAD5 as the primary antibody. L, liver extracts. Numbers at left are in kilodaltons.

the embryo, we analyzed extracts from dissected hatched larvae (stage 35) on Western blots. Figure 7 demonstrates that XLFB1 could be detected in the anterior and posterior middle portions by monoclonal antibody XAD5 but that it was absent in the head and tail. This restricted appearance of XLFB1 in the embryo was also observed in gel retardation assays with the monoclonal antibodies (data not shown).

Since the appearance of transcription factor XLFB1 during embryogenesis may not directly reflect the transcriptional activation of the corresponding gene, we analyzed RNA samples derived from various stages of embryogenesis by using a probe specific for XLFB1a mRNA in RNase protection experiments. While we did not detect any maternal XLFB1a RNA in unfertilized eggs, the first embryonic expression of XLFB1a transcripts was evident shortly after the midblastula transition at the gastrula stage (Fig. 8A, lane

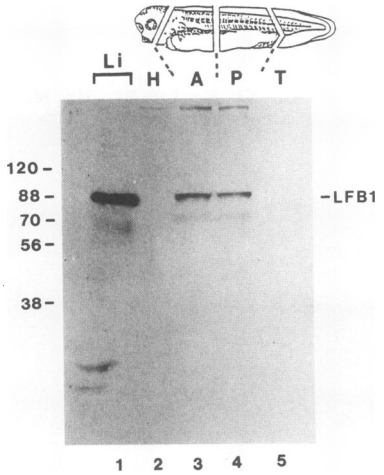

FIG. 7. Western blotting to detect the spatial expression of XLFB1 in *Xenopus* embryos. Two-day-old *Xenopus* hatched larvae (stage 35) were dissected into four parts, as shown, and extracts derived from the head (H), anterior middle portion (A), posterior middle portion (P), and tail (T), containing 60 μg of protein, were analyzed by Western blotting with XAD5 as the primary antibody. An extract made from adult *Xenopus* liver was used as a control (Li). Numbers at left are in kilodaltons.

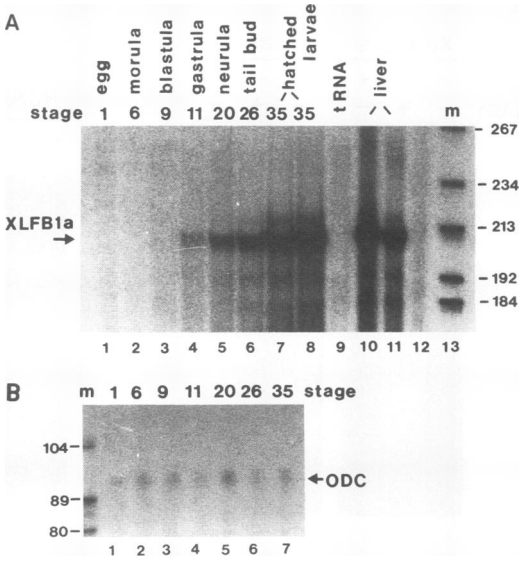

FIG. 8. RNase protection analysis to detect XLFB1a mRNA during *Xenopus* embryogenesis. (A) RNase protection analysis with 100 μg of total RNA from whole embryos at the indicated developmental stages (34) was performed with a 206-bp fragment of XLFB1a cDNA (lanes 1 to 8). As a control, tRNA and total RNA from adult *Xenopus* liver were used (lane 9 and lanes 10 and 11, respectively). A molecular mass marker (m) was loaded in lane 13 (numbers are in nucleotides). (B) RNase protection analysis with 10 μg of the same RNA preparation as that used in panel A but carried out with a 91-bp probe from the housekeeping gene ornithine decarboxylase (ODC) (lanes 1 to 7). m is as defined for panel A.

4). The abundance of the XLFB1a mRNA increased thereafter by a factor of about 10 up to the stage of 2-day-old hatched larvae, reaching a level comparable to that observed for frog liver RNA. As expected, the amount of ornithine decarboxylase mRNA, known to remain constant throughout *Xenopus* embryogenesis (43), did not vary significantly in the various embryonic RNA samples (Fig. 8B).

DISCUSSION

Evolutionary conservation of transcription factor LFB1. Using a rat LFB1 cDNA probe, we isolated *Xenopus* cDNA clones derived from two related genes. The finding of two closely related genes in *X. laevis* is not surprising, as most genes identified in this species so far have been found to be duplicated (e.g., vitellogenin genes [47] and albumin genes [48]), possibly because of a genome duplication that occurred during the evolution of the *Xenopus* genus (21).

On the basis of the sequence comparison, it is clear that both the XLFB1a and the XLFB1b genes are distinct from mammalian LFB1-related LFB3, identified recently as a related transcription factor in mammals (3, 10, 31, 37). The overall homology between the *Xenopus* amino acid sequence and rat LFB3 is only 48%, compared with 64% for LFB1. In addition, activation domain I, which is absent in LFB3, is partially conserved in the *Xenopus* sequence. However, a common feature of the *Xenopus* XLFB1a and XLFB1b proteins and LFB3 in mammals is the absence of proline-rich activation domain II (32), which is characteristic for mammalian LFB1. Further support that we have cloned a *Xenopus* LFB1 homolog was provided by monoclonal antibody RAD1, which was derived from an immunization with the activation domain of the *Xenopus* protein, since this anti-

body reacts with rat LFB1 but not LFB3 (Fig. 4B). In addition, the cross-reaction of RAD1 with the rat and human proteins (Hep-G2 cells; data not shown) further documented the conservation of LFB1 during vertebrate evolution.

A sequence comparison between the rat and *Xenopus* proteins identified two highly conserved regions, both involved in DNA binding, i.e., the homeodomain and POU-A-related domain (Fig. 2). This high conservation is consistent with our previous observation that LFB1 binding site HP1 in the *Xenopus* albumin promoter is fully functional in mammalian systems (41).

A relatively high homology of 64% was also found in the N-terminal dimerization domain, which constitutes another typical structure of the LFB1 transcription factor. Recent studies of the dimerization domain of rat LFB1 have shown that leucine residues 5, 16, and 26 are necessary to stabilize the dimer, whereas terminal positions of the dimerization domain and leucine residue 21 are less important (35). These results are in agreement with the conservation of leucine residues 5, 16, and 26 between *X. laevis* and the rat, whereas 21 is absent from *X. laevis*.

In addition to the three conserved domains involved in dimerization and DNA binding, we identified another stretch of high homology just preceding the POU-A-related domain (aa 58 to 97; underlined in Fig. 2B) rich in acidic amino acids. The functional importance of this segment is not clear, as deletion of this sequence in rat LFB1 did not alter DNA binding (32). Transactivation experiments established that the cloned *Xenopus* XLFB1a factor stimulates a reporter construct containing HP1 (Fig. 3). This activation requires the C-terminal portion of XLFB1a, as a truncated protein (aa 1 to 293) is not functional. These results agree with those of similar experiments done with rat LFB1 (10, 32, 36). With an in vitro transcription system, the rat LFB1 activation region has been subdivided into two functional domains (Fig. 2), i.e., activation domains I and II (32). However, the highest homology between the rat and *Xenopus* proteins is found between activation domains I and II. This region, which extends from aa 382 to 531 and which we define as the conserved activation domain (AD conserved in Fig. 2), is also highly conserved between rats and humans (2) and contains the essential portion of the activation domain of rat LFB1 (36). On the basis of our data, we assume that the C-terminal portion of the conserved activation domain is not absolutely required for transactivation, as the C-terminal deletion clone of XLFB1b (aa 1 to 410) has a transactivation potential similar to that of XLFB1a (Fig. 3). However, a more detailed dissection of the activation domain, with special emphasis on the evolutionarily conserved segments, is required.

Transcription factor XLFB1 has a broader tissue distribution than originally assumed. Using monoclonal antibodies, we demonstrated that XLFB1 is present not only in the liver but also in the stomach, small intestine, colon, and kidney (Fig. 5). Recent experiments with mammalian systems demonstrated the presence of the LFB1 protein in the kidney (5, 10, 31) and, on the RNA level, LFB1 transcripts have also been detected in the stomach (4). Therefore, the tissue distribution of LFB1 has been maintained during vertebrate evolution and involves tissues of endodermal as well as mesodermal origin. However, we do not know what target genes are activated in nonhepatic tissues and what cell types besides hepatocytes contain LFB1 (compare references 4, 5, and 28).

Our data revealed that in *X. laevis*, LFB1 is encoded by the XLFB1a and XLFB1b genes. As we have not found group b cDNA clones encoding the entire open reading frame for XLFB1b but rather have found two fragments, both of which contain potential intron sequences with in-frame stop codons, the existence of the b protein is not yet established. Assuming that the b cDNA clones are splicing variants rather than splicing intermediates, both would encode truncated proteins. The translational product of the b1 cDNA clone would represent just the dimerization domain (Fig. 2B). As a synthetic peptide containing the rat dimerization domain can form heterodimers with LFB1 in vitro (32), a truncated protein containing the dimerization domain might interfere with the full-length transcription factor in vivo and thus modulate its activity. The protein made from the b2 cDNA clone would contain a truncated activation domain. Our transfection data imply that this truncated transcription factor (aa 1 to 410) has a transactivation potential similar to that of the full-length protein (compare Fig. 3A and D). However, it is possible that the truncated factor transactivates differentially when a more complex promoter is used in the reporter construct.

Expression of XLFB1 during early development in *X. laevis*. We detected LFB1 transcripts shortly after the midblastula transition (Fig. 8), a result indicating that LFB1 is encoded by the earliest expressed genes in the embryo. When we searched for the XLFB1 protein, we detected it no earlier than in the 2-day-old hatched larvae (Fig. 6), in which organogenesis is ongoing. This apparent delay between protein and mRNA expression may have been due to the fact that the methods used to detect the protein are considerably less sensitive. The amounts of XLFB1 transcripts accumulating between the gastrula and hatched-larvae stages reached levels comparable to those found in the adult liver. As the transcripts were not evenly distributed in the embryo (data not shown), we assume that certain areas in the middle portion of the embryo, in which the highest levels can be found, have LFB1 mRNA levels considerably higher than those found in hepatocytes. This idea would be consistent with the notion that a higher level of XLFB1 may be required to trigger the activation of LFB1-dependent promoters during embryogenesis, compared with the level required for the maintenance of ongoing LFB1-dependent transcription in hepatocytes. A similar observation has been made for the embryonic expression of muscle-specific transcription factor MyoD (16, 38).

When the distribution of the XLFB1 protein in dissected embryos was analyzed, the factor was only detected in the middle portion of the embryo, in which the liver diverticulum, stomach, gut, and pronephros (kidney anlagen) are localized (Fig. 7). This result suggests that XLFB1 plays a role early during organogenesis in the tissues in which it is also present in the adult.

In conclusion, our data provide the first evidence that a gene coding for a transcription factor known to be involved in liver-specific gene control is activated very early during development prior to organogenesis. We speculate that this tissue-specific transcription factor triggers the determination and/or differentiation of specific cell types. With the amphibian system, it should now be possible to define the inducing factors and the signal transduction pathways leading to the embryonic appearance of this tissue-specific transcription factor.

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