FTZ-F1-Related Orphan Receptors in *Xenopus laevis*: Transcriptional Regulators Differentially Expressed during Early Embryogenesis

HEIDRUN ELLINGER-ZIEGELBAUER,¹ ABDELMADJID K. HIHI,² VINCENT LAUDET,³
HANSJÖRG KELLER,² WALTER WAHLI,² AND CHRISTINE DREYER^{1*}

Max-Planck-Institut für Entwicklungsbiologie, D-72011 Tübingen, Federal Republic of Germany¹; Institut de
Biologie Animale, Université de Lausanne, CH-1015 Lausanne, Switzerland²; and Unité d'Oncologie
Moléculaire, Institut Pasteur, 59019 Lille Cédex, France³

Received 27 October 1993/Returned for modification 1 December 1993/Accepted 10 January 1994

Orphan receptors of the FTZ-F1-related group of nuclear receptors (xFF1r) were identified in *Xenopus laevis* by isolation of cDNAs from a neurula stage library. Two cDNAs were found, which encode full-length, highly related receptor proteins, xFF1rA and B, whose closest relative known so far is the murine LRH-1 orphan receptor. xFF1rA protein expressed by a recombinant vaccinia virus system specifically binds to FTZ-F1 response elements (FRE; PyCAAGGPyCPu). In cotransfection studies, xFF1rA constitutively activates transcription, in a manner dependent on the number of FREs. The amounts of at least four mRNAs encoding full-length receptors greatly increase between gastrula and early tailbud stages and decrease at later stages. At early tailbud stages, xFTZ-F1-related antigens are found in all nuclei of the embryo.

The nuclear hormone receptor superfamily includes receptors for steroid and thyroid hormones, vitamin D and retinoic acid, which regulate gene expression in the presence of their respective ligands by binding to *cis*-acting DNA sequences called hormone response elements (HRE) (53). These nuclear receptors, which control different developmental and physiological processes, share several structural and functional features. They are composed of six domains, A to F, of which the most highly conserved Zn-finger-containing C domain is responsible for specific DNA binding (25). In addition to ligand binding, the E domain appears to be involved in dimerization and transcriptional activation or repression (12, 53).

Recently, a number of receptors have been identified for which a ligand has not yet been found or may not exist. However, these orphan receptors are structurally related to other nuclear receptors and, thus, are included in the nuclear receptor superfamily (27, 41). This superfamily can be divided into subfamilies on the basis of either characteristic amino acid sequence motifs of its members (53), their evolutionary relationships (27), or functional criteria, such as the ability to bind to distinct HREs (27, 42, 46). Classical steroid hormone, or type I, receptors function as homodimers and bind to palindromically arranged half-sites of 6 bp invariably spaced by 3 nucleotides. Type II receptors, which include the receptors for thyroid hormone (TR), all-*trans*- and 9-*cis*-retinoic acid (RAR), 9-*cis*-retinoic acid (RXR), vitamin D (VDR), peroxisome proliferators (PPAR), and ecdysone (EcR), bind and transactivate through HREs composed mainly of directly repeated 6-bp half-site motifs spaced by 1 to 8 nucleotides. Palindromes and inverted palindromes may also be functionally recognized by some receptors. In contrast to the classical steroid receptors, members of the type II family preferentially function as heterodimers with RXRs (3, 20, 22, 33, 36, 59, 60), or *ultraspiracle* (48, 58). The orphan receptors DmFTZ-F1 (31), mELP (51), NGFI-B (57), and Rev-ErbA α (17) define

yet another class of receptors (type III). They most probably bind as monomers to extended, unrepeatable half-site motifs. A domain adjacent to the C-terminal end of the DNA binding region participates in DNA binding by interaction with sequences upstream of the AGGTCA motif, which is the consensus half-site element recognized by both the estrogen receptor and type II receptors (37). In an attempt to identify additional nuclear receptors, we have screened a *Xenopus laevis* neurula cDNA library with a mixture of oligonucleotides encoding a consensus sequence from part of the first Zn finger region of TRs and RARs. Two types of cDNAs encoding nuclear receptors were isolated: one type encodes a *X. laevis* RAR of the γ 2 subtype (7), and a second type, which we now report, encodes orphan receptors related to the *Drosophila melanogaster* FTZ-F1 transcription factor (DmFTZ-F1) that is involved in the regulation of *fushi tarazu* (*ftz*) expression in a seven-stripe pattern in early *D. melanogaster* embryos (31).

The FTZ-F1-related receptors of *X. laevis* (xFF1r), by their structure and also by their function, belong to the newly emerging FTZ-F1 group of receptors, whose evolution is discussed. The temporally regulated expression of xFF1r orphan receptors suggests possible mechanisms of gene control by these factors during early development of *X. laevis*.

MATERIALS AND METHODS

cDNA library screening and cDNA sequence analysis. A λ gt10 cDNA library from *X. laevis* stage 17 neurula (21) was screened with a mixture of 48-mer oligonucleotides each encoding a consensus peptide from the first Zn finger of the DNA-binding domain of known nuclear receptors for thyroid hormones and retinoic acid, and the isolated cDNAs were characterized as previously described (7). The Microgenie program (Beckman) and the University of Wisconsin Genetics Computer Group program package (5) were used for analysis of nucleotide sequences and amino acid comparison. The GenBank and EMBL data bases were searched by using the FASTA and TFASTA programs. Amino acid sequences were compared with the Clustal V package. The phylogenetical tree

* Corresponding author. Mailing address: Postfach 2107, D-72011 Tübingen, Federal Republic of Germany. Phone: 0049-7071-601471. Fax: 0049-7071-601449.

was calculated by using the Fitch least square algorithm and confirmed by the neighbor-joining and phylogenetic analysis using parsimony (PAUP [47]) methods as previously described (29). The bootstrap procedure was used for statistical analysis (9).

RNA analysis. Isolation of total RNA from staged embryos, Northern (RNA) blotting, and hybridization with ^{32}P -labelled probes were done as described previously (8). Poly(A)⁺ RNA was selected with an mRNA isolation kit (Stratagene).

RNAse protection assays were performed by the method of Krieg and Melton (24), except that only RNase A at 20 $\mu\text{g}/\text{ml}$ was used. The RNA samples were either total RNA from 2.5 embryo equivalents or 10 pg of sense RNA synthesized in vitro using the cDNAs as templates. The sizes of protected fragments were estimated from a DNA sequence ladder. The antisense RNA probes for Northern blots and RNase protection represented a *PvuII-SacI* fragment from the 5'-untranslated region (5' probe), a *BglII-EcoRI* fragment (3' probe for Northern blot), and a *PvuII-EcoRI* fragment of the xFF1rA cDNA (3' probe for RNase protection; arrows in Fig. 1A).

Plasmids. For protein expression in bacteria, different parts of the cDNA xFF1rA, as indicated in Fig. 1A, were cloned in pQE (Qiagen) and pGEX (Pharmacia) vectors. For cloning of pQE16-xFF1rA[AB] which encodes domain A/B coupled N terminally to the mouse dihydrofolate reductase (DHFR), a DNA fragment encoding xFF1rA codons 4 to 51 was amplified by PCR by using primers with restriction enzyme sites for either *BamHI* or *BglII* and cloned into the *BamHI* site of pQE16. pGEX-xFF1rA[AB], which encodes a fusion protein of glutathione-S-transferase (GST) with domain A/B, was constructed by cloning the same PCR fragment into the *BamHI* site of pGEX-2T. To construct pGEX-xFF1rA[DEF], a cDNA fragment, encoding amino acids 181 to 501 of xFF1rA with a Klenow-repaired *StyI* site at the 5' end and a *SmaI* site at the 3' end, was cloned into *SmaI*-cut pGEX-2T. This construct encodes amino acids 181 to 501 fused C terminally to GST and was termed GST-xFF1rA[DEF]. To construct pQE17-xFF1rA[DEF Δ], a *BamHI-BglII* fragment from pGEX-xFF1rA[DEF] was cloned into *BamHI-BglII*-cut pQE17. The encoded protein contains amino acids 181 to 394.

Expression vector pSG5-xFF1rA contained the cDNA sequences downstream of the unique *SacI* site in the 5' untranslated region.

Reporter constructs are based on the gCAT plasmid, which contains the rabbit β -globin promoter in front of the bacterial chloramphenicol acetyltransferase (CAT) gene. The double-stranded oligonucleotides containing the FRE-fDE1 element(s) were cloned either blunt-ended into the Klenow-repaired *BamHI* site of gCAT (gCAT-FRE, gCAT-FRE₂, gCAT-FRE₄) or as a *HindIII-BamHI* fragment after a first cloning step in pBluescript SK (gCAT-FRE₃). For preparation of a recombinant vaccinia virus vector, the cDNA encoding xFF1rA was first cloned as a *SacI* fragment into the *SacI* site of pGEX-KG (16). Ligation of an *EcoRI* fragment from this recombinant into *EcoRI*-cut pHGS-1 yielded pHGS-1 xFF1rA, which encodes xFF1rA in frame with the start methionine of the 11-kDa protein of the vaccinia virus. Recombinant vaccinia virus was prepared as described by Bertholet et al. (2), except that HeLa cells were used. All plasmid DNA was purified with Qiagen ion-exchange columns.

Oligonucleotides. Oligonucleotides are listed with the nucleotides added for cloning purposes in lowercase letters: FRE-fDE1, 5'-gcgaattcCGTCTCAAGGTCGCCGAGTAgat tcgc-3'; FRE, 5'-gatcTCTCAAGGTCA-3'; NBRE, 5'-gatcTT TAAAGGTCA-3'; RARE, 5'-gcgaattcAGGGTTCACCGA

AAGTTCACCTCgaattcgc-3'; RevRE, 5'-gatcAAAGTAGGT CA-3'.

Preparation of protein extracts. For the preparation of nuclear extracts of tailbud stage embryos, nuclei were isolated essentially as described by Gorski et al. (14). Briefly, about 1,500 embryos were homogenized in 20 ml of buffer H (10 mM Tris HCl [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.15 mM spermine, 0.5 mM spermidine, 2 M sucrose, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1.4 μg of aprotinin per ml, 2 μg of leupeptin per ml, 1 μg of pepstatin per ml) with a motor-driven Teflon-glass homogenizer. The release of intact nuclei was monitored by microscopic examination. The homogenate was filtered through nylon cloth, layered over cushions of buffer H, and centrifuged at 107,000 $\times g$ for 35 min in a SW27.1 rotor. The nuclear pellet was resuspended in 400 μl of buffer N (20 mM Tris HCl [pH 7.5], 0.4 M KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 1.4 μg of aprotinin per ml, 2 μg of leupeptin per ml, 1 μg of pepstatin per ml), and nuclear proteins were extracted by gentle agitation for 50 to 60 min at 4°C. After centrifugation at 150,000 $\times g$ for 30 min at 4°C in a TL-100 centrifuge, the supernatant was frozen in liquid nitrogen and stored in aliquots at -80°C.

HeLa cells were infected with the recombinant xFF1rA vaccinia virus at a multiplicity of infection of 10. After 12 h, the cells were washed with phosphate-buffered saline (PBS) and detached by incubation for 10 min at 37°C in a buffer containing 10 mM Tris HCl (pH 7.5), 1 mM EDTA, and 150 mM NaCl. The cells were harvested and centrifuged for 20 s at 4°C at 14,000 $\times g$. Four times the packed-cell volume of extraction buffer (20 mM Tris HCl [pH 7.5], 0.4 M KCl, 2 mM dithiothreitol, 20% glycerol, 1 μg of each protease inhibitor per ml) was added, and the cells were submitted to five cycles of freezing and thawing and subsequently centrifuged for 1 h at 100,000 $\times g$ and 0°C. The supernatant was kept at -70°C.

Gel mobility shift assays. Gel mobility shift assays were performed in 20 μl of binding buffer containing 10 mM Tris HCl (pH 7.5), 80 mM KCl, 10% glycerol, 0.2 mM EDTA, 2 mM dithiothreitol, 2 μg of poly(dIdC), 0.5 mM phenylmethylsulfonyl fluoride, 1.4 μg of aprotinin per ml, 2 μg of leupeptin per ml, 1 μg of pepstatin per ml. Where indicated, the KCl concentration was 40 mM and bovine serum albumin was added to 4 mg/ml. The oligonucleotide probes were labelled either with [γ - ^{32}P]dATP and T₄-polynucleotide kinase or by a filling-in reaction with Klenow enzyme in the presence of [α - ^{32}P]dATP. Either 2 μg of whole-cell extract prepared from vaccinia virus-infected HeLa cells or 4 μl of nuclear extract of embryos at the early tailbud stage (approx. 1.5 mg/ml) was incubated in binding buffer for 15 min on ice with or without unlabelled competitor oligonucleotide. After the preincubation, 15 to 20 fmol of ^{32}P -oligonucleotide was added and the incubation was continued for 30 min at room temperature. For supershift assays, 0.05% Nonidet P-40 was included and the indicated amount of antiserum was added 15 min after the addition of the probe. DNA-protein complexes were electrophoresed on a 5% polyacrylamide gel (30:1) in 0.5 $\times$ TBE (1 $\times$ TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA) containing 5% glycerol at 20 mA at 4°C.

Antibodies. The parts of the bacterially expressed proteins used as antigens to generate rabbit antisera, anti-xFF1rA[AB] and anti-xFF1rA[DEF Δ], and the mouse antiserum anti-xFF1rA[DEF] are represented as lines beneath the predicted xFF1rA protein in Fig. 1A. The A/B domain of the xFF1rA protein was expressed as a DHFR-fusion protein, and parts of the DEF domains (DEF Δ in Fig. 1A) were expressed without

DHFR in the pQE system and used for the immunization of rabbits. The insoluble proteins were purified under denaturing conditions on Ni-charged nitriloacetic acid columns (Qiagen). After electrophoresis on a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel, the proteins were visualized by CuCl_2 staining (32) and electroeluted or were renatured in the gel by treating it with PBS containing 6 or 3 M urea or no urea.

For immunizations, 150 to 200 μg of the electroeluted proteins were used per injection. Gel slices containing the renatured protein were freeze-dried, minced, and rehydrated. An amount equivalent to 500 μg of protein was injected.

The GST-fusion proteins GST-xFF1rA[DEF] and GST-xFF1rA[AB] were expressed as described previously (8). After removal of the GST part by site-specific proteolysis, the

FIG. 1. Sequence analysis of xFF1r cDNA clones. (A) Schematic representation and restriction maps of xFF1r cDNA clones. The restriction map of the 2,079-bp-long cDNA xFF1rA is shown at the top, and that of the related cDNA xFF1rB is indicated beneath and arranged according to its homology to xFF1rA. The open reading frame within the xFF1rA cDNA is indicated by a thick bar, and the predicted xFF1rA protein is depicted in the lower part of the figure, with its subdomain structure according to Krust et al. (25) and Ueda et al. (52). Probes specific for the xFF1rA cDNA are indicated by arrows and are explained in Materials and Methods. Regions of the xFF1rA protein used as antigens are indicated by lines. (B) Nucleotide and predicted amino acid sequence of the xFF1rA cDNA. In-frame stop codons in the 5'-untranslated region are underlined. The DNA binding domain and regions II and III within the E domain (54) are boxed. The FTZ-F1 box (52) is underlined twice. (C) Comparison of subdomains of the xFF1rA deduced amino acid sequence with selected members of the nuclear receptor superfamily. The amino acid sequences of the DNA binding domain and of regions II and III were compared, and the percent amino acid identity is indicated. The numbers above each bar represent the first and last amino acids of the respective subdomains. mLRH-1, mouse liver receptor homologous protein 1 (GenBank accession number M81385); mELP, mouse embryonal long terminal repeat binding protein (51); bAd4BP, bovine Ad4 binding protein (18); DmFTZ-F1, *D. melanogaster* ftz factor 1 (30); DHR39, *D. melanogaster* hormone receptor 39 (1), also described as ftz factor 1 β (40); mRXR γ , mouse retinoid X receptor γ (33); xRAR γ 2, *X. laevis* retinoic acid receptor γ 2 (7); rGR, rat glucocorticoid receptor (38). (D) Phylogenetical tree connecting all members of the FTZ-F1 group of nuclear receptors based on sequence comparison of the C, D and E domains. The black dot indicates the dichotomy between insect and vertebrate genes. The arrow indicates the duplication of the ELP and LRH-1 ancestral gene in vertebrates. The solidity of each branch of the tree was calculated by the bootstrap procedure (9) and is indicated as a percentage.

insoluble DEF protein was prepared for immunization of mice by gel electrophoresis. A polyclonal rabbit serum against the AB domain of xFF1rA (anti-xFF1rA[AB]), a rabbit serum against part of the DEF domain (anti-xFF1rA[DEFA]), and a mouse antiserum against the DEF domain of xFF1rA (anti-xFF1rA[DEF]) were obtained. For affinity purification of the polyclonal rabbit xFF1rA[AB]-antiserum, the fusion protein GST-xFF1rA[AB] was coupled to N-hydroxy-succinimide-activated Sepharose (HiTrap; Pharmacia). The anti-xFF1rA[DEFA] rabbit serum was affinity purified by using the antigen bound to a Western blot (immunoblot). These polyclonal antisera cannot distinguish between the isoforms xFF1rA and xFF1rB. Their antigens are therefore referred to as xFF1r.

Immunoprecipitation after in vitro transcription and trans-

lation and immunostaining of embryos was performed as described previously (8).

Transfections. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 mg of glucose per ml and supplemented with 10% fetal calf serum, 20 U of Nystatin per ml, 20 μ g of gentamicin per ml, 0.1 mg of streptomycin per ml, and 100 U of penicillin per ml. For transfections, 4×10^5 to 4.5×10^5 cells were seeded into 20-cm² dishes in DMEM without phenol red and with 10% fetal calf serum depleted of endogenous lipophilic compounds by treatment with dextran-coated charcoal (4). The following day, the cells were transfected by the calcium phosphate precipitation technique (15) with 3 μ g of CAT reporter plasmid, the indicated amounts of pSG5-based effector plas-

mids, 1 µg of CMV-β (35) to control for transfection efficiency, and pBluescript plasmid to give a total of 10 µg DNA. The amount of pSG5-based expression vector was kept constant by adding nonrecombinant pSG5 plasmid, if required. The medium was changed 6 and 24 h after transfection. Cell extracts were prepared 48 hr after transfection by freeze-thawing in 100 µl of 0.25 M Tris HCl (pH 7.8)–1 mM dithiothreitol per plate. After normalizing for β-galactosidase activity, cell extracts were assayed for CAT activity as described by Gorman et al. (13). CAT activity was quantified by extraction of radioactive material from adequate pieces of the thin-layer chromatography plates by liquid scintillation counting.

Nucleotide sequence accession numbers. The sequences described in this paper have been assigned GenBank accession numbers U05001 and U05003.

RESULTS

Isolation and characterization of cDNAs encoding *X. laevis* FTZ-F1-related orphan receptors. Screening of an *X. laevis* neurula cDNA library in λgt10 (21) with consensus oligonucleotides as previously described (7) resulted in isolation of cDNAs that encoded new members of the *Xenopus* nuclear receptor superfamily. The cDNA inserts of these recombinant phages were named xFF1rA and xFF1rB for reasons given below (Fig. 1A). The xFF1rA cDNA is 2,079 nucleotides long and contains an open reading frame of 501 amino acids preceded by three in-frame stop codons. No polyadenylation signal is found in the short 3'-untranslated region of this cDNA, indicating that it is most probably not full length. This is also suggested by Northern blot analysis which reveals transcripts of 2.2 kb and longer (see below). Another cDNA (1,564 bp) is 93% identical in its nucleotide sequence to xFF1rA, and it was therefore named xFF1rB. The high similarity of this cDNA and of the encoded protein (96%) suggests that xFF1rA and xFF1rB are homologs resulting most likely from the tetraploidization event in *X. laevis* (23).

Comparison of the amino acid sequences deduced from these cDNAs with other members of the nuclear receptor superfamily revealed a high similarity to the orphan receptor FTZ-F1 of *D. melanogaster* (31), and therefore we termed them *Xenopus* FTZ-F1-related A and B (xFF1rA, xFF1rB).

Drosophila FTZ-F1 is a prototype of a subclass of orphan receptors, comprising in addition to FTZ-F1 its subsequently described late isoform (30), DHR39 or DmFtz-F1β (1, 40), an unpublished murine liver receptor homolog (mLRH-1; GenBank accession number M81385), the murine embryonal long terminal repeat binding protein (mELP [51]), the bovine adrenal Ad4-binding protein (bAd4BP [18]) and the murine steroidogenic factor 1 (mSF-1 [19]). Apart from the DNA binding domain, this receptor subclass has a characteristic conserved amino acid sequence, called the FTZ-F1 box (Fig. 1B and C). It lies adjacent to the DNA binding domain and is required for high affinity and sequence-specific binding. The FTZ-F1 box is 25 amino acids long and is 100% identical between xFF1rA, xFF1rB, mLRH-1, and DmFTZ-F1. The FTZ-F1 boxes of mELP and bAd4BP differ by only one conservative exchange. DHR39 seems to be a more distantly related member, as the amino acid sequence of its C domain is only 64% identical to the corresponding sequence of xFF1rA, or of other FTZ-F1-related factors (Fig. 1C). Similarly, its FTZ-F1 box amino acid sequence is only 48% identical with that of xFF1rA.

The amino acid sequence of the P box, which is positioned at the base of the first Zn finger, is ESCKG in all FTZ-F1

box-containing receptors. It is responsible for the specific recognition of the 6-bp half-site motif of the target sequence.

Furthermore, two sequences in the putative ligand-binding domain called region II and region III, which have been used to classify nuclear hormone receptors (54), are highly conserved, except for DHR39 (Fig. 1C). Interestingly, the mELP protein lacks a recognizable region III, suggesting that it has a truncated ligand-binding domain. This may explain its function as a repressor (50). The mELP and mSF-1 are apparently expressed as splice variants of the same gene (19). xFF1rA is highly similar to mLRH-1, having 84% identical and 91% similar amino acids. Thus, it is likely that they are homologs. Taken together, xFF1rA and xFF1rB are clearly members of the FTZ-F1-related subgroup of nuclear receptors.

We have performed phylogenetical tree reconstruction to further confirm the FTZ-F1-related subgroup of nuclear receptors and to analyze the relationship between members of the subgroup. The sequences of the receptors without the nonconserved A/B domains were aligned, and phylogenetical trees were constructed either with the distance matrix method (see reference 27 for references) or with the parsimony analysis (see reference 45 for a review). Both methods gave the same result, which is depicted in Fig. 1D. The tree was tested by using the statistical bootstrap procedure that permits an estimation of the solidity of its branches.

The tree clearly shows the monophyly of the FTZ-F1 group, with the exception of DHR39, which is a divergent member of the group (i.e., the monophyly of the DHR39 with the others is supported by only 66% of confidence in the bootstrap analysis). From the tree it is also evident that the xFF1rA and xFF1rB are immediate relatives and most likely are the *Xenopus* homologs of the mLRH-1 orphan receptor. On the other hand, the mELP and the homologous bAd4BP are encoded in a different locus. Finally, the mELP/mSF-1 and mLRH-1 loci appear to be the result of a duplication of the ancestral FTZ-F1 gene in vertebrates.

The orphan receptor xFF1rA binds specifically to an extended single AGGTCA half-site motif, the FTZ-F1 response element (FRE). All of the FTZ-F1-related receptors tested previously bind DNA elements from which the consensus sequence 5'-PyCAAGGPyCPu-3' can be derived (1, 18, 26, 31, 40, 51). Compared to the classical AGGTCA half-site, the consensus FRE has three additional nucleotides at the 5' end, which are important for interaction with FTZ-F1-related receptors. Since, on the basis of amino acid sequence, xFF1rA belongs to this subgroup of receptors, we used gel mobility shift assays to test its binding specificity. Vaccinia virus-expressed xFF1rA protein bound specifically to both an idealized FRE containing the core sequence TCAAGGTCA, and to TCAAGGTCG, which is the FRE of the *D. melanogaster* ftz gene (FRE-fDE1; 31). Antisera against either the A/B domain (Fig. 2B, lane 5) or the DEF domain (Fig. 2B, lane 7) supershifted the band shift complex, confirming the presence of xFF1rA in the FRE complex. Almost complete competition for binding of the labelled FRE probes was achieved with a fivefold molar excess of unlabelled FRE (Fig. 2A, lanes 4 to 6), whereas the very similar NGFI-B response element (NBRE; TAAAGGTCA), which differs in only one of the three nucleotides flanking the AGGTCA motif, competed only at a 100-fold molar excess and the RevErbA response element (RevRE; AAAGTAGGTCA), which differs in the three flanking nucleotides from the FRE, did not compete (Fig. 2A). Furthermore, elements with two half-sites such as in the retinoic acid response element (RARE) from the RARβ gene, the thyroid hormone response element (TRE), the estrogen response element (ERE), and the peroxisome proliferator

FIG. 2. Gel mobility shift assays of xFF1rA proteins. (A) Binding specificity of vaccinia virus-expressed xFF1rA. Protein extracts containing xFF1rA (lanes 2 to 9) or mock extract (WR, lane 1) were incubated with radiolabelled FRE. Complexes competed with the indicated amounts of unlabelled FRE (lanes 3 to 5), NGFI-B response element (NBRE; lanes 6 and 7), or RevErbA response element (RevRE; lanes 8 and 9). (B) Test for the presence of xFF1rA in the band shift complex by antibody-induced supershift assay. Binding of radiolabelled FRE-fDE1 to the xFF1rA protein (arrowhead) was inhibited by FRE-fDE1 (lanes 2 and 3), but not by a 250-fold excess of RARE (lane 4). The xFF1rA-FRE complex was incubated with 1 μ l of anti-xFF1rA[AB] rabbit serum (lane 5), 1 μ l of anti-xFF1rA[DEF] mouse serum (lane 7), or 1 μ l of preimmune serum as a control (lane 6).

response element (PPRE) did not compete for binding of the xFF1rA to the FRE (Fig. 2B, lane 4; and results not shown). Thus, xFF1rA binds specifically to the FRE, like the other FTZ-F1-related receptors, further confirming that xFF1rA is a member of this receptor subgroup. Taken together, our results suggest that the three nucleotides upstream of the AGGTCA motif are crucial for the distinction between FRE, NBRE, and RevRE.

xFF1rA is a constitutive transcriptional activator. To define the transcriptional regulatory properties of the xFF1rA receptor, HeLa cells were cotransfected with reporter plasmids containing from one to four copies of the FRE and an expression plasmid for xFF1rA (Fig. 3A).

The xFF1rA receptor activated transcription from FRE-containing reporter genes (Fig. 3A). A small but significant transcriptional activation of the reporter plasmid containing one copy of an FRE was observed in the presence of xFF1rA but not without the receptor (Fig. 3B). Transcriptional activation was dramatically increased at the level of both absolute activity and fold induction when the reporter plasmid with two FREs was used. Addition of further FREs in the reporter plasmids resulted in a decrease of the absolute CAT activity but a further increase of the fold induction, since the basal activity in the absence of receptor had decreased even more. Activation of transcription was obtained in the absence of exogenously added ligands or activators. Similar results were obtained when the TK promoter was used instead of the globin promoter (data not shown). The xFF1rA receptor was always found in the nuclei of transfected cells (data not shown).

Taken together, these data indicate that the xFF1rA orphan

receptor acts as a constitutive transcriptional activator and that multiple FREs result in synergistic stimulation of transcription.

xFF1rA mRNAs are expressed in a complex pattern during early embryogenesis of *X. laevis*. Expression of xFF1rA was analyzed at the RNA level during early development of *X. laevis* by Northern blot analysis and by RNase protection analysis (Fig. 4 and 5). At least four transcripts of different lengths could be detected on Northern blots of poly(A)⁺ RNA (Fig. 4A, B, and C). Antisense RNA probes specific for either the whole xFF1rA cDNA (Fig. 4C) or its 3' end (Fig. 4B) revealed almost identical patterns of transcripts of 3.75, 3, 2.5, and 2.2 kb. The amount per embryo of all four transcripts increases between neurula and early tailbud stages (compare lane 2 with lane 3 of Fig. 4B and 5C) and decreases at the late tailbud stage (compare lane 3 with lane 4 of Fig. 4B and 5C). A 5'-specific antisense RNA probe of xFF1rA (Fig. 1A) also hybridized to transcripts of 3.75, 3, and 2.5 kb (Fig. 2A); however, it did not detect the RNA of 2.2 kb seen in Fig. 4B and 5C. Using this 5' probe, the 3-kb transcripts increase only slightly in amount between the late neurula and early tailbud stages (compare lane 2 with lane 3 of Fig. 4A) and then remain constant up to the late tailbud stage (compare lane 3 with lane 4 of Fig. 4A). The lower level of signal detection by the 5' probe could indicate that the population of 3-kb transcripts seen in Fig. 4B and 5C may comprise 5'-end splice variants.

To identify possible splice sites, RNase protection analysis was performed with total RNA of embryos at different stages of development (Fig. 5). With the probe comprising the 3' end of the cDNA xFF1rA, one protected fragment of 400 nucle-

FIG. 3. The xFF1rA orphan receptor activates transcription of FRE-containing reporter genes without the addition of exogenous ligands. (A) Schematic representation of reporter and effector constructs showing the number and orientations of the FRE-FDE1 elements, with the consensus FRE shown in boldface letters. globin prom, rabbit β -globin promoter; CAT, chloramphenicol acetyltransferase. A putative leucine heptad region (LLLL) present in xFF1rA is indicated. (B) Transcriptional activation by xFF1rA depends on the number of response elements. HeLa cells were transfected with 3 μ g of the reporter plasmid as indicated and either 6 μ g of pSG5 or 4 μ g of pSG5-xFF1rA. CAT activities ($\pm$ standard deviation) from duplicate transfections are shown as bars, and the fold induction for each reporter construct is shown at the bottom. The results were reproduced in at least three independent experiments.

otides was detected (Fig. 5, lanes 7 to 10), which is of the same size as the fragment obtained after hybridization with control sense RNA derived from the cDNA clone (Fig. 5, lane 12). The expression profile of the RNAs giving rise to this fragment closely resembles that of the summed profiles of the four

transcripts detected by Northern blot analysis (Fig. 4B and 5C), with a great increase during neurulation (Fig. 5, lanes 7 and 8) and a maximum at the early tailbud stage (Fig. 5, lane 9).

The 5' probe which consists of 5'-untranslated sequences encoded by the xFF1rA cDNA (Fig. 1A) gave rise to two fragments (Fig. 5, lanes 3 to 6), one of 323 nucleotides corresponding to the expected size (compare with lane 11) and another one of 207 nucleotides, which reveals a splice site between the *Pvu*II site and the *Sac*I site in the 5' end of the xFF1rA cDNA. The amount of the 323-nucleotide fragment increased only slightly during the course of development. In contrast, the expression pattern of the mRNA splice variants giving rise to the 207-nucleotide fragment correlates well with the Northern blot analysis (Fig. 4B and 5C).

Taken together, these data show that first, at least four transcripts encoding xFF1rA proteins differently spliced in the 5'-untranslated region are expressed during the early development of *X. laevis*, with a great increase of the steady-state level between early gastrula and neurula, a maximum at the early tailbud stages, and a decrease at later stages. On Northern blots, this maximum is seen per embryo as well as relative to the amount of actin mRNA (Fig. 4A, B, and C). At least three of these transcripts most probably encode a full-length xFF1rA protein, because they are detected by both the 3' and the 5' probes.

xFTZ-F1-related protein is found in *X. laevis* embryos. To investigate the expression and the function of the xFF1rA protein, antisera were raised against bacterially expressed fusion proteins containing different regions of the xFF1rA protein (Fig. 1A). The resulting rabbit antisera against the A/B domain (anti-xFF1rA[AB]) and against part of the DEF domain (anti-xFF1rA[DEF Δ]) and a mouse serum against the DEF domain (anti-xFF1rA[DEF]), were tested by immunoprecipitation of in vitro-translated xFF1rA. All three sera specifically precipitated xFF1rA (Fig. 6).

FIG. 4. Northern blot analysis reveals a complex pattern of expression of xFF1r mRNAs in *Xenopus* embryos at different stages of development. (Upper panels) Antisense RNA probes corresponding to either 5'-untranslated sequences (A), the 3' end of the xFF1rA cDNA (B), or the whole xFF1rA cDNA (C) as specified in Fig. 1A, were hybridized to poly(A)⁺ RNA equivalent to 10 (A and B) or 5 (C) embryos of the stages indicated below each lane. (Lower panels) Rehybridization of the same filters with chicken β -actin cDNA, reacting with α -, β -, and γ -actin of *X. laevis*, as indicated on the right. Embryos were staged by the method of Nieuwkoop and Faber (39); g, gastrula stage 10.5; n, midneurula stages 16 to 17; et, early tailbud stages 21 to 23; t, late tailbud stages 28 to 30. The positions of RNA molecular weight standards are indicated on the right.

FIG. 5. RNase protection analysis reveals a splice site in the 5'-untranslated region. Antisense RNA probes corresponding to either 5'-untranslated sequences (lanes 3 to 6) or the 3' end of the xFF1rA cDNA (lanes 7 to 10), as indicated in Fig. 1A, were hybridized to total RNA equivalent to 2.5 embryos of the stages as indicated and were processed as described in Materials and Methods. en, early neurula stage 13; n, late neurula stage 18; et, early tailbud stage 23; t, late tailbud stage 28. As a positive control, the 5' probe (lane 11) and 3' probe (lane 12) were hybridized to 10 pg of sense RNA synthesized in vitro. Lane 1, undigested probes; lane 2, mixture of both antisense RNA probes digested after hybridization to 5 μ g of yeast tRNA; all signals in lane 2 result from the 3' probe (not shown). The sizes of the undigested probes and protected fragments are indicated on the right; nt, nucleotides.

In HeLa cells transiently transfected with pSG5-xFF1rA, cell nuclei were stained by the anti-xFF1rA[AB] and by the anti-xFF1rA[DEF Δ] rabbit serum (data not shown).

Since transcription of xFF1rA mRNAs greatly increased during early development, we investigated whether functional xFF1r protein is also present in *Xenopus* embryos. For this purpose, band shift assays were performed with a nuclear extract of embryos at the early tailbud stage and radiolabelled FRE-fDE1 as a probe. A specific band shift complex was detected, which is due to endogenous xFF1r protein since the complex has the same mobility as the complex formed with the vaccinia virus-expressed recombinant xFF1rA (Fig. 7A) and its formation is inhibited by an excess of unlabelled FRE but not with an excess of RARE (Fig. 7B). In addition, the complex was completely supershifted by the mouse anti-xFF1rA[DEF] serum and by the rabbit anti-xFF1rA[DEF Δ] serum but not by the preimmune control serum (Fig. 7B, lanes 4 to 6).

Further support that *Xenopus* embryos at the early tailbud stage do express xFF1r orphan receptor protein is given by the detection of a nuclear antigen in tailbud stage embryos

FIG. 6. Characterization of xFF1rA antisera by immunoprecipitation. (A) [35 S]methionine-labelled proteins translated in vitro from Brome mosaic viral mRNAs (BMV, lane 1) and xFF1rA mRNA (FF1r, lane 2) were separated by SDS-polyacrylamide gel electrophoresis and revealed by fluorography. (B) Immunoprecipitation of [35 S]-labelled FF1rA or BMV protein as a control (7×10^5 cpm each) using the following antibodies: 0.5 μ l of immune serum of rabbit anti-xFF1rA [AB] (lanes 1 and 3); rabbit anti-xFF1rA[DEF Δ] (lanes 4 and 6); mouse anti-xFF1rA[DEF] (lanes 7 and 8); and 0.5 μ l of preimmune serum (PI) of rabbit xFF1rA[AB] (lane 2) or rabbit xFF1rA[DEF Δ] (lane 5). The positions and sizes (in kilodaltons) of marker proteins are indicated on the right. Note that the anti-A/B serum only precipitated two protein bands with an apparent molecular mass close to 55 kDa (lane 3), whereas sera against the DEF region also precipitated partial products resulting from internal translational starts (compare A, lane 2, with B, lanes 6 and 8). None of the preimmune sera precipitated the receptor (lanes 2 and 5).

by whole-mount staining with affinity-purified anti-xFF1rA [DEF Δ] antibodies (Fig. 8A) and by staining of frozen sections with the same antiserum and with the anti-A/B antiserum (data not shown). The antigens are found in all nuclei of the early tailbud embryos as seen by counterstaining with 4'-6-diamidino-2-phenylindole (DAPI) (not shown). As in transfected cells expressing xFF1rA, the nucleoli are devoid of the antigen (Fig. 8).

DISCUSSION

Evolution of the FTZ-F1 group within the nuclear hormone receptor superfamily. We have identified FTZ-F1-related nuclear orphan receptors in a lower vertebrate. The xFF1rA and B isoforms may represent pseudoalleles resulting from the evolutionary duplication of the *X. laevis* genome. All vertebrate FTZ-F1-related nuclear receptors, the ones described herein included, are homologs of the DmFTZ-F1 protein (31). The homology between the DmFTZ-F1 and its vertebrate counterparts becomes most obvious from the comparison of the DNA binding domain, including domain C and the adjacent FTZ-F1 box, and the conserved regions II and III of the E domain (54). The comparatively low percent amino acid identity within the E domains might indicate that the FTZ-F1-related receptors have no ligand in common, in agreement with their constitutive function as transcriptional regulators.

Within the vertebrate FTZ-F1-related receptors, xFF1rA is 84% identical on the amino acid level to its closest relative mLRH-1, forming the first subgroup. Similarly, in the second

FIG. 7. FRE-binding protein is found in embryos of *X. laevis*. (A) A specific complex (arrowhead) of the same mobility was formed between FRE-fDE1 and either vaccinia virus-expressed xFF1rA (lanes 1 and 2) or nuclear protein extracted from embryos of early tailbud stages 21 to 23 (lanes 3 and 4). The upper band seen in lane 1 was observed only after freezing and thawing of the nuclear extract. (B) Gel mobility shift assay of nuclear extract from embryos at the early tailbud stages 21 to 23. Radiolabelled FRE-fDE1 was incubated with nuclear extract and analyzed in the absence (lane 1) and presence (lane 2) of a 25-fold molar excess of FRE-fDE1 or a 25-fold excess of RARE (lane 3). Induction of supershift was tested by using 0.1 μ l anti-xFF1rA[DEF] mouse serum (ms DEF; lane 4), 0.1 μ l of anti-xFF1rA[DEF Δ] rabbit serum (rs DEF; lane 6), or 0.1 μ l of preimmune serum of the xFF1rA[DEF Δ] rabbit (rs PI; lane 5).

subgroup, the mELP and the bAd4BP are closely related up to about the middle of domain E; however, mELP shows no similarity to region III of other receptors. A mouse homolog of bAd4BP, mSF-1, has recently been identified as a splice variant originating from the mELP gene, which does contain region III (19). Thus the *Drosophila* FTZ-F1 gene could be viewed as the arthropod homolog of both subgroups which arose by a gene duplication specific to the vertebrate lineage. Such gene duplications have occurred during nuclear receptor gene evolution, as previously illustrated by the RXR or chicken ovalbumin upstream promoter (COUP) groups of receptors (6, 11) as well as in other transcription factor families such as the *ets*, HMG, or Hox families (28, 29, 44).

Our analysis reveals that the FTZ-F1-related genes form a distinct and new group of nuclear receptors. Interestingly, this group belongs to subfamily III (GR, ER, KNI, and EcR/VDR groups) on the basis of the C domain comparison, whereas it belongs to subfamily II (RXR, COUP, HNF4, and TLL groups) on the basis of the Ti-DM domain comparison for the construction of a phylogenetical tree (27). It is not known if this observation, already made for the VDR, EcR, and NGFI-B, reflects different evolutionary origins of presently associated domains or if it is due to a high convergence of one of the domains, for instance, the short C domain. Thus, the precise placement of the FTZ-F1-related receptors in the

superfamily must await the sequencing of other members of the group, especially from "primitive" organisms. Also, we cannot presently decide whether DHR39 (1, 40) is a member of the FTZ-F1 subgroup, because statistical analysis resulted in 66% confidence for the monophyly between the two insect receptors (Fig. 1D). No vertebrate homolog of DHR39 has been isolated so far, despite the fact that FTZ-F1 and DHR39 apparently diverged before the appearance of vertebrates.

The orphan receptor xFF1rA is an activator of transcription. The vertebrate FTZ-F1-related receptors function as activators (bAd4BP [18], mSF-1 [19]) and as repressors (mELP [51]) of transcriptional activity. In *D. melanogaster*, FTZ-F1 can bind to the *zebra* element, which contains both positive and negative regulatory elements and is located 5' upstream of the *fz* gene (31, 49). Therefore, we selected the FRE-fDE1 motif from the *zebra* element as the response element for most of our functional studies. This FRE-fDE1 consisting of a monomeric HRE consensus half-site extended by three nucleotides at its 5' side (TCAAGGTCG) is specifically bound in vitro by xFF1rA. xFF1rA, unlike the RXR-dependent RAR and other type II receptors, does not appear to require cofactors for binding to an FRE in vitro. We have compared the mobility of complexes formed between β RARE and xRAR γ 2 in the presence of hRXR β to that of complexes formed between FRE-fDE1 and xFF1rA. The latter migrated significantly faster, indicating that

FIG. 8. xFTZ-F1-related nuclear antigens in embryos. Embryos at the early tadpole stage 23 were stained with affinity-purified anti-xFF1rA[DEFA] (A) or preimmune serum (B). The dorsal part of transverse sections from the region of the anterior neural tube is shown. nt, neural tube; n, notochord; s, somite. The yolk platelets are seen on these black and white prints because of their yellow autofluorescence. Bar, 100 μ m.

xFF1rA binds as a monomer (data not shown). This is consistent with the monomeric structure of the FRE and the results from DmFTZ-F1 (52), mELP (50), and bAd4BP (18). Similar observations about RevErbA α have been published (17). Nevertheless, potential interfaces for protein-protein interactions have been noted in the E domains of all of these orphan receptors, with the exceptions of mELP and DHR39short, a C-terminally truncated form of DHR39 (1).

In cotransfection experiments using FRE-containing reporter genes, xFF1rA acts as a transcriptional activator. Although a single copy of the FRE-fDE1 was sufficient for binding in the band shift assay and yielded a weak yet reproducible stimulation of transcription, two or more copies were much more efficient for transcriptional enhancement. In functional assays of the mELP (51) and the bAd4BP (18), only reporter constructs containing eight or four copies of the FRE, respectively, were used. However, more recently, in R2C cells transfected with reporter constructs containing either one or two copies of an FRE, the latter were more efficiently activated by an endogenous factor, probably identical with SF-1 (10, 34). Therefore the other members of the group may also require several copies of the FRE for strong regulatory effects on the transcription of target genes. The higher efficiency of multiple FREs suggests a possible cooperativity by unknown mechanisms between xFF1rA receptors. Multiple copies of functional FRE within the promoters of chromosomal target genes for FTZ-F1-related receptors have not been described. In the 5' upstream region of CYP genes that are potential target genes of Ad4BP, multiple elements each containing only one AGGTCA/G consensus half-site are arranged at a distance, yet only one strict FRE consensus per gene was found (26). Interestingly, the CYP21 (steroid 21 hydroxylase) gene, in addition to a single FRE, contains a single NBRE in its 5' upstream region (56).

Expression of FTZ-F1-related orphan receptors in *X. laevis* embryos. The expression of the orphan receptor xFF1rA has been studied during embryogenesis of *X. laevis*. On Northern blots, at least four different transcripts greatly increase in amount after the onset of zygotic transcription and reach a maximum during early organogenesis. The complex pattern may potentially be explained by the existence of two closely related but different genes in *X. laevis*, xFF1rA and B, and alternative splicing of primary transcripts. In agreement with

this notion, a single product was obtained after reverse-transcription PCR (RT-PCR) with primers that were deliberately chosen from regions of the E domain that are identical between xFF1rA and B (data not shown). On Northern blots and by RT-PCR we also detected xFF1r transcripts in oocytes, liver, and kidney (data not shown).

On developmental Northern blots (Fig. 3) and by RT-PCR (data not shown) a maximum number of transcripts encoding the full-length receptors is observed during early tailbud stages. Nuclear extracts from embryos at this developmental stage also contain functional FRE-binding protein that is recognized by antisera raised against xFF1rA. Our immunohistological data suggest that the FTZ-F1-related receptors are found in all nuclei of the embryo. In transfected HeLa cells, xFF1rA antigens were localized in the nuclei and this result was not influenced by charcoal treatment of the serum added to the culture medium or by omission of phenol red, indicating that nuclear translocation was not dependent on exogenous ligands (data not shown).

Conclusions. At least seven orphan receptors, including the two described herein, are members of the FTZ-F1-related receptor subgroup. Five of these receptors have been isolated from vertebrates and all of them share a common ancestor with the *D. melanogaster* FTZ-F1 receptor. All these receptors seem to bind as monomers to a single AGGTCA/G motif preceded by PyCA. The contacts between these PyCA nucleotides seem to involve the FTZ-F1 box, located adjacent to the DNA binding domain C (55). As response elements, the FTZ-F1-related receptors share the 6-bp half-site monomeric motif extended by three nucleotides with NGFI-B and RevErbA α , but the additional nucleotides (17, 55, 56) differ between the three types of response elements, as do the crucial amino acids adjacent to the C domain of the three receptors. Therefore, several distinct groups of nuclear receptors that bind DNA as a monomer are likely to emerge and may be constitutively active (17, 43). Despite the binding of monomers in vitro, cooperative effects may enhance transcriptional regulation. The expression pattern of the xFF1r receptors, together with that of the other members of the FTZ-F1 group, indicates that these transcription factors are required in early embryonic development but also in late embryogenesis and in adult animals.

ACKNOWLEDGMENTS

We thank Ricardo Wittek for advice on the vaccinia virus system and Douglas Melton for the cDNA library. We thank Brigitte Gläser for expert technical assistance, Kathrin Brenner and her colleagues for photographic work, and Nicolas Mermod, Beatrice Devergne, and Sheryl van der Poel for critically reading the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft and the Swiss National Science Foundation.

REFERENCES

- Ayer, S., N. Walker, M. Mosammaparast, J. P. Nelson, B. Shilo, and C. Benyajati. 1993. Activation and repression of *Drosophila* alcohol dehydrogenase distal transcription by two steroid hormone receptor superfamily members binding to a common response element. *Nucleic Acids Res.* **21**:1619–1627.
- Bertholet, C., R. Drillien, and R. Wittek. 1985. One hundred base pairs of 5' flanking sequence of a vaccinia virus late gene are sufficient to temporally regulate late transcription. *Proc. Natl. Acad. Sci. USA* **82**:2096–2100.
- Bugge, T. H., J. Pohl, O. Lonnoy, and H. Stunnenberg. 1992. RXR α , a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J.* **11**:1409–1418.
- Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**:173–181.
- Devereux, J., P. Haeblerli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395.
- Dreyer, C., H. Keller, A. Mahfoudi, V. Laudet, G. Krey, and W. Wahli. 1993. Positive regulation of the peroxisomal β -oxidation pathway by fatty acids through activation of peroxisome proliferator-activated receptors (PPAR). *Biol. Cell* **77**:67–76.
- Ellinger-Ziegelbauer, H., and C. Dreyer. 1991. A retinoic acid receptor expressed in early development of *Xenopus laevis*. *Genes Dev.* **5**:94–104.
- Ellinger-Ziegelbauer, H., and C. Dreyer. 1993. The pattern of retinoic acid receptor γ (RAR γ) expression in normal development of *Xenopus laevis* and after manipulation of the main body axis. *Mech. Dev.* **41**:31–46.
- Felsenstein, J. 1988. Phylogenesis from sequences: inference and reliability. *Annu. Rev. Genet.* **22**:521–565.
- Fitzpatrick, S. L., and J. S. Richards. 1993. *Cis*-acting elements of the rat aromatase promoter required for cyclic adenosine 3',5'-monophosphate induction in ovarian granulosa cells and constitutive expression in R2C Leydig cells. *Mol. Endocrinol.* **7**:341–354.
- Fjose, A., S. Nornes, U. Weber, and M. Mlodzik. 1993. Functional conservation of vertebrate seven-up related genes in neurogenesis and eye development. *EMBO J.* **12**:1403–1414.
- Forman, B. M., and H. H. Samuels. 1990. Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol.* **4**:1293–1301.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044–1051.
- Gorski, K., M. Carneiro, and U. Schibler. 1986. Tissue-specific in vitro transcription from the mouse albumin promoter. *Cell* **47**:767–776.
- Graham, F., and A. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–457.
- Hakes, D. J., and J. E. Dixon. 1992. New vectors for high level expression of recombinant proteins in bacteria. *Anal. Biochem.* **202**:293–298.
- Harding, H. P., and M. A. Lazar. 1993. The orphan receptor rev-erbA α activates transcription via a novel response element. *Mol. Cell. Biol.* **13**:3113–3121.
- Honda, S., K. Morohashi, M. Nomura, H. Takeya, M. Kitajima, and T. Omura. 1993. Ad4BP regulating steroidogenic P-450 gene is a member of the steroid hormone receptor superfamily. *J. Biol. Chem.* **268**:7494–7502.
- Ikeda, J., D. S. Lala, X. Luo, E. Kim, M.-P. Moisan, and K. L. Parker. 1993. Characterization of the mouse *FTZ-F1* gene, which encodes a key regulator of steroid hydroxylase gene expression. *Mol. Endocrinol.* **7**:852–860.
- Keller, H., D. Dreyer, J. Medin, A. Mahfoudi, K. Ozato, and W. Wahli. 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. USA* **90**:2160–2164.
- Kintner, C. R., and D. A. Melton. 1987. Expression of *Xenopus* N-CAM mRNA in ectoderm is an early response to neural induction. *Development* **99**:311–325.
- Kliwer, S. A., K. Umehono, D. J. Mangelsdorf, and R. M. Evans. 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D₃ signalling. *Nature (London)* **355**:446–449.
- Kobel, H. R., and L. Du Pasquier. 1986. Genetics of polyploid *Xenopus*. *Trends Genet.* **2**:310–315.
- Krieg, P. A., and D. A. Melton. 1987. *In vitro* RNA synthesis with SP6 polymerase. *Methods Enzymol.* **155**:397–415.
- Krust, A., S. Green, P. Argos, V. Kumar, P. Walter, J. Bornert, and P. Chambon. 1986. The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO J.* **5**:891–897.
- Lala, D. S., D. A. Rice, and K. L. Parker. 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of *fushi tarazu*-factor I. *Mol. Endocrinol.* **6**:1249–1258.
- Laudet, V., C. Hänni, J. Coll, F. Catzeflies, and D. Stehelin. 1992. Evolution of the nuclear receptor gene superfamily. *EMBO J.* **11**:1003–1013.
- Laudet, V., C. Niel, M. Duterque-Coquillaud, D. Leprince, and D. Stehelin. 1993. Evolution of the *ets* gene family. *Biochem. Biophys. Res. Commun.* **190**:8–14.
- Laudet, V., D. Stehelin, and H. Clevers. 1993. Ancestry and diversity of the HMG box superfamily. *Nucleic Acids Res.* **21**:2493–2501.
- Lavorgna, G., F. D. Karim, C. S. Thummel, and C. Wu. 1993. Potential role for a FTZ-F1 steroid receptor superfamily member in the control of *Drosophila* metamorphosis. *Proc. Natl. Acad. Sci. USA* **90**:3004–3008.
- Lavorgna, G., H. Ueda, J. Clos, and C. Wu. 1991. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of *fushi tarazu*. *Science* **252**:848–851.
- Lee, C., A. Levin, and D. Branton. 1987. Copper staining: a five-minute protein stain for sodium dodecylsulfate-polyacrylamide gels. *Anal. Biochem.* **166**:308–312.
- Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J. Y. Chen, A. Staub, J. M. Garnier, S. Mader, and P. Chambon. 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR and TR heterodimerizes to bind to target sequences. *Cell* **68**:377–395.
- Lynch, J. P., D. S. Lala, J. J. Peluso, W. Luo, K. L. Parker, and B. A. White. 1993. Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. *Mol. Endocrinol.* **7**:776–786.
- MacGregor, G. R., and C. T. Caskey. 1989. Construction of plasmids that express *E. coli* β -galactosidase in mammalian cells. *Nucleic Acids Res.* **17**:2365.
- Marks, M. S., P. L. Hallenbeck, T. Nagata, J. H. Segars, E. Appella, V. M. Nikodem, and K. Ozato. 1992. H-2RIIBP (RXR β) heterodimerization provides a mechanism for combinatorial diversity in the regulation of retinoic acid and thyroid hormone responsive genes. *EMBO J.* **11**:1419–1435.
- Martinez, E., F. Givel, and W. Wahli. 1991. A common ancestor DNA motif for invertebrate and vertebrate hormone response elements. *EMBO J.* **10**:263–268.
- Miesfeld, R., S. Rusconi, P. J. Godowski, B. A. Maler, S. Okret, A. C. Wikström, J. A. Gustafsson, and K. R. Yamamoto. 1986. Genetic complementation of a glucocorticoid receptor deficiency by expression of a cloned receptor cDNA. *Cell* **46**:389–399.
- Nieuwkoop, P. D., and J. Faber. 1967. Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam.
- Ohno, C. K., and M. Petkovich. 1992. FTZ-F1 β , a novel member of the *Drosophila* nuclear receptor superfamily. *Mech. Dev.* **40**:13–24.
- O'Malley, B. W., and O. M. Conneely. 1992. Orphan receptors: in

- search of a unifying hypothesis of activation. *Mol. Endocrinol.* **6**:1359–1369.
42. **Parker, M. G.** 1993. Steroid and related receptors. *Curr. Opin. Cell Biol.* **5**:499–504.
 43. **Paulsen, R. E., C. A. Weaver, T. J. Fahrner, and J. Milbrandt.** 1992. Domains regulating transcriptional activity of the inducible orphan receptor NGFI-B. *J. Biol. Chem.* **267**:16491–16496.
 44. **Schubert, F. R., K. Nieselt-Struwe, and P. Gruss.** 1993. The Antennapedia-type homeobox genes have evolved from three precursors separated early in metazoan evolution. *Proc. Natl. Acad. Sci. USA* **90**:143–147.
 45. **Stewart, C. B.** 1993. The powers and pitfalls of parsimony. *Nature (London)* **361**:603–607.
 46. **Stunnenberg, H. G.** 1993. Mechanisms of transactivation by retinoic acid receptors. *Bioessays* **15**:309–315.
 47. **Swofford, D. L.** 1992. PAUP: Phylogenetic Analysis Using Parsimony, version 3.0s. Computer program and manual distributed by the Center for Biodiversity, Illinois Natural History Survey, Champaign.
 48. **Thomas, H. E., H. G. Stunnenberg, and A. F. Stewart.** 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and ultraspiracle. *Nature (London)* **362**:471–475.
 49. **Topol, J., C. R. Dearwolf, K. Prakash, and C. S. Parker.** 1991. Synthetic oligonucleotides recreate *Drosophila fushi tarazu* zebra-stripe expression. *Genes Dev.* **5**:855–867.
 50. **Tsukiyama, T., and O. Niwa.** 1992. Isolation of high affinity cellular targets of the embryonal LTR binding protein, an undifferentiated embryonal carcinoma cell-specific repressor of Moloney leukemia virus. *Nucleic Acids Res.* **20**:1477–1482.
 51. **Tsukiyama, T., H. Ueda, S. Hirose, and O. Niwa.** 1992. Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. *Mol. Cell. Biol.* **12**:1286–1291.
 52. **Ueda, H., G. C. Sun, T. Murata, and S. Hirose.** 1992. A novel DNA-binding motif abuts the zinc finger domain of insect nuclear hormone receptor FTZ-F1 and mouse embryonal long terminal repeat-binding protein. *Mol. Cell. Biol.* **12**:5667–5672.
 53. **Wahli, W., and E. Martinez.** 1991. Superfamily of steroid nuclear receptors: positive and negative regulators of gene expression. *FASEB J.* **5**:2243–2249.
 54. **Wang, L. H., S. Y. Tsai, R. G. Cook, W. G. Beattie, M. J. Tsai, and B. W. O'Malley.** 1989. COUP transcription factor is a member of the steroid receptor superfamily. *Nature (London)* **340**:163–166.
 55. **Wilson, T. E., T. J. Fahrner, and J. Milbrandt.** 1993. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third paradigm of nuclear receptor-DNA interaction. *Mol. Cell. Biol.* **13**:5794–5804.
 56. **Wilson, T. E., A. R. Mouw, C. A. Weaver, J. Milbrandt, and K. L. Parker.** 1993. The orphan receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Mol. Cell. Biol.* **13**:861–868.
 57. **Wilson, T. E., R. E. Paulsen, K. A. Padgett, and J. Milbrandt.** 1992. Participation of non-zinc residues in DNA binding by two nuclear orphan receptors. *Science* **256**:107–110.
 58. **Yao, T. P., W. S. Segraves, A. E. Oro, M. McKeown, and R. M. Evans.** 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. *Cell* **71**:63–72.
 59. **Yu, V. C., C. Delsert, B. Anderson, J. M. Holloway, O. V. Devary, A. M. Näär, S. Y. Kim, J. M. Boutin, C. K. Glass, and M. G. Rosenfeld.** 1991. RXR β : a coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. *Cell* **67**:1251–1266.
 60. **Zhang, X. K., B. Hoffmann, P. B. V. Tran, G. Graupner, and M. Pfahl.** 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature (London)* **355**:441–446.