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Elements and Factors Involved in Tissue-Specific and Embryonic Expression of the Liver Transcription Factor LFB1 in *Xenopus laevis*

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LFB1 (HNF1) is a tissue-specific transcription factor found in the livers, stomachs, intestines, and kidneys of vertebrates. By analyzing the promoter of the *Xenopus* LFB1 gene, we identified potential autoregulation by LFB1 and regulation by HNF4, a transcription factor with a tissue distribution similar to that of LFB1. Injection of LFB1 promoter-chloramphenicol acetyltransferase constructs into *Xenopus* eggs revealed embryonic activation that is restricted to the region of the developing larvae expressing endogeneous LFB1. Proper embryonic activation was also observed with a rat LFB1 promoter. Deletion analysis of the *Xenopus* and rat promoters revealed that in both promoters embryonic activation is absolutely dependent on the presence of an element that contains CCNCTCTC as the core consensus sequence. Since this element is recognized by the maternal factor OZ-1 previously described by N. Ovsenek, A. M. Zorn, and P. A. Krieg (Development 115:649–655, 1992), we might have identified the main constituents of a hierarchy that leads via LFB1 to the activation of tissue-specific genes during embryogenesis.

Tissue-specific expression of genes is achieved to a large extent by the interaction of transcription factors with *cis*-acting elements present in the promoters and enhancers of genes that are expressed differently in different tissues. These transcription factors themselves are tissue specific. This restricted expression pattern is established sometime during embryogenesis, and we assume that it plays a key role in the differentiation processes. Molecular analysis of early vertebrate development has established a complex hierarchy of regulatory factors involved in establishing the body plan (for recent reviews, see references 12 and 15). Some of the genes encoding these factors are the homeobox genes known to be conserved between *Drosophila melanogaster* and mammals (for a recent review, see reference 26). There is increasing evidence that these genes form a very complex regulatory network in which specific genes influence the activities of other genes. Typically these regulatory genes are expressed in distinct areas of the embryo and may be active only transiently during embryogenesis. There is, at least in the case of *D. melanogaster*, clear evidence that some transcription factors are already present in fertilized eggs, and it is assumed that these maternal factors are responsible for initiating gene activation in early embryogenesis (for a recent review, see reference 16). So far no direct link between the early active transcription factors and the expression of tissue-specific transcription factors could be made. In one approach to this question, we analyzed the regulatory elements and factors involved in embryonic activation of the promoter of the liver transcription factor LFB1 (HNF1) in *Xenopus laevis*.

Previous data had shown that LFB1 plays a major role in the expression of genes specifically transcribed in hepatocytes. Support for this conclusion comes from the observations that a functional LFB1-binding site (HP1) is present in the promoters of several liver-specific genes (9, 14, 21) and that the binding site HP1 is sufficient to direct liver-specific transcription in vitro (24, 32, 38). In addition, HP1 is the only regulatory element which has been conserved during evolution in the albumin promoters of mammals and *X. laevis* (39). Furthermore, the presence of LFB1 is positively correlated with the differentiation state of hepatoma cells (5). Recent data have shown that LFB1 is not restricted to the liver but can also be found in the kidney, stomach, colon, and small intestine (2, 3, 10, 28). However, in these nonhepatic tissues, the target genes have not been identified yet.

By sequencing cloned *Xenopus* LFB1 cDNAs, we could show that LFB1 is encoded by two genes (XLFB1a and XLFB1b) and that the structures of transcription factor LFB1 in *X. laevis* and in mammals are very similar (2). Using a cloned cDNA as a hybridization probe, we established that LFB1 gene transcripts appear shortly after mid-blastula transition. Thus, the gene encoding LFB1 is expressed early in embryogenesis. Localizing LFB1 protein in the hatched *Xenopus* larvae, we could show that LFB1 is restricted to the middle sections, where the organs containing LFB1 in the adult animals are present (2), indicating early tissue-specific expression of LFB1 in the embryo. Concerning regulation of LFB1 in mammals, functional analysis of the LFB1 promoter has recently established that HNF4, another liver transcription factor, can activate the LFB1 promoter (22, 45).

In this report, we describe the functional properties of the LFB1 promoter of *X. laevis* and define the elements involved in embryonic activation of the LFB1 promoter by microinjection of reporter constructs into fertilized *Xenopus* eggs. Most notably, the mammalian (rat) LFB1 promoter can also be properly regulated in developing amphibian embryos.

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MATERIALS AND METHODS

Plasmid constructions and sequencing. The *Xenopus* LFB1 promoter constructs AB (positions −886 to −46), A (positions −886 to −489), and B (positions −494 to −46) were cloned in the vector pCAT-Enhancer (Promega). For the deletion constructs, the *Xenopus* LFB1 promoter was subcloned into the *Bam*HI and *Hin*dIII sites of pEU-CAT (36), using the *Sau*3AI site at position −743 and a *Hin*dIII linker added at position −57 generated by limited *Bal* 31 digestion. Additional 5′ or 3′ deletions were made by *Bal* 31 digestion and addition of a *Bam*HI or *Hin*dIII linker, respectively (18). The exact border of the deletion was determined by sequencing. The rat LFB1 promoter construct was derived by insertion of the sequence from positions −1536 (*Pst*I) to −133 (*Hin*dIII linker after *Bal* 31) from the clone pHNF1-CAT/-2kb (kindly provided by Tian and Schibler [45]) into the *Pst*I and *Hin*dIII sites of pEU-CAT. The 5′ deletions were essentially made as described above for the *Xenopus* promoter. The LFB1 expression vector (donated by B. Denecke) was generated by insertion of the *Hin*cII-*Bam*HI fragment of RSVB1 (46) (kindly provided by G. Ciliberto) into Rc/CMV (Invitrogen). The HNF4 expression vector was constructed by digesting the HNF4 cDNA construct pf7 (42) with *Apa*I and *Not*I and inserting the HNF4 cDNA into the expression vector Rc/CMV. The HNF3 expression vector (provided by T. Drewes) was constructed by digesting the HNF-3A cDNA construct pLH3 (23) with *Bam*HI. The 1.6-kb insert was subcloned in the vector pBluescript II SK$^+$, and the *Hin*dIII and *Xba*I sites of this vector were used to insert the HNF3 cDNA into the expression vector Rc/CMV. The cDNA clones pf7 and pLH3 were generously provided by J. E. Darnell.

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.

Extract preparation. *Xenopus* liver extract and nuclear extracts from rat livers were prepared as described previously (2). Embryo extracts were obtained as described by Ovsenek et al. (34).

Oligonucleotide synthesis. Oligonucleotides were synthesized as complementary strands to give double-stranded sequences with *Hin*dIII, *Bgl*II, or *Bam*HI overhangs upon annealing. The double-stranded sequences including the overhangs are as follows: HP1, the LFB1-binding site of the *Xenopus* albumin promoter (39), 5′-AGCTTGAGGTTAA TAATTTTCCAGATC-3′; H1/3, the *Xenopus* LFB1 promoter (see Fig. 1), 5′-GATCCAGGTTACTGTGTATTTG TTTTGGTATTAACAGATC-3′; H4, HNF4-binding site of the human α1-antitrypsin promoter (41) 5′-AGCTTAGCA AACAGGGGCTAAGTCCACTGGCTGGATC-3′; b1wt, B-activator binding site of the *Xenopus* vitellogenin B1 promoter (20), 5′-AGCTTGGTGCACATGCGCCAGATC-3′; ERE, the estrogen-responsive element of the *Xenopus* A2 vitellogenin gene (18), 5′-GATCCGTCAGGTCACAGTGA CCTGATGGATC-3′; and OZ-Xen., the *Xenopus* LFB1 promoter (Fig. 1), 5′-GATCTGTATCTCTCTTCCCCTCTCTG GTTTGGATC-3′. For the H4 oligonucleotide representing the HNF4 site of the *Xenopus* LFB1 promoter, the complementary strands were 5′-GATCCACTTTGTGGGGTCCAA AGTTCAGTAA-3′ and 5′-CAAGTTACTGAACTTTGGAC CCCACAAAGTG-3′, whereas the OZ element of the neural cell adhesion molecule (N-CAM) promoter (34) was blunt ended with 5′-GTTTTGTGTCCCCCTCTCAGGTGT-3′ as the upper strand.

Baculovirus expression and purification of recombinant XLFB1 protein. To construct an N-terminally His-tagged cDNA encoding XLFB1, the expression vector Rc/CMV-XLFB1a1 (2) was cut with *Hin*dIII, treated with Klenow polymerase, and recut with *Xba*I. The cDNA insert containing the entire open reading frame was cloned between the *Sma*I and *Xba*I sites of MH_6-A/B-NT. MH_6-A/B-NT is a pBluescript II SK$^+$ derivative containing an *Nhe*I site followed by sequences encoding the peptide MSHHHHHH and restriction sites for *Spe*I, *Bam*HI, *Sma*I, *Xba*I, *Eco*RI, and *Nhe*I, allowing insertion of coding sequences. The His-tagged cDNA was cloned as an *Nhe*I fragment into the *Nhe*I site of the baculovirus transfer vector pBlueBac (Invitrogen). Because of the presence of 5′ untranslated and linker sequences, the His-tagged protein contains another 11 amino acids (TSGSPSLATER) preceding the authentic Met codon.

Recombinant baculoviruses were generated by cotransfection of Sf9 cells with the transfer vector and linear viral DNA (BaculoGold linearized) as described by the supplier (Pharmingen). Recombinant viruses directing the expression of XLFB1 were identified by infection of Sf21 cells and subsequent Western blotting (immunoblotting) of whole-cell lysates with monoclonal antibody XAD5 (2). For large-scale production, Sf21 cells were grown as suspension cultures in Grace's insect medium supplemented with yeastolate (3.62 g/liter), lactalalbumin hydrolysate (3.62 g/liter), 0.05% Pluronic F68 (GIBCO BRL) and 10% fetal calf serum. About 5×10^8 Sf21 cells were collected by centrifugation, resuspended in 50 ml of medium lacking fetal calf serum and Pluronic F68, and infected at a multiplicity of infection of 10. After 1 h, cells were diluted to 1.5×10^6 cells per ml with complete medium lacking Pluronic F68 only.

Cells were harvested by centrifugation 44 h after infection, washed two times with phosphate-buffered saline, resuspended in two times the original packed cell volume of buffer I (10 mM HEPES-KOH [pH 7.9], 15 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol [DTT], 5 mM NaF), and incubated for 5 min on ice. After centrifugation (5 min, 8,000 × *g*, 4°C), the supernatant was saved, adjusted to 20 mM HEPES-KOH (pH 7.9), 200 mM NaCl, 5% (vol/vol) glycerol, 0.2 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 0.8 mM imidazole-HCl (pH 7.0), and stored on ice. The pellet was resuspended in 2 packed cell volumes of buffer II (20 mM HEPES-KOH [pH 7.9], 10% [vol/vol] glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EGTA, 2 mM DTT, 5 mM NaF) and cells were broken in a Dounce homogenizer. After 20 min on ice, insoluble material was removed by centrifugation (1 h, 200,000 × *g*, 4°C). The soluble fraction was adjusted to 0.8 mM imidazole-HCl (pH 7.0), combined with the first supernatant, and incubated with 1-ml bed volume of Ni^{2+}-nitrilotriacetic acid-agarose (Diagen) for 1 h at 4°C. The gel was washed three times with buffer III (20 mM HEPES-KOH [pH 7.9], 100 mM KCl, 5% [vol/vol] glycerol, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 5 mM NaF, 2 mM DTT, 0.8 mM imidazole-HCl [pH 7.0]), transferred to a column, and washed with 3 bed volumes of buffer IV (20 mM HEPES-KOH [pH 7.9], 50 mM KCl, 5% [vol/vol] glycerol, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 2 mM DTT, 5 mM imidazole-HCl [pH 7.0]). Bound His-tagged XLFB1 was eluted with 3 bed volumes of buffer IV containing 80 mM imidazole-HCl (pH 7.0) and stored in aliquots at −80°C. To all buffers, protease inhibitors (0.1 mM 4-[2-aminoethyl]-benzolsulfonylfluoride [AEBSF], 0.1 mM benzamidine, 1 μg of leupeptin per ml, 1

μg of pepstatin A per ml, and 0.5 μg of aprotinin per ml) were added immediately prior to use.

Band shift assays. The conditions used for the LFB1 band shift assays (see Fig. 2) were defined previously (2, 20).

The OZ band shift was performed as described by Ovsenek et al. (34). The binding reactions contained one egg equivalent of whole-cell extract mixed with 200 ng of poly(dI-dC) and 200 ng of *Hin*fI-digested pBluescript II SK$^+$ in a solution of 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol.

Transient transfections and CAT assays. The dedifferentiated rat hepatoma cell line C2, grown in a 1:1 mixture of Dulbecco minimal essential medium and Ham F12 medium with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% fetal calf serum, was transfected with 5 μg of reporter plasmid and 200 ng of the expression vector by the calcium phosphate precipitation method (30). Protein (100 to 200 μg) was used for chloramphenicol acetyltransferase (CAT) assays (18).

Embryological methods. *Xenopus* embryos were obtained by artificial fertilization as previously described (1). Prior to injection, the embryos were dejellied in 2% cysteine-HCl (pH 8.0) and then washed in 25 mM NaCl. Embryos were injected in 1× NAM (normal amphibian medium [40]) containing 5% Ficoll at the one- or two-cell stage with 250 pg of plasmid DNA. The embryos were transferred to 1/12 NAM 1 h after injection. At stage 37/38 (33), the embryos were dissected as described in Results. The different parts of the embryos were homogenized in 0.25 M Tris (pH 7.8), and equal amounts of protein were used in the CAT assay (18).

Nucleotide sequence accession number. The nucleotide sequence of the *Xenopus* LFB1 promoter has been deposited in GenBank data bank under accession number L09605.

RESULTS

The LFB1 promoters of *X. laevis* and mammals are highly homologous. To analyze the regulation of the LFB1 gene in *X. laevis*, we isolated genomic clones encoding LFB1 (50). By sequencing we concluded that these clones contain the b gene including the 5′ end of the cloned cDNA XLFB1b (2). In Fig. 1, we present the sequence upstream of the ATG initiation codon of XLFB1b. To locate the transcription start sites, we performed RNase protection experiments with an antisense probe covering sequences upstream of the initiation codon. Using the antisense RNA from positions −46 to −494, we observed a protected fragment of 170 nucleotides (49), indicating the presence of a major transcription initiation site at position −216 upstream of the ATG codon (B start in Fig. 1). However, at least one additional transcription initiation site is present at a position further upstream, since substantial amounts of the antisense RNA gave a protected fragment corresponding to full protection of the genomic sequence. This is consistent with our previous cDNA cloning that resulted in the b1 cDNA clone that extends to position −509 upstream of the ATG codon (2).

By searching for known regulatory elements that might mediate liver-specific expression of LFB1, we identified a potential HNF4-binding site at about position −260, close to the major start site, and two binding sites for LFB1 further upstream at position −510 (Fig. 1).

Comparing the sequence of the *Xenopus* LFB1 promoter with those of the rat (45) and mouse (22) LFB1 promoters recently described, we observed a high similarity of about 80% over 102 bp in the region containing the B start site and the potential HNF4-binding site (underlined in Fig. 1). No further sequence similarities of the *Xenopus* promoter to the mouse and rat promoters were evident, including the 3,517-bp 5′-flanking sequence of the rat LFB1 promoter deposited by the group of R. Cortese in the EMBL data bank (accession no. X67649). An alignment of the *Xenopus* and mouse LFB1 promoter sequences, given in Fig. 2, reveals that at the 5′ end of the matched region the potential HNF4-binding site of the *Xenopus* sequence is identical to the central 14 bp of the C site of the mouse sequence known to contain a functional HNF4-binding site (22). The similarity between *Xenopus* and mouse sequences extends 3′ into the region where binding of HNF3 and AP1 in the mouse sequence has been described (22). Most notably, the potential LFB1-binding sites located at position −510 of the *X. laevis* promoter are not found in the mouse and rat promoters.

Identification of the binding sites for HNF4 and LFB1. Sequencing of the *Xenopus* promoter had revealed at position −260 a potential HNF4-binding site (Fig. 1). To prove that this sequence binds HNF4, we used a double-stranded oligonucleotide (H4) extending from positions −251 to −276 in a band shift assay with *Xenopus* liver extract. The formation of complexes was inhibited by H4 and by an oligonucleotide containing the HNF4-binding site present in the human α1-antitrypsin promoter (41), whereas no competition was observed with an unrelated oligonucleotide (data not shown). The binding of HNF4 is in agreement with the recent report for the mouse (22) and rat (45) promoter, where the corresponding sequence also binds HNF4 (Fig. 2).

To verify the potential LFB1-binding sites at position −510, we used *Xenopus* LFB1 protein expressed in the baculovirus system in band shift assays. The data given in Fig. 3 demonstrate that this recombinant protein generated a retarded complex (lane 1) with the HP1 oligonucleotide containing the LFB1-binding site and that this complex was supershifted (lane 2) by the monoclonal antibody XAD1 specifically recognizing LFB1 (2). The recombinant LFB1 displayed sequence-specific DNA binding, because it did not bind to the ERE (lanes 11 and 12). Using the labeled oligonucleotide H1/3, extending from positions −524 to −495 in the LFB1 promoter (Fig. 1), as the probe for binding to the recombinant LFB1, a retarded band was observed (lane 3) that was supershifted by the antibody XAD1 (lane 4). This proves that the sequence extending from positions −524 to −495 is able to bind LFB1. However, the binding affinity of LFB1 to H1/3 is lower than that of the well-characterized LFB1-binding site HP1 (20, 39), since H1/3 inhibited binding less efficiently than HP1 (lanes 5 to 8). The specificity of competition is documented by the addition of an unrelated oligonucleotide (ERE) that did not prevent the formation of complexes (lanes 9 and 10). Specific binding of LFB1 to H1/3 was also observed with either *Xenopus* or rat liver extracts (data not shown). Competition experiments with various oligonucleotides again confirmed that the LFB1-binding site found in the LFB1 promoter is a relatively weak binding site. All these data establish that the sequence from positions −524 to −495 of the LFB1 promoter contains a bona fide binding site for LFB1 although with an affinity lower than that of the well-characterized binding site HP1 (38, 39).

Distinct parts of the *Xenopus* LFB1 promoter mediate inducibility by HNF4 and LFB1. The presence of binding sites for HNF4 and LFB1 in the *Xenopus* promoter suggests that these transcription factors might influence the expression of the LFB1 gene. To analyze whether such a regulatory network exists, we constructed LFB1 promoter-CAT fusion

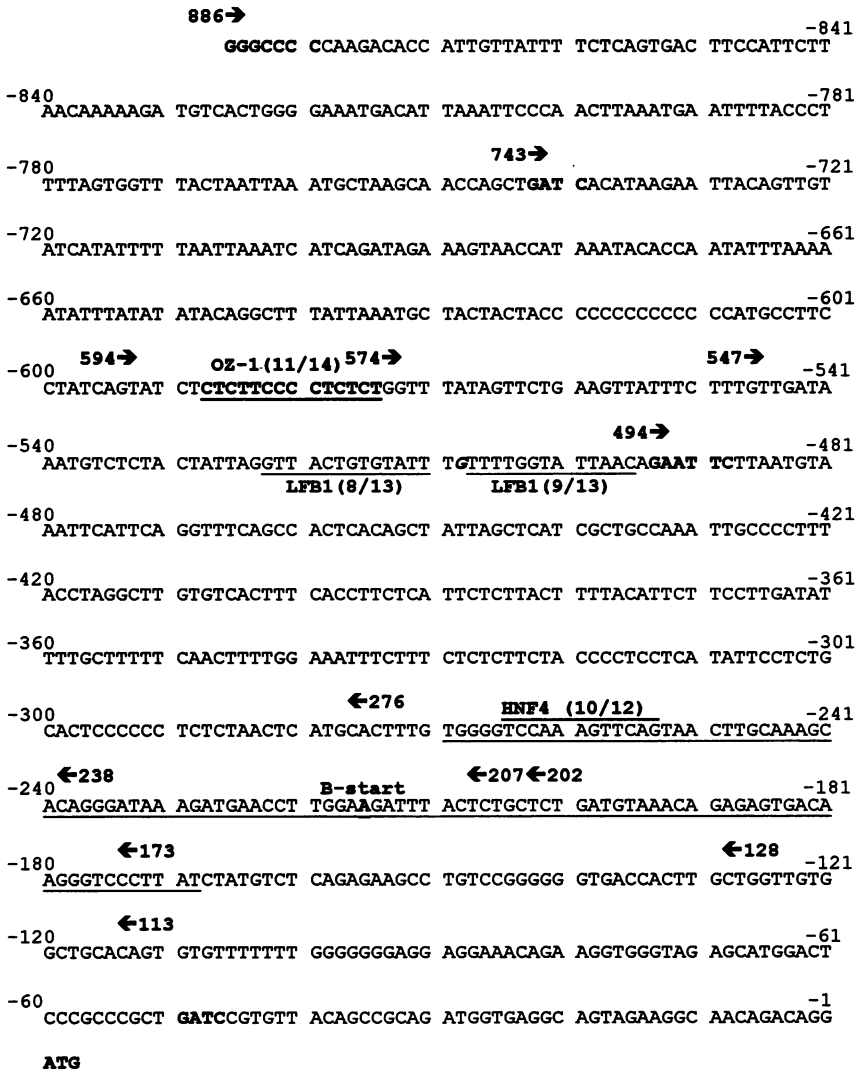

FIG. 1. *Xenopus* LFB1 promoter sequence. The potential binding sites for OZ-1 (34), LFB1 (27), and HNF4 (42) are marked, and the number of bases matching those of the consensus sequence is given (for example, 11/14 means that the *Xenopus* sequence matches the consensus sequence in 11 of 14 bp). The translation start codon (ATG) and the transcription start site (B start) are indicated. The region which is highly conserved between the *Xenopus* and mouse promoters is underlined (positions −169 to −270). The 5′ and 3′ borders of the deletion clones are marked by arrows. The restriction sites for *Dra*II (position −886), *Sau*3AI (positions −743 and −50), and *Eco*RI (position −494) are given in bold letters.

genes as reporter constructs for transfection experiments. For recipient cells, we chose the dedifferentiated C2 rat hepatoma cells known to lack the transcription factors HNF4 and LFB1 (5, 22). Using an LFB1 promoter-CAT reporter construct containing the sequence from positions −886 to −46 (promoter AB construct) of the *Xenopus* promoter (Fig. 1), we obtained a three- or fivefold activation, by cotransfection of an expression vector encoding HNF4 or LFB1, respectively. The addition of an expression vector coding for HNF3 had no stimulatory effect (Fig. 4, Prom AB) whereas a reporter construct containing an HNF3-binding site (23) was properly activated by HNF3 (data not shown). Thus, we conclude that the *Xenopus* LFB1 promoter is inducible by HNF4 and by LFB1, but not by HNF3.

Because the LFB1- and HNF4-binding sites are located adjacent to potential transcription start sites, we made two LFB1 promoter constructs containing either the HNF4- or LFB1-binding sites. The promoter B construct extending from positions −494 to −46 containing the HNF4-binding site and the transcription start site (B start [Fig. 1]) is induced only by the transcription factor HNF4 and unaffected by either LFB1 or HNF3 (Fig. 4, Prom B). On the other hand, the promoter A construct containing the sequence from positions −886 to −489 including the LFB1-binding sites (Fig. 1) can be activated only by LFB1 (Fig. 4, Prom A). The activity of the promoter A construct supports the notion that this fragment contains a transcription start site distinct from the B start.

Embryonic activation of the *Xenopus* LFB1 promoter in injected *Xenopus* embryos. In our previous investigation, we had established that LFB1 is transcriptionally activated in early development at mid-blastula transition of *X. laevis* (2). We wondered whether regulatory sequences in the promoter region that are responsible for embryonic activation of this

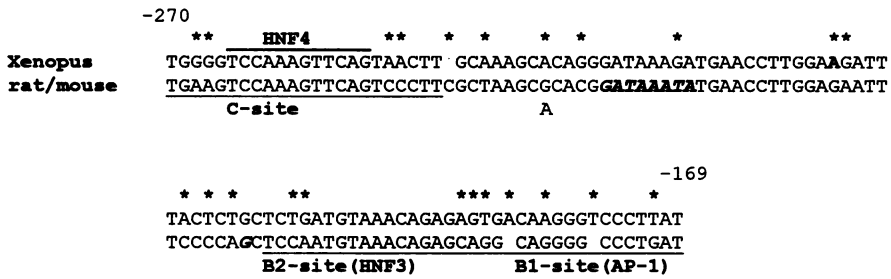

FIG. 2. Highly homologous region between the *Xenopus* and mouse LFB1 promoters. Mismatches are indicated by stars. The sequence is numbered according to the numbering of the *Xenopus* promoter sequence in Fig. 1. The single difference (G to A) in this area between the rat and mouse (22) LFB1 promoters is indicated (45). The binding sites for HNF4 (C site), HNF3 (B2 site), and AP-1 (B1 site) are underlined in the mouse sequence as defined previously (22). The TATA box-like sequence (GATAAATA), as defined in the rat promoter (45), is indicated by bold italic letters. The transcription start sites of *Xenopus* (bold) and rat (bold italic) promoters are marked. The highly conserved HNF4-binding site is indicated.

gene can be identified. In addition, we were interested in identifying regulatory elements that restrict the expression of LFB1 to the middle section of the embryo where the tissues expressing LFB1 are located (2). Therefore, we injected fertilized *Xenopus* eggs with LFB1 promoter-CAT constructs and analyzed the activities of these reporter constructs 2 days later in hatched larvae that were dissected into three fragments corresponding to the heads, middle parts, and tails of the larvae. As exemplified in Fig. 5A, injection of an LFB1 promoter-CAT construct extending from −886 to −46 (promoter AB as used for the transfection assay) resulted in a high CAT activity in the middle part of the embryo whereas about a 6- and 20-fold lower activity was present in the head and tail, respectively. In contrast, the promoter construct containing the region from −494 to −46 (promoter B) gave low CAT activities in all three parts of the embryo (Fig. 5B), suggesting that essential elements for embryonic activation had been removed. To prove the spatial specificity of the embryonic activation of LFB1-CAT reporter constructs, we injected a reporter construct containing the simian virus 40 enhancer and promoter (pSV2CAT). In this case, the highest CAT activity was found in the head, intermediate activity was found in the middle section, and low activity was found in the tail (Fig. 5C). By using the cloning vector pEU-CAT lacking any promoter (36), no significant CAT activity (<0.3%) could be seen in any sample (data not shown). On the basis of these

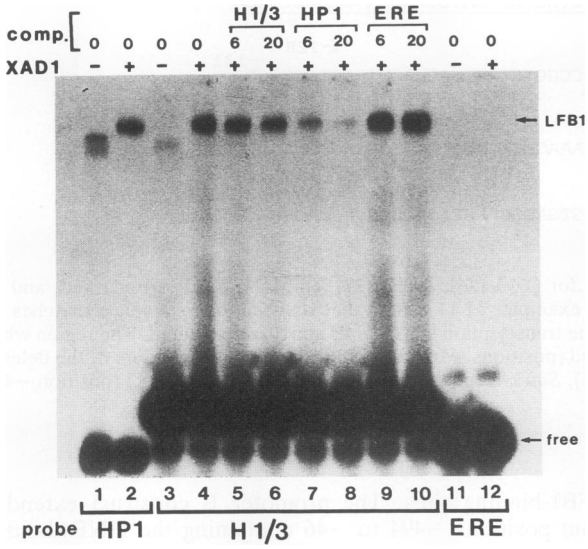

FIG. 3. LFB1 binds to the *Xenopus* LFB1 promoter in band shift assays. For gel retardation assays, radioactively labeled HP1 (10^4 cpm) (lanes 1 and 2), H1/3 (4×10^4 cpm) (lanes 3 to 10), or ERE (4×10^4 cpm) (lanes 11 and 12) were incubated with 15 ng of recombinant LFB1 made in the baculovirus system. The presence (+) or absence (−) of monoclonal antibody XAD1 specific for LFB1 is indicated over the lanes. The amounts (in nanograms) of competitor oligonucleotides (comp.) are given above the lanes. The autoradiogram has been overexposed to visualize the bands in lanes 7 and 8. From a shorter exposure of the same gel, we determined the remaining shifted activity by densitometry. By using H1/3 as the competitor gave residual activities of 65 (lane 5) and 35% (lane 6) for 6 ng and 20 ng of competitors, respectively. Using HP1 as the competitor, values of 19 (lane 7) and 5% (lane 8) were calculated. By using a 200-fold excess of unlabeled H1/3, complete self-competition was observed (data not shown).

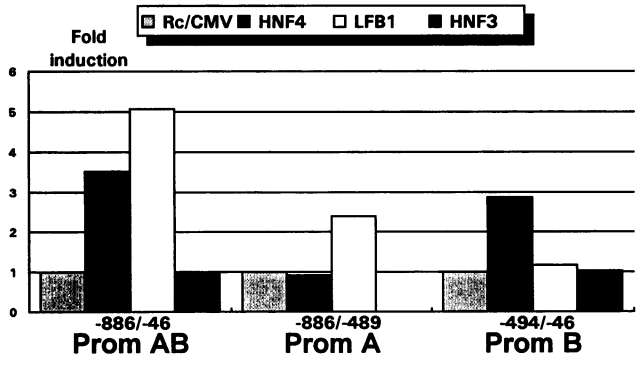

FIG. 4. Transactivation of the *Xenopus* LFB1 promoter in C2 cells by LFB1, HNF4, and HNF3. CAT assays were performed with extracts from C2 cells transfected with 5 μg of the LFB1 promoter-CAT constructs (promoter [Prom] AB, A, or B) and 100 ng of the Rc/CMV expression vector encoding either LFB1, HNF4, or HNF3. The averages of two or three independent experiments are given. The fold induction values are compared with the CAT conversion values for Rc/CMV alone, which is set at 1. Fold induction values for individual experiments are shown as follows, with the values given in parentheses after the conditions: promoter AB and either HNF4 (3.9, 5.0, and 1.8), LFB1 (3.3, 8.3, and 3.6), or HNF3 (0.7 and 1.2); promoter A and either HNF4 (0.6 and 1.5) or LFB1 (1.5 and 3.4); and promoter B and either HNF4 (3.4, 2.9, and 2.3), LFB1 (1.4 and 1.0), or HNF3 (1.4 and 0.7). Numbers indicate the positions of the promoter fragments, with the numbering as in Fig. 1.

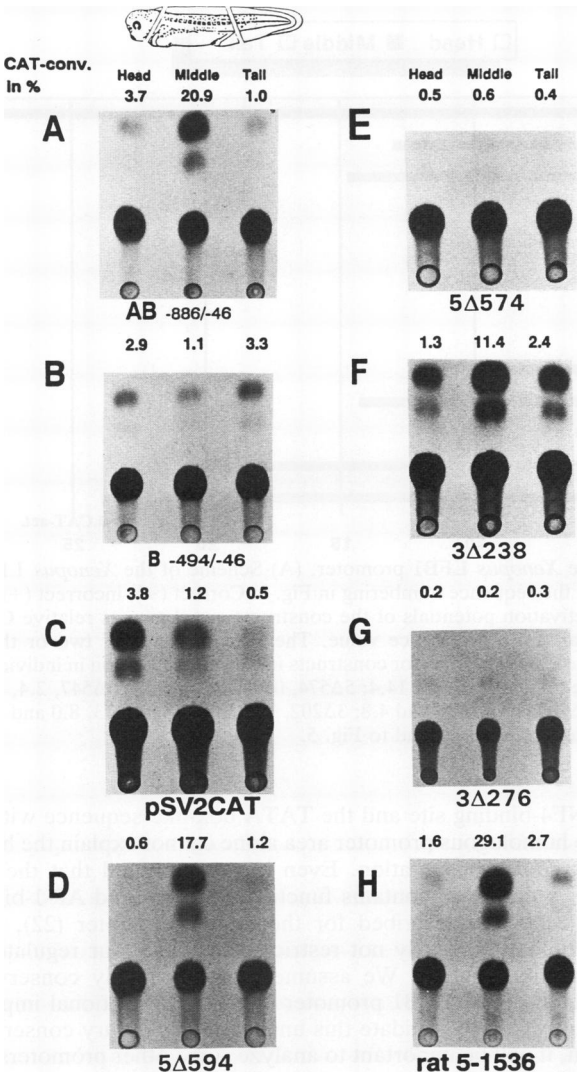

FIG. 5. Microinjection of LFB1 promoter deletion constructs. Promoter-CAT constructs of the *Xenopus* and rat LFB1 promoters were microinjected into fertilized *Xenopus* eggs. At stage 37/38, the larvae were dissected into three pieces (head, middle part, and tail), as shown schematically in the upper left-hand part of the figure. The pieces of 10 to 15 embryos were pooled, embryo extracts were prepared, and equal amounts of protein were used in the CAT assay. The percent CAT conversion (CAT-conv.) is given. Numbers of the constructs indicate their 5′ or 3′ end according to the numbering shown in Fig. 1 and Fig. 7A. The pSV2CAT construct was used as a control. The promoter AB (−886 to −46) and B (−494 to −46) constructs were cloned in the vector pCAT-Enhancer (Promega), and all other promoter deletion constructs were cloned in pEU-CAT (36).

experiments, we conclude that the *Xenopus* LFB1 promoter can be activated upon injection into the fertilized *Xenopus* egg in a way that reflects the activation of the endogenous gene. The observation that a deletion construct of the LFB1 promoter has lost its regulatory potential implies that the regulatory elements responsible for embryonic activation can be identified. Therefore, we generated a series of 5′ deletions in the *Xenopus* LFB1 promoter with the pEU-CAT vector and injected the corresponding constructs into fertilized eggs to analyze their function. As shown in Fig. 5D and summarized in Fig. 6, deletion constructs up to position −594 retained embryonic activation restricted to the middle part of the embryo, whereas a further deletion of another 20 nucleotides (construct 5Δ574) destroyed the ability for embryonic activation completely (Fig. 5E and Fig. 6).

To define the 3′ border of the sequence involved in embryonic activation of the LFB1 promoter, we injected reporter constructs with various 3′ deletions into fertilized eggs. As summarized in Fig. 6, deletion up to −207 (construct 3Δ207) retained embryonic activation, whereas significant loss of regulation was observed when another 31 nucleotides were removed (construct 3Δ238). The reduced regulation of this reporter construct was due to the increased activity found in the tail, since in three independent experiments the percentages of acetylated chloramphenicol in the tail were 3.5% for the 3Δ238 construct and 0.6% for the 3Δ207 construct. Upon a further deletion of another 38 nucleotides (position −276), the activity was lost in all three parts of the embryo (Fig. 5G and Fig. 6). In conclusion, the disappearance of proper embryonic activation in the 3′ deletion clones correlates with the deletion of the B start site in the deletion clone 3Δ238, while regulated expression is completely lost upon removal of the conserved region including the HNF4-binding site.

The rat LFB1 promoter is activated in injected *Xenopus* embryos. To analyze whether regulatory elements involved in embryonic activation of LFB1 have been conserved in vertebrate evolution, we injected a rat LFB1 reporter construct into fertilized *Xenopus* eggs. The rat LFB1 promoter fragment used was kindly provided by U. Schibler (45). As shown in Fig. 5H, the rat LFB1 promoter-CAT construct extending to position −1536 mediated an embryonic expression pattern similar to those of the functional *Xenopus* promoter constructs. To narrow down the promoter area involved in embryonic activation of the rat LFB1 promoter, we tested a series of 5′ deletion constructs. As Fig. 7B demonstrates, deletion up to −425 (construct 5-425) still gave a proper control in embryonic activation of the rat LFB1 promoter. On average, the activities of these reporter constructs in the middle sections were three- to eightfold higher than those for the tail sections (Fig. 7B). These values are significantly lower than the ones for the *Xenopus* constructs, which were in the range of 6- to 28-fold (Fig. 6B). Embryonic activation of the rat LFB1 promoter was lost in the deletion constructs 5-410 and 5-360 (Fig. 7B). Therefore, the 5′ border of a regulatory element essential for embryonic activation is located between −425 and −410 of the rat LFB1 promoter. This is some 110 bp upstream of the promoter area that is highly similar between the *Xenopus* and rat LFB1 promoters.

Embryonic activation of the injected LFB1 gene requires the OZ element. Close inspection of the sequences of the *Xenopus* and rat LFB1 promoters revealed some similarity at the 5′ borders where the potential for embryonic activation is lost: in both genes, the sequence TNCCNCTCTC was present (in the *Xenopus* promoter starting at −585 [Fig. 1]; in the rat promoter starting at −411, [Fig. 7A]). This element is surprisingly similar to the OZ element (GTNTCCCCCTCT CA) that has very recently been identified as a regulatory sequence essential for embryonic activation of the GS17 and N-CAM promoter in *X. laevis* (34). In fact, 11 and 9 bp of the 14-bp OZ consensus sequence are conserved in the *Xenopus* and rat promoters, respectively (see Fig. 8).

Because the OZ element has been characterized as a

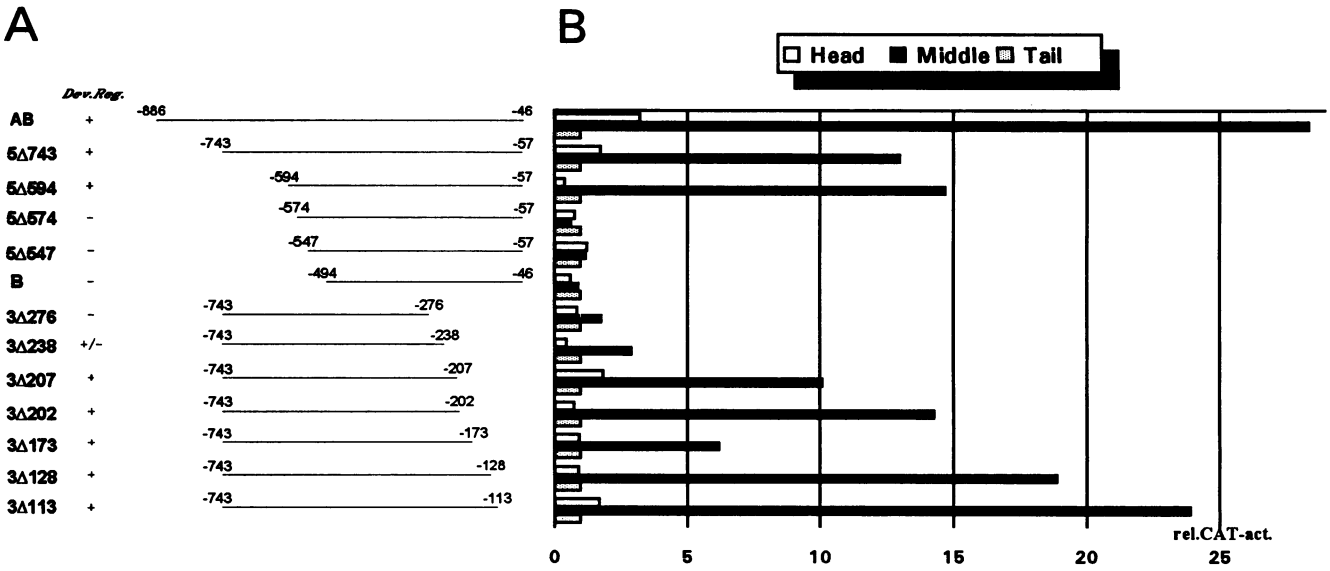

FIG. 6. Activation potentials of microinjected deletion constructs of the *Xenopus* LFB1 promoter. (A) Scheme of the *Xenopus* LFB1 promoter constructs used for microinjection. The numbering is according to the sequence numbering in Fig. 1. Correct (+), incorrect (+/−), and no (−) developmental activation (*Dev. Reg.*) are indicated. (B) The activation potentials of the constructs are shown as relative CAT activities in the head, middle, and tail sections, using the value in the tail as the reference value. The average value of two or three experiments, each representing a pool of 10 to 15 embryos, is shown. Relative CAT activities for constructs in the middle section in individual experiments were as follows: AB, 21.3 and 35.4; 5Δ743, 21.1, 14.0, and 3.8; 5Δ594, 15.0 and 14.4; 5Δ574, 0.4, 0.2, and 1.3; 5Δ547, 2.4, 1.0, and 0.3; B, 1.5 and 0.3; 3Δ276, 0.7, 3.8, and 0.7; 3Δ238, 2.4, 4.8, and 1.6; 3Δ207, 17.9, 7.6, and 4.8; 3Δ202, 19.8 and 8.8; 3Δ173, 8.0 and 4.3; 3Δ128, 27.1 and 10.7; 3Δ113, 14.5 and 33.4. For description of cloning vectors, see the legend to Fig. 5.

regulatory element binding to the maternal protein OZ-1 of the *Xenopus* egg (34), we analyzed whether the region of the *Xenopus* LFB1 promoter from −588 to −574 (OZ-Xen) has the binding properties defined for OZ (34). Gel retardation assays revealed that the oligonucleotide containing OZ-Xen interacts with a protein present in *Xenopus* egg extracts and that the formation of the complex can be inhibited specifically by the unlabeled oligonucleotide containing the OZ-1-binding site present in the N-CAM promoter (data not shown). However, the gel retardation assays using the OZ element as a probe did not show the same degree of specificity as corresponding experiments using the binding sites for LFB1 or HNF4, since unrelated oligonucleotides gave some weak competition (data not shown). Nevertheless, it is most reasonable to assume that the regulatory element we have found to be essential for embryonic activation of the *Xenopus* and rat LFB1 gene (Fig. 6 and 7) corresponds to the OZ element described by Ovsenek et al. (34).

DISCUSSION

Homology within the LFB1 promoter. Comparing the LFB1 promoter of *X. laevis* with those of the rat and mouse, we observed an unusually high similarity of about 80% within 102 bp of the promoter sequence (Fig. 2). In all other comparisons of promoters between *X. laevis* and mammals, the similarities have been restricted to short elements of about 10 bp corresponding to binding sites of evolutionary conserved transcription factors (e.g., cardiac actin genes [31], albumin genes [39], and keratin genes [43]). We believe that the extensive similarity between the *Xenopus* and mammalian LFB1 promoters is attributable to a common evolutionary origin. Therefore, we interpret the similarity as homology in its precise meaning (37). The presence of an HNF4-binding site and the TATA box-like sequence within the homologous promoter area alone cannot explain the high degree of conservation. Even if it is assumed that the *X. laevis* promoter contains functional HNF3- and AP-1-binding sites, as described for the mouse promoter (22), the homology is clearly not restricted to these four regulatory elements (Fig. 2). We assume that this highly conserved stretch of the LFB1 promoter is of some additional importance. To fully validate this unusual evolutionary conservation, it will be important to analyze some other promoters of regulatory genes conserved in amphibian and mammalian species.

Factors involved in the tissue-specific expression of LFB1. Our data establish that the *Xenopus* LFB1 promoter can be activated in transfection experiments by the tissue-specific transcription factors LFB1 and HNF4 (Fig. 4). The action of LFB1 on its own promoter implies an autoregulatory loop, possibly ensuring continuous expression upon initial activation. Such an autoregulatory control has been detected for other tissue-specific transcription factors including C/EBP (7), HNF3 (35), MyoD (44), Pit-1 (6), and GATA-1 (13, 47). Since for the rat and mouse promoters, potential autoregulation has not been demonstrated so far (22, 45), LFB1 autoregulation may be a feature restricted to the *Xenopus* system. The potential regulation of the LFB1 promoter by HNF4 in *Xenopus* embryos (this study), rat (45), and mouse (22) may be considered as a control to establish and/or maintain tissue-specific expression of LFB1, because the tissue distribution pattern of transcription factor HNF4 is very similar to that of LFB1 (41). Thus, at least in *Xenopus* embryos, tissue-specific expression of LFB1 might be controlled by two distinct tissue-specific factors.

Activation of injected genes in developing *Xenopus* embryos. The main goal of our investigation was to define the elements and factors involved in the early activation of LFB1 gene

FIG. 7. Elements involved in the embryonic activation of the rat LFB1 promoter in *Xenopus* embryos. (A) The nucleotide sequence of the rat LFB1 promoter is given (45). The translation start codon ATG, the transcriptional start site, and the regulatory elements (the HNF4-binding site referred to as TRHE in the original report and the TATA box-like sequence) are indicated (45). The ends of the deletion clones are marked by arrows, and the common 3′ end of the constructs, at position −133, is indicated (solid diamond). The OZ element and the highly conserved sequence of the *Xenopus* and rat promoters is underlined (Fig. 2). The number of bases matching those of the consensus sequence is given (see legend to Fig. 1). (B) The activation potentials of the constructs are shown as relative CAT activities in the head, middle, and tail sections, using the value in the tail as the reference value. The 5′ deletion constructs are named according to the numbering shown in panel A. Up to position −621, the sequence is as in reference 45, and upstream the sequence is as deposited in the EMBL data bank (accession no. X67649) by the group of R. Cortese. The relative CAT activities for constructs in the middle section were as follows: 5-1536, 10.8 and 1.8; 5-1315, 4.5, 6.6, 5.8, and 7.0; 5-975, 10.4 and 6.6; 5-734, 3.9, 4.1, 4.1, and 0.9; 5-621, 1.9, 1.7, 5.1, 1.1, 1.3, and 5.1; 5-461, 1.0, 4.5, 1.9, and 3.8; 5-425, 13.1, 1.7, 1.9, and 1.2; 5-410, 0.8, 1.3, 0.9, and 1.2; and 5-360, 1.1, 0.6, 1.0, and 0.9.

Xen. LFB1	ctcttCCcCTCTCt
rat LFB1	tggtgCCtCTCTCc
GS 17	gtctcCCcCTCTCa
N-CAM	gtgtcCCcCTCTCa
mouse LFB1	gggtcCCaCTCTCc
γ2 crystallin	tttttCCcCTCTCt
keratin A1	cagcaCCaCTCTCt
core	CCNCTCTC

FIG. 8. Potential OZ elements in various LFB1 promoters. The OZ elements in the *Xenopus* (Xen.) and rat LFB1 promoters are from Fig. 1 and 7, respectively. The OZ elements of GS17 and N-CAM promoters (34) are given. A sequence similar to the OZ element present in the mouse LFB1 promoter is also included. Potential OZ elements present in the mouse LFB1 promoter (250 bp upstream of the transcription start site on the lower strand [22]), the mouse γ2-crystallin promoter (88 bp upstream of the transcription start site on the lower strand [25]), and the *Xenopus* keratin A1 promoter (231 bp upstream of the transcription start site on the lower strand [29]). The core sequence present in all potential OZ elements is given in capital letters.

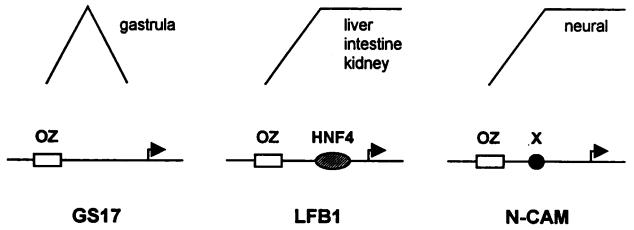

FIG. 9. A model explaining the distinct embryonic activation of genes in *Xenopus* embryos. The basic structures of the GS17, LFB1, and N-CAM promoters are illustrated. Above each construct, the pattern of its activity over time and tissue restriction of expression is given. The element of the N-CAM promoter marked with X indicates a postulated binding site for a factor mediating nervous system-specific expression. For further details, see text.

transcription during *Xenopus* embryogenesis. On the basis of previous reports that promoter constructs can be properly activated during early embryogenesis upon injection into fertilized *Xenopus* eggs (17, 19, 30, 48), we injected LFB1 promoter-CAT constructs into developing *Xenopus* embryos. By using this approach, we detected proper expression of the LFB1 promoter in dissected 3-day-old larvae (Fig. 5). Regulated expression of injected genes in *Xenopus* tadpoles has been reported at even much later stages of development (21 days after injection) for the γ2-crystallin gene (4). Most likely, the activities of the injected genes are predominantly derived from episomal gene copies, as stable transgenic animals are rarely obtained with *X. laevis* (e.g., references 1 and 11).

We show that the injection of rat LFB1 promoter constructs leads to expression patterns similar to those observed for the *Xenopus* constructs, although the mammalian promoter in the amphibian system is less active in the middle section of the larvae than the injected *Xenopus* promoter is (compare Fig. 6 with Fig. 7B). Such proper embryonic control of a mammalian promoter in the developing *Xenopus* embryo has also been observed for the γ2-crystallin promoter of the mouse (4). Therefore, the *Xenopus* system seems to be also a very useful system in defining regulatory elements involved in embryonic activation of mammalian genes. Clearly, corresponding experiments with mammalian embryos would be much more time-consuming and would require substantially more technical support and be much more expensive.

Factors involved in embryonic activation of LFB1. Deletion analysis of the LFB1 promoter revealed at the 5′ end of both the *Xenopus* and rat LFB1 promoters a regulatory element essential for embryonic activation (Fig. 6 and Fig. 7B). Band shift competition experiments (data not shown) indicate that the regulatory element in the *Xenopus* promoter has the binding properties described very recently for the OZ element (34). As shown in Fig. 8, the mouse LFB1 promoter also contains a sequence that is very similar to the OZ element. The OZ element has initially been identified in the 5′-flanking region of the GS17 and N-CAM genes which are both activated early in *Xenopus* embryogenesis (34). As shown in Fig. 8, we found potential OZ elements (CCNC TCTC) in the promoter regions of the *Xenopus* keratin A1 gene and the mouse γ2-crystallin gene which have also been shown to be activated upon injection into developing *Xenopus* embryos (4, 17). This finding implies that the OZ element might be a major control element for early embryonic activation.

The GS17 gene is activated at the mid-blastula transition and expressed only until late gastrula stage (19), whereas all the other genes containing OZ elements are expressed in specific tissues throughout the lifetime of the organism. On the basis of the distinct tissue-specific expression patterns of these genes, we postulate that the OZ element is required only for the onset of gene activation during embryogenesis and is not responsible for tissue-specific expression. Thus, the function of the OZ element may be restricted to embryogenesis. This assumption is in agreement with the functional analysis of various rat LFB1 promoter constructs, since deletion of the region that we now know to contain the OZ element (−414 to −400 in Fig. 7A) does not contribute to the activity of the promoter in in vitro transcription or in transfected cell lines (45).

Concerning tissue-specific expression of LFB1, we assume that the restricted expression of LFB1 is due to HNF4, as the binding site for this transcription factor is present in the *Xenopus* and rat promoter constructs (Fig. 2). It will be interesting to see whether this transcription factor is already present in the egg or is also transcriptionally activated in early embryogenesis. Because HNF4 is a member of the steroid hormone receptor superfamily and represents an orphan receptor (42), it seems possible that an as yet unidentified ligand may regulate HNF4 activity. It is most attractive to speculate that such a ligand might function as a morphogen during early development. In the case of the *Xenopus* gene, LFB1 itself may be also involved in the tissue-specific regulation of LFB1, because the corresponding binding site is within this promoter (Fig. 1).

Since 40 bp of the 3′ end of the conserved promoter area can be removed without impairing regulation (Fig. 6B), we conclude that embryonic activation does not require the entire highly conserved promoter region.

A potential hierarchy of genes in vertebrate development. Since the OZ-1 factor that interacts with the OZ element accumulates during oogenesis and is therefore present as a maternal factor in the egg (34), we have potentially the main constituents of the hierarchy at hand that lead to the activation and tissue-specific expression of some genes during embryogenesis. According to our working hypothesis, OZ-1 would activate after mid-blastula transition a whole series of distinct genes that contain OZ elements in the promoter (Fig.

9). Some of these OZ-1-dependent genes encode structural proteins, such as N-CAM, keratin, and γ2-crystallin, whereas others are regulatory factors, such as GS17 and LFB1. We speculate that a gene such as GS17 that contains only an OZ element in its promoter is only transiently activated at the gastrula stage (Fig. 9). Its activity would disappear as OZ-1 activity is lost. In contrast, the LFB1 gene that contains additional regulatory elements in its promoter would remain active, as these other regulatory elements are now functional (Fig. 9). We assume that the HNF4-binding site plays a crucial role in this process and restricts the expression to the liver, intestine, and kidney. In other genes activated by OZ-1, such as N-CAM, distinct elements and factors may mediate different expression patterns (Fig. 9).

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