MOLECULAR AND CELLULAR BIOLOGY, May 1994, p. 3166–3175
0270-7306/94/$04.00+0
Copyright © 1994, American Society for Microbiology
Vol. 14, No. 5

The *Drosophila* Nuclear Receptors FTZ-F1α and FTZ-F1β Compete as Monomers for Binding to a Site in the *fushi tarazu* Gene

CAROLYN K. OHNO,[1,2] HITOSHI UEDA,[3] AND MARTIN PETKOVICH[1,2,4]*

Cancer Research Laboratories[1] and Departments of Biochemistry[2] and Pathology,[4] Queen's University, Kingston, Ontario, Canada K7L 3N6, and Genetic Stock Research Center, National Institute of Genetics and Department of Genetics, The Graduate University for Advanced Studies, Mishima, Shizuoka-ken 411, Japan[3]

Received 17 November 1993/Returned for modification 26 January 1994/Accepted 23 February 1994

The striped pattern of *fushi tarazu* (*ftz*) expression found in the blastoderm of the *Drosophila melanogaster* embryo is generated largely through complex interactions between multiple transcription factors that bind to the zebra element of the *ftz* gene. A motif in the zebra element, the FTZ-F1 recognition element (F1RE), has been shown to bind a transcription factor, FTZ-F1α, that is a member of the nuclear receptor family. We recently identified a second, related member of this family, FTZ-F1β, that also binds to this motif. To investigate the possibility that FTZ-F1α and FTZ-F1β coregulate *ftz* transcription through the F1RE, we have studied the DNA binding properties of FTZ-F1α and FTZ-F1β. We demonstrate that recombinant FTZ-F1α and FTZ-F1β proteins produce similar in vitro DNase I footprint patterns on a 14-nucleotide region of the zebra element and bind to this site with similar affinities and sequence specificities. Using wild-type and N-terminally truncated receptors, we have determined that FTZ-F1α and FTZ-F1β both bind as monomers to the 9-bp F1RE in the zebra element, as well as to an imperfect inverted F1RE repeat present in the *Drosophila* alcohol dehydrogenase gene. A polyclonal antibody raised against FTZ-F1β identifies a predominant F1RE-binding component in embryonic nuclear extracts. Although FTZ-F1α is also present in these extracts, FTZ-F1α and FTZ-F1β do not appear to form heterodimers with each other. Cotransfection assays in mammalian cell culture indicate that both receptors contribute to the net transcriptional activity of a reporter gene through their direct interaction with the F1RE. These data suggest that FTZ-F1α and FTZ-F1β likely coregulate common target genes by competition for binding to a 9-bp recognition element.

During early development of the *Drosophila* embryo, generation of the segmented body pattern is controlled by a cascade of genetic and molecular events involving both maternal and zygotic factors (1, 34, 41). The zygotic segmentation gene, *ftz*, is expressed in the early blastoderm in a distinct seven-stripe pattern which is the result of precise, temporally and spatially restricted transcription (12). The minimal promoter and upstream sequences required for this *ftz* expression pattern in the mesodermal primordia have been mapped to the 0.7-kb zebra element (at positions −669 to +73 of the *ftz* gene) (17, 18). Through biochemical analyses, a number of nuclear proteins which interact with *cis*-acting DNA motifs within this region have been identified (14, 45). Included among these *trans*-acting nuclear factors are *tramtrack* (*ttk*)/FTZ-F2 (5, 15), *caudal* (6), FTZ-F1 (herein referred to as FTZ-F1α) (28), and FTZ-F1β (35).

FTZ-F1α and FTZ-F1β belong to the superfamily of nuclear receptors that bind to signalling molecules which include steroid hormones, thyroid hormone, vitamin D, and retinoids and in turn alter the transcription of specific target genes (references 2, 26, and 42 and references therein). As is characteristic of other members of this family, each has a highly conserved DNA binding domain comprising two zinc finger motifs. Whether FTZ-F1α and FTZ-F1β bind specific ligands is presently unknown, although both show some conservation in their C-terminal regions with other ligand-binding members of this family (35).

* Corresponding author. Mailing address: Cancer Research Laboratories, Botterell Hall, Queen's University, Kingston, Ontario, Canada K7L 3N6. Phone: (613) 545-6791. Fax: (613) 545-6830.

FTZ-F1α and FTZ-F1β are expressed both maternally and zygotically during early embryonic development. Two FTZ-F1α isoforms that differ in their N-terminal regions have been identified, suggesting that splicing variants of this receptor may be specifically regulated in a manner similar to that seen for the retinoic acid (reference 37 and references therein), thyroid hormone (20), and progesterone (46) receptors. These FTZ-F1α isoforms are differentially expressed in early (2- to 4-h) and late (14- to 22-h) embryos (27). The expression of FTZ-F1α transcripts throughout the early blastoderm disappears in 4-h embryos but reappears in older embryos in a more spatially restricted manner, with high levels of expression in the proventriculus and anal plate (35). In contrast, FTZ-F1β appears to be expressed continuously throughout embryonic development, although its expression at later stages appears to be limited to the central nervous system (35).

Mutations in the loci for either FTZ-F1α or FTZ-F1β have not yet been characterized; however, several studies suggest that these nuclear receptors may play an important role in the regulation of *ftz* gene expression. FTZ-F1α was identified in *Drosophila* nuclear extracts by its sequence-specific interaction with an FTZ-F1 recognition element (F1RE), found between positions −299 and −267 in the zebra element of the *ftz* promoter (28). Overlapping positive and negative regulatory elements have been mapped to this region of the zebra element by studies using promoter deletion constructs in transformant flies (7, 45). Constructs harboring mutations in the F1RE showed that this site was important for the efficient generation of stripes 1, 2, 3, and 6 (49). The *Drosophila FTZ-F1β* gene was cloned in our laboratory by its sequence homology with FTZ-F1α. At the amino acid level, the zinc

finger DNA binding domain of FTZ-F1β most closely resembled that of FTZ-F1α, and in our previous studies we found that both receptors could bind efficiently to F1RE (35). In addition, Ad4BP (19) and ELP/SF-1 (24, 47), the mammalian homologs of FTZ-F1α, exhibit a high degree of specificity for elements in mammalian genes that conform to the F1RE consensus, indicating that both the DNA binding domains and the cognate element evolved in a concerted manner. These findings are consistent with the possibility that both FTZ-F1α and FTZ-F1β are potential regulators of the *ftz* gene, a hypothesis also supported by the observation that spatial domains of FTZ-F1α and FTZ-F1β mRNA transcripts overlap with those of *ftz* in early embryos (35).

In the present study, we examined the possibility that FTZ-F1α and FTZ-F1β might interact. First, we wanted to further define and compare the DNA binding specificities of FTZ-F1α and FTZ-F1β, and second, we wanted to examine whether these receptors formed homo- or heterodimers. Finally, we compared the transcription-activating functions of these receptors alone or in combination to determine whether FTZ-F1α and FTZ-F1β could act synergistically or antagonistically on the *ftz* F1RE. We show that FTZ-F1α and FTZ-F1β bind as monomers either to oligonucleotides corresponding to the F1RE or to a similar site present as an inverted repeat in the *Drosophila* alcohol dehydrogenase (*Adh*) gene which was demonstrated previously to bind FTZ-F1α and FTZ-F1β (3). We also show that antagonism between these two receptors contributes to the net F1RE-dependent transcription of a reporter gene in cotransfection assays. Our results suggest that common target genes in *Drosophila melanogaster* may be coregulated at the transcriptional level by a mechanism of competition between FTZ-F1α and FTZ-F1β monomers for binding to a common element.

MATERIALS AND METHODS

Plasmid constructs. A full-length cDNA encoding FTZ-F1α (28) (FTZ-F1 early isoform) was subcloned into the mammalian expression vector pTL2 (a derivative of pSG5 [11] that contains an expanded *Bgl*II-*Kpn*I-*Sac*I-*Pst*I-*Sma*I-*Not*I-*Xho*I-*Hin*dIII-*Bam*HI-*Eco*RI polylinker sequence and was constructed by T. Lufkin). The mammalian expression vector for FTZ-F1β was constructed by subcloning the *Sac*I-*Apa*I cDNA fragment of clone H2a described previously (35), encoding the 808-amino-acid open reading frame, into pBluescriptSK (Stratagene), and subsequently the *Sac*I-*Kpn*I fragment from this intermediate vector was cloned into pTL1 (a vector identical to pTL2 described above except that the polylinker sequence is inverted) to generate TLFTZ-F1β. The reporter vectors F1RE-SV40-Luc and 2F1RE-SV40-Luc contain one and two copies, respectively, of the 33-bp F1RE-binding site upstream of the simian virus 40 (SV40) early promoter driving the firefly luciferase reporter gene. The reporter constructs were created by blunt-end ligation of the synthetic F1RE site into the *Sma*I site of pGL2-promoter (Promega).

Truncated FTZ-F1α constructs, FF1α1 and FF1α3, contain *Eco*RI cDNA fragments encoding amino acids 192 to 1043 and 575 to 1043, respectively, subcloned into pGem7Zf+ (Promega). The FF1α2 construct encoding amino acids 328 to 1043 was generated by ligation of a *Bgl*II-*Sac*I linker (5′-GATCTCCACCATGAGCT-3′) containing a mammalian consensus translation initiation sequence (23) into *Bgl*II-*Sac*I-digested TLFTZ-F1α. The FF1β1 and FF1β2 truncation constructs encoding amino acids 144 to 808 and 328 to 808 of FTZ-F1β, respectively, contain FTZ-F1β cDNA *Eco*RI fragments subcloned into pBluescriptSK.

For DNase I protection assays, a 0.9-kb *Pst*I fragment was isolated from *ftz* subclone BS2.5 (a gift from A. Laughon) and cloned into the *Pst*I site of pBluescriptSK. A 0.3-kb *Bsi*HKA fragment corresponding to positions −297 to +28 relative to the *ftz* transcriptional start site was isolated from this intermediate plasmid and inserted into the *Hin*cII site of pBluescriptSK (with the *Bsi*HKA site made blunt ended with Klenow fragment) to generate the plasmid ftzSK-8.

Bacterial expression of FTZ-F1α and FTZ-F1β and production of FTZ-F1β antiserum. Expression of malE–FTZ-F1α and malE–FTZ-F1β fusion proteins in *Escherichia coli* was performed as previously described (35). Expression of FTZ622 (corresponding to an FTZ-F1α minimal DNA binding domain, amino acids 507 to 622) in *E. coli* by the T7 phage expression system was as described elsewhere (50).

For the production of the rabbit polyclonal anti-FTZ-F1β antibody, malE–FTZ-F1β fusion protein was purified by affinity chromatography on an amylose resin according to the manufacturer's directions (New England Biolabs). The fusion protein was concentrated by microconcentration (Microcon 30; Amicon, Inc.) and resuspended in phosphate-buffered saline (PBS). Purified protein (150 μg) was injected with Freund's adjuvant (GIBCO) into two New Zealand White rabbits. Additional boosters (50 μg) were given at 3-week intervals. Preimmune and immune sera were tested in gel mobility shift assays.

In vitro transcription and translation. Each plasmid containing receptor cDNA was linearized with the appropriate restriction enzyme and used as a template for in vitro transcription of capped RNA transcripts with T7 or Sp6 RNA polymerase according to the manufacturer's specifications (Promega or Ambion). In vitro translation was performed in rabbit reticulocyte lysate (Promega) as instructed by the manufacturer in the presence of [^{35}S] methionine (ICN) or unlabeled methionine, and the labeled translation products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.

Gel mobility shift assays. DNA binding by bacterially expressed FTZ-F1α and FTZ-F1β fusion protein or the FTZ622 peptide was assessed by gel mobility shift assays as described elsewhere (35, 50). For in vitro-translated proteins, DNA binding assays were carried out in 16-μl reaction volumes in 1× binding buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.4; 50 mM KCl; 1 mM dithiothreitol, 5% glycerol) containing 2 μg of poly(dI-dC), 1 μg of sheared salmon sperm DNA, 2 μl of unlabeled in vitro-translated protein in rabbit reticulocyte lysate, and 60 fmol of double-stranded end-labeled F1RE or Adh oligonucleotide probes (1.5×10^4 cpm). Binding reaction mixtures were incubated for 10 min at room temperature prior to addition of the labeled binding site and a further incubation for 30 min at room temperature. Protein-DNA complexes were resolved on a 4.5% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer, pH 7.4, at 4°C. Gels were fixed, dried, and subjected to autoradiography. Nuclear extracts from 0- to 12-h *Drosophila* embryos (Promega) (5 μg of protein) and/or 1 μl of a 1:4 dilution of crude preimmune serum or anti-FTZ-F1β serum was added to some samples. Saturation binding analysis was performed in quadruplicate with a constant amount of FTZ-F1α and FTZ-F1β fusion protein and various concentrations of DNA (145 to 0.75 nM). Quantitation of free and bound DNA was performed with a Molecular Dynamics Computing Densitometer by volume integration with Image Quant V3.3 software (Molecular Dynamics). Dissociation con-

stant (K_d) values for FTZ-F1α and FTZ-F1β were calculated by Scatchard analysis (40).

DNase I protection assays. A 324-bp *Eco*RI-*Xho*I fragment from plasmid ftzSK-8 was labeled at the *Eco*RI site with Klenow fragment and [α-^{32}P]dATP. Binding reactions were carried out on ice for 15 min in a 20-μl volume in 1× footprint buffer (25 mM HEPES, pH 7.6; 5 mM $MgCl_2$; 34 mM KCl) containing 1 μg of poly(dI-dC), 10 μg of bovine serum albumin (BSA), 5 ng of end-labeled DNA, and 1 μl of a 1:10, 1:5, or 1:1 dilution of bacterial fusion protein. DNase I (Sigma) was freshly diluted at 0.055 μg/μl in DNase I dilution buffer (11 mM HEPES, pH 7.6; 27.5 mM $CaCl_2$; 0.11 μg of BSA per μl), and 1 μl of diluted DNase I was added to each sample. After a 3-min incubation on ice, reactions were terminated by the addition of 200 μl of stop buffer (100 mM Tris, pH 7.5; 10 mM EDTA; 100 mM NaCl; 0.1% SDS), 10 μg of yeast tRNA, and 100 μg of self-digested proteinase K (Boehringer Mannheim). After incubation at 37°C for 30 min, samples were extracted with phenol-$CHCl_3$-isoamyl alcohol (24:24:1) followed by $CHCl_3$ alone. DNA was recovered by ethanol precipitation, resuspended in loading dye (80% deionized formamide, 1× TBE, 0.1% bromophenol blue), and resolved by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. Sequencing reactions were performed as described by Maxam and Gilbert (32).

Cell culture and transient transfections. The human hepatoma cell line Hep3B (22) was maintained in Minimal Essential Medium (MEM) (GIBCO) with 10% fetal calf serum (GIBCO). Cells were plated at 50% confluency in 12-well plates (Corning) and transiently transfected with 1.6 μg of total DNA by the calcium phosphate precipitation method (39). Each well received 0.4 μg of 2F1RE-SV40-Luc, F1RE-SV40-Luc, or pGL2-promoter or pGL2-basic (Promega) reporter plasmid; 1.0 μg of combined pTL1, TLFTZ-F1α, and TLFTZ-F1β expression vectors; and 0.2 μg of pCH110 (Pharmacia), a β-galactosidase expression vector which was included to control for differences in transfection and harvesting efficiencies (38). Cells were incubated with DNA precipitate for 16 h, washed with PBS, and incubated for 24 h in fresh MEM prior to harvesting. Cells were rinsed twice with PBS, and cytoplasmic extracts were prepared by addition of 60 μl of lysis buffer {1% [vol/vol] Triton X-100; 25 mM glycylglycine, pH 7.8; 15 mM $MgSO_4$; 4 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]; 1 mM dithiothreitol} to each well. Cell extracts were spun in a Microfuge for 5 min to remove cell debris. Luciferase activity was assayed as described previously (8) and normalized to β-galactosidase activity (39).

RESULTS

FTZ-F1β DNA binding activity in embryonic nuclear extracts. We first wanted to demonstrate that FTZ-F1β binding activity existed in embryonic cell nuclear extracts. This would (i) confirm that FTZ-F1β protein was expressed in the embryo and (ii) allow comparison of the relative electrophoretic mobilities of protein-DNA complexes formed between FTZ-F1β derived from embryonic nuclei or synthesized in vitro and the F1RE. In Fig. 1, results of gel mobility shift assays demonstrating that multiple retarded complexes were formed by 0- to 12-h *Drosophila* embryonic nuclear extract with the labeled F1RE are shown. Two of these complexes exhibited mobilities similar to those of single retarded complexes formed by FTZ-F1α and FTZ-F1β proteins generated by in vitro translation (Fig. 1, lanes 2 to 4).

To identify the major F1RE DNA-binding component as FTZ-F1β, a polyclonal antibody (anti-FF1β) was raised against

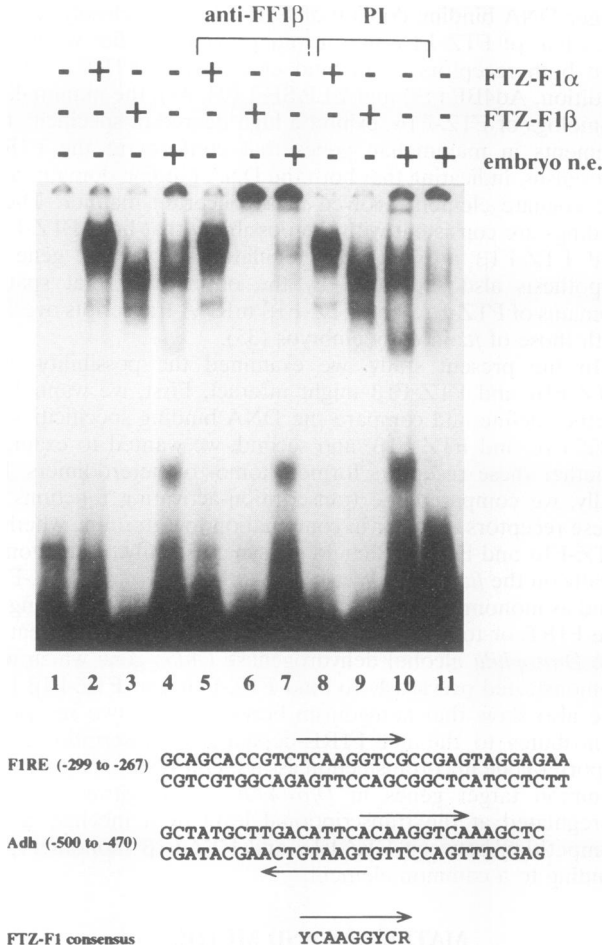


FIG. 1. Identification of an FTZ-F1β binding activity in *Drosophila* embryonic nuclear extracts. In vitro-synthesized FTZ-F1α or FTZ-F1β (in 2 μl of reticulocyte lysate) or a 0- to 12-h mixed embryonic nuclear extract (embryo n.e.), as indicated by the plus (presence) and minus (absence) signs, was incubated with ^{32}P-labeled F1RE (lanes 1 to 10) or Adh (lane 11) oligonucleotides (sequences of the binding site probes are shown at the bottom) and analyzed by gel mobility shift assay on a 4.5% polyacrylamide gel. Lane 1 contains unprogrammed reticulocyte lysate. Anti-FTZ-F1β serum (anti-FF1β) (lanes 5 to 7) or preimmune serum (PI) (lanes 8 to 10) was added to the binding reaction mixtures. The 9-bp FTZ-F1 consensus binding sequence is denoted by an arrow and is shown below the other sequences. The double-stranded 33-bp F1RE binding site (positions −299 to −267 relative to the *ftz* transcriptional start site) contains a single FTZ-F1 consensus half-site sequence. The 31-bp Adh binding site (positions −500 to −470 relative to the *Adh* transcriptional start site) is composed of an imperfect inverted repeat with a 3′ half-site containing 1 nonconsensus bp and a 5′ half-site containing 2 nonconsensus bp. The Adh half-sites overlap by 1 bp.

a malE-FTZ-F1β bacterial fusion protein and used in the gel mobility shift assay. While preimmune serum produced no effect on the number of retarded bands (Fig. 1, lanes 8 to 10), the anti-FF1β antibody added to the binding reaction mixtures specifically recognized the in vitro-translated FTZ-F1β, as well as the predominant F1RE-binding component of the embryonic nuclear extracts, and resulted in a supershifted DNA-protein complex (Fig. 1, lanes 6 and 7). The anti-FF1β antibody did not cross-react with in vitro-translated FTZ-F1α (Fig. 1, lane 5). There was an apparent stabilization of protein-DNA complex formation as a result of incubation with

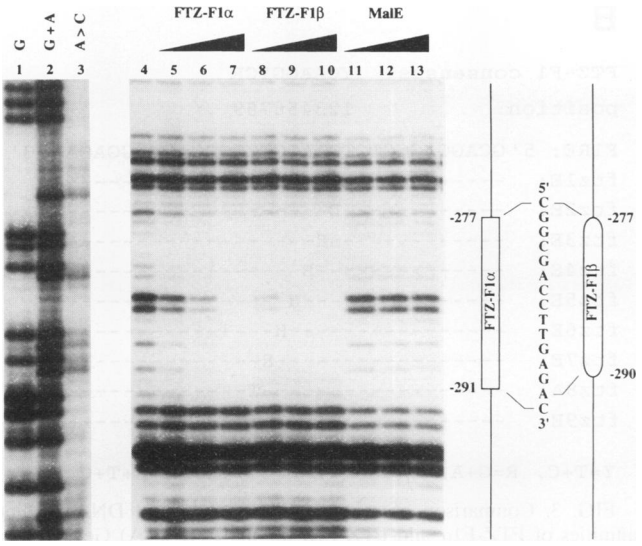


FIG. 2. FTZ-F1α and FTZ-F1β binding to the F1RE at site I in the *ftz* promoter. A 324-bp *Eco*RI-*Xho*I *ftz* fragment corresponding to positions −296 to +28 relative to the *ftz* transcription start site was labeled with [α-^{32}P]dATP at the *Eco*RI site and incubated with increasing amounts (0.35, 0.7, and 3.5 μg) of bacterial extract containing malE–FTZ-F1α (FTZ-F1α) (lanes 5 to 7), malE–FTZ-F1β (FTZ-F1β) (lanes 8 to 10), or malE (MalE) (lanes 11 to 13) fusion proteins and subjected to limited DNase I digestion. The control (lane 4) contains no protein. Maxam and Gilbert sequencing reactions are shown in lanes 1 to 3. The DNase I footprint produced by FTZ-F1α spans 15 bp from positions −277 to −291 and overlaps with the footprint produced by FTZ-F1β from positions −277 to −290 (shown at the right).

rabbit serum, particularly of those complexes formed with nuclear extracts. While the remaining F1RE-binding components are uncharacterized, in other studies early and late FTZ-F1α binding activities have been found in embryonic nuclear extracts and have been identified as isoforms of FTZ-F1α by using antibodies that recognize both isoforms (27, 49).

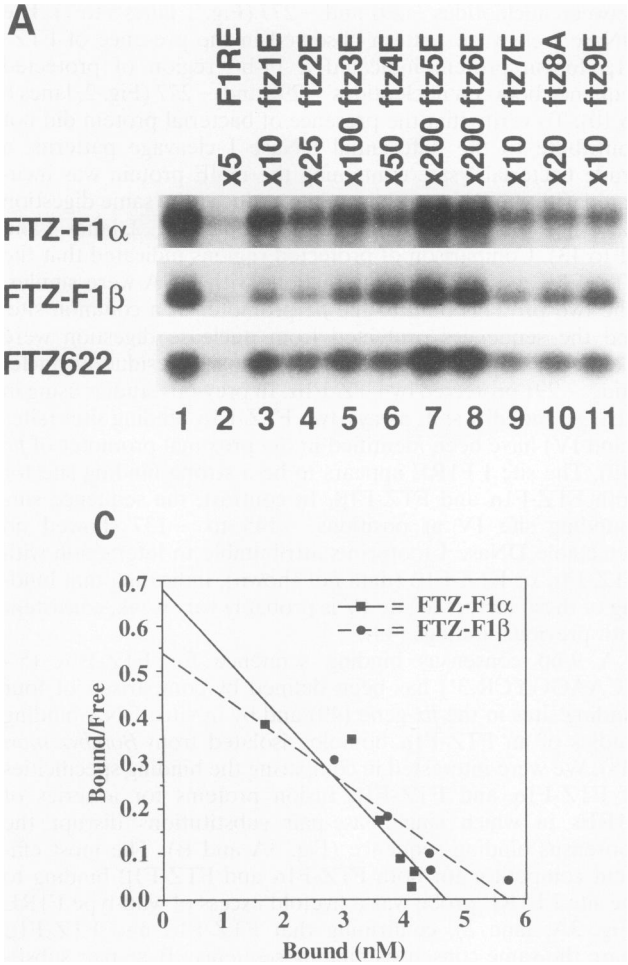
Interestingly, Ayer et al. (4) have recently identified two factors which specifically interact with a site in the promoter of the *Adh* gene: FTZ-F1α and DHR39 (which is identical to FTZ-F1β) (35). An upstream region of the *Drosophila* distal *Adh* gene contains an imperfect inverted repeat of the FTZ-F1 consensus binding sequence (Fig. 1). When this site was incubated with nuclear extracts, the pattern of retarded bands was similar to that for the *ftz* F1RE binding site (Fig. 1, compare lanes 4 and 11). This suggested that the F1RE and Adh sites formed similar complexes with the components of nuclear extracts, in particular FTZ-F1α and FTZ-F1β.

Comparison of FTZ-F1α and FTZ-F1β DNA sequence recognition and binding affinities. Since both FTZ-F1α and FTZ-F1β specifically recognize the F1RE, DNase I footprinting was used to compare the interactions of the receptors with the corresponding fragment of the *ftz* promoter containing this element. Crude lysates from *E. coli* expressing malE–FTZ-F1α or malE–FTZ-F1β fusion proteins were used in a DNase I protection analysis with a DNA probe encompassing a region of the *ftz* proximal promoter (Fig. 2). Equivalent amounts of FTZ-F1α and FTZ-F1β protein, as determined by SDS-PAGE (data not shown), were used in the binding reactions. Increasing concentrations of FTZ-F1α fusion protein produced a 15-bp DNase I protection pattern in the *ftz* promoter sequence between nucleotides −291 and −277 (Fig. 2, lanes 5 to 7). The DNase I cleavage pattern observed in the presence of FTZ-F1β fusion protein indicated a 14-bp region of protected sequence between nucleotides −290 and −277 (Fig. 2, lanes 8 to 10). To verify that the presence of bacterial protein did not contribute to the differential DNase I cleavage patterns, a crude bacterial lysate containing the malE protein was incubated with the DNA probe, and it produced the same digestion pattern as did a no-protein control sample (Fig. 2, lanes 4 and 11 to 13). Comparison of protected regions indicated that the FTZ-F1α and FTZ-F1β interactions with DNA were similar. The two proteins bind to the *ftz* promoter at a common site, and the sequences protected from nuclease digestion were identical except for one additional cytosine residue at nucleotide −291 protected by FTZ-F1α. In previous studies using in vitro gel mobility shift assays, two FTZ-F1α binding sites (sites I and IV) have been identified in the proximal promoter of *ftz* (49). The site I F1RE appears to be a strong binding site for both FTZ-F1α and FTZ-F1β. In contrast, the sequence surrounding site IV at positions −145 to −137 showed no detectable DNase I footprints attributable to interaction with FTZ-F1α or FTZ-F1β (data not shown), indicating that binding of these factors to site IV is probably very weak, consistent with previous findings (49).

A 9-bp consensus binding sequence for FTZ-F1α (5′-YCAAGGYCR-3′) has been defined by comparison of four binding sites in the *ftz* gene (49) and by in vitro DNA binding studies of an FTZ-F1α homolog isolated from *Bombyx mori* (48). We were interested in comparing the binding specificities of FTZ-F1α and FTZ-F1β fusion proteins for a series of F1REs in which single-base-pair substitutions disrupt the consensus binding sequence (Fig. 3A and B). The most efficient competitor for both FTZ-F1α and FTZ-F1β binding to the site I F1RE probe was a fivefold excess of wild-type F1RE (Fig. 3A, lane 2), confirming that FTZ-F1α and FTZ-F1β share the same consensus binding sequence. Base-pair substitutions at positions 3 to 6 significantly affected the ability of these oligonucleotides to compete with wild-type F1RE for receptor binding (Fig. 3A, lanes 5 to 8). For example, a 200-fold excess of competitor having substitutions at position 5 or 6 no longer appeared to bind receptor proteins. FTZ622, a protein product corresponding to the FTZ-F1α DNA binding domain which has previously been shown to bind with a high degree of specificity to the F1RE (50), showed a competition pattern identical to that of FTZ-F1α fusion protein, indicating that the malE portion of the chimeric protein was not interfering with DNA binding specificity (Fig. 3A).

Overall, the competition patterns for FTZ-F1α and FTZ-F1β were very similar, although some differences between FTZ-F1α and FTZ-F1β DNA binding were observed. The ftz1E and ftz2E mutant F1RE binding sites which introduce substitutions of R (purine) for Y (pyrimidine) and D (G+A+T) for C at positions 1 and 2, respectively, of the consensus sequence were more effective at competing for DNA binding with FTZ-F1β than FTZ-F1α recombinant proteins. Minor differences in competition patterns between FTZ-F1α and FTZ-F1β were also noted with the mutant competitors, ftz3E, ftz4E, and ftz9E, which disrupt positions 3, 4, and 9, respectively. The results showing the strong competition differences suggest that FTZ-F1β may have an affinity for binding sites containing R and D residues at binding site positions 1 and 2, respectively. Thus, at positions 1 and 2 some degeneracy is permitted for FTZ-F1β binding activity.

We compared the DNA binding affinities of the F1RE to these nuclear receptors by Scatchard analysis (Fig. 3C). Saturation binding was monitored by a gel mobility shift assay with



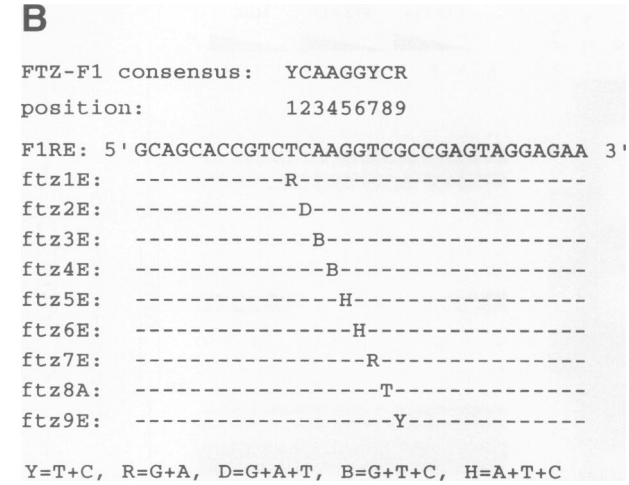


FIG. 3. Comparison of sequence specificities and DNA binding affinities of FTZ-F1α and FTZ-F1β for the F1RE. (A) Gel mobility shift assay with mutant oligonucleotide competitors. Bacterial FTZ-F1α or FTZ-F1β fusion protein or FTZ622 peptide (corresponding to the DNA binding domain of FTZ-F1α) was incubated with a fixed amount of ^{32}P-labeled F1RE binding site and various amounts of unlabeled mutant competitor oligonucleotides (between 5- and 200-fold excess binding site, as indicated). The mutant oligonucleotides (sequences are shown in panel B) disrupt the FTZ-F1 consensus sequence. The DNA-protein complexes resolved on a 0.8% agarose gel are shown. (B) Series of mutant oligonucleotides used in the competition assay. The sequence of the 33-bp F1RE binding site (positions −299 to −267 in *ftz*) is shown in comparison with mutant oligonucleotides containing single-base-pair substitutions which disrupt the 9-bp FTZ-F1 consensus binding sequence. (C) Scatchard plot of saturation binding of ^{32}P-labeled F1RE DNA binding site to bacterial FTZ-F1α or FTZ-F1β fusion protein. Points represent the means for quadruplicate binding reactions. K_d values for FTZ-F1α and FTZ-F1β are 6.9 and 10.5 nM, respectively.

bacterial recombinant FTZ-F1α and FTZ-F1β fusion proteins. The apparent K_d values for FTZ-F1α and FTZ-F1β binding to the F1RE oligonucleotides were 6.9 and 10.5 nM, respectively. These K_d values are in agreement with the binding activities of FTZ-F1α and FTZ-F1β in footprint analyses and binding specificity studies.

FTZ-F1α and FTZ-F1β bind to DNA as monomers. Nine conserved heptad repeats of hydrophobic residues in the ligand-binding domains of nuclear receptors have been proposed to form a leucine zipper-like motif necessary for dimerization (9, 10). These motifs are identifiable in the ligand-binding domain of FTZ-F1β and to a lesser extent in that of FTZ-F1α, suggesting that FTZ-F1α and FTZ-F1β may potentially form dimers. A series of N-terminal truncation mutants of FTZ-F1α and FTZ-F1β (Fig. 4) were constructed to detect possible dimeric complexes. Wild-type and mutant proteins were produced by in vitro translation in a rabbit reticulocyte lysate and visualized by SDS-PAGE (data not shown). The translation products, when assayed for specific DNA binding to the site I F1RE, formed single retarded complexes, while mock-translated reticulocyte lysate did not show specific binding complexes (Fig. 5, lane 1). The truncated FTZ-F1α receptor, FF1α3 (amino acids 575 to 1043), did not bind DNA (Fig. 5A, lane 5) as a result of deletion of the zinc finger DNA binding domain, although this truncated receptor still contains the regions immediately C terminal to the DNA binding domain that may be important for dimer formation in the retinoid X receptor (RXR) (29). Deletion of amino acids 1 to 192 or 1 to 328 in FTZ-F1α (mutants FF1α1 and FF1α2) or amino acids 1 to 144 or 1 to 323 in FTZ-F1β (mutants FF1β1 and FF1β2) did not detectably affect DNA binding activity (Fig. 5, lanes 2 to 4 in both panels). In mixing experiments to detect dimer formation, full-length and truncated receptors were incubated together prior to incubation with the DNA probe (Fig. 5A, lanes 6 to 9, and Fig. 5B, lanes 5 to 7). Only bands corresponding to complexes containing the full-length or truncated proteins were detected, and no intermediate mobility complexes corresponding to heterodimers appeared, indicating that both FTZ-F1α and FTZ-F1β were binding to DNA as monomers.

In addition, we tested whether FTZ-F1α and FTZ-F1β could form monomers or dimers upon binding to the Adh site, which contains an imperfect inverted repeat of the consensus binding sequence (see the legend to Fig. 1). The inverted repeat of the Adh site closely resembles classical steroid/thyroid hormone/retinoic acid receptor response elements, which contain a direct or inverted repeat of a 6-bp half-site sequence, whereas the site I F1RE contains a single 9-bp consensus binding sequence for FTZ-F1α and FTZ-F1β. Identical patterns of gel mobility shift complexes were observed with both the Adh and the F1RE binding sites (Fig. 5A, compare lanes 1 to 9 with lanes 10 to 18; Fig. 5B, compare lanes 1 to 7 with lanes 8 to 14). In Fig. 5B, lanes 8 to 14, a nonspecific intermediate band is apparent; however, this band is present at the same position in all lanes and is therefore

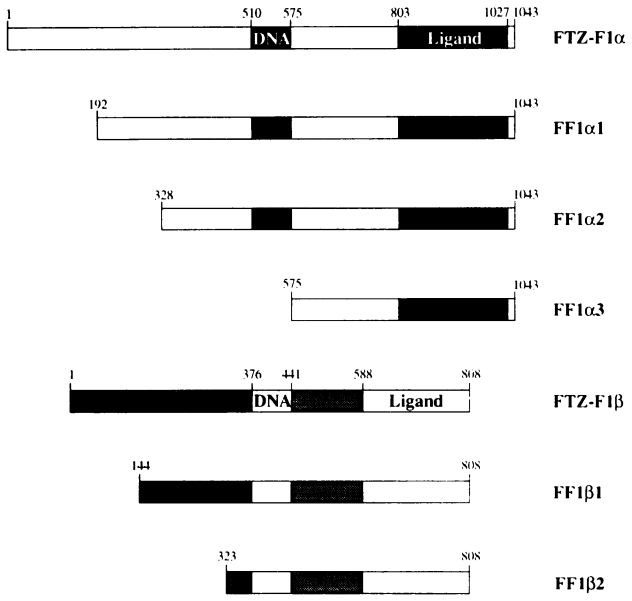


FIG. 4. Schematic of wild-type and N-terminal-truncation mutants of FTZ-F1α and FTZ-F1β. The DNA and ligand binding domains are indicated, and amino acid positions are shown.

unlikely to correspond to a receptor dimer complex. These results demonstrate that FTZ-F1α and FTZ-F1β do not form homodimers upon binding to either the F1RE or the palindromic Adh site and confirm our results obtained with nuclear extracts (Fig. 1).

To investigate the possibility of FTZ-F1α and FTZ-F1β heterodimer formation, in vitro-translated full-length and truncated receptors were tested in combination for binding to the F1RE and Adh sites (Fig. 6). The absence of additional DNA-protein complexes with different mobilities in mixing experiments indicates that there is no detectable FTZ-F1α–FTZ-F1β heterodimer formation. This also appears to be true for complexes formed with extracts from embryonic nuclei (Fig. 1) or transfected Hep3B cells which coexpress FTZ-F1α and FTZ-F1β (see Fig. 7C).

The *Drosophila* homolog of the vertebrate RXR, usp (16, 36, 43), has been shown to form a heterodimer with the ecdysone receptor upon binding to DNA (44, 54). In addition, usp can substitute for RXR heterodimer formation with the vertebrate nuclear receptors, retinoic acid receptor (RAR), vitamin D receptor, thyroid receptor, and peroxisome proliferator activator receptors (21, 54). Studies with in vitro-translated usp indicate that usp does not affect the mobilities of the FTZ-F1α–DNA or FTZ-F1β–DNA complexes, suggesting that usp does not interact with these receptors on either the F1RE or the Adh element (data not shown).

FTZ-F1α and FTZ-F1β exhibit different transcriptional activities. To establish the functional relevance of FTZ-F1α and FTZ-F1β binding activities to the F1RE, the transcriptional activities of these receptors were assessed in transient cotransfection assays. Schneider cell lines from *D. melanogaster* express high levels of endogenous FTZ-F1α and FTZ-F1β (35a). Also, *ftz* promoter-reporter gene constructs exhibited very low levels of activity when transfected into these cell lines. We therefore chose to perform transfection experiments with the human hepatoma cell line Hep3B. Cells were transiently transfected with a reporter plasmid containing one or two

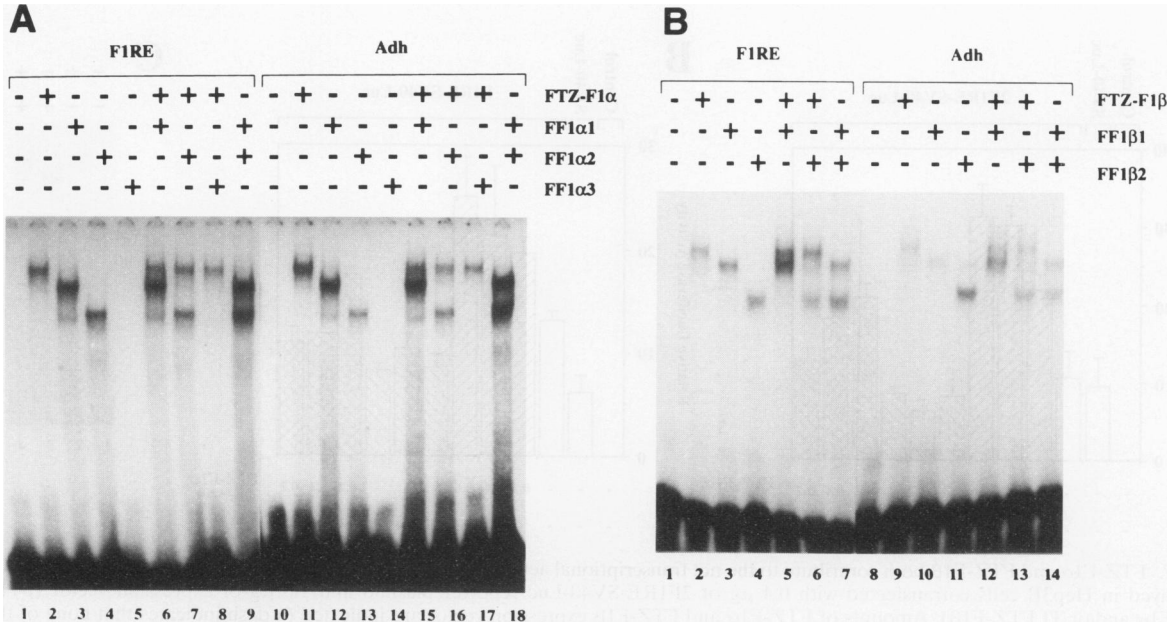


FIG. 5. Monomers of FTZ-F1α and FTZ-F1β bind to two related binding sites. (A) Protein-DNA complexes formed by in vitro-translated full-length and N-terminally truncated FTZ-F1α receptors (as shown in Fig. 4), alone or in combination with F1RE (lanes 1 to 9) or Adh (lanes 10 to 18) binding sites, were assessed by gel mobility shift assay as described in the legend to Fig. 1. A specific binding complex was not detected with the FTZ-F1α truncation mutant, FF1α3, lacking the DNA binding domain (lanes 5 and 14). Control lanes, 1 and 10, contain 2 μl of unprogrammed reticulocyte lysate. Approximately equal amounts of in vitro translation product were added to all reaction mixtures. (B) Binding of full-length or N-terminally truncated FTZ-F1β receptors (as shown in Fig. 4) alone or combined with F1RE (lanes 1 to 7) or Adh (lanes 8 to 14) binding sites. A faint nonspecific complex in lanes 8 to 14 migrates between the FF1β1 and FF1β2 complexes with the Adh probe and corresponds to a component of the reticulocyte lysate. +, receptor present; −, receptor absent.

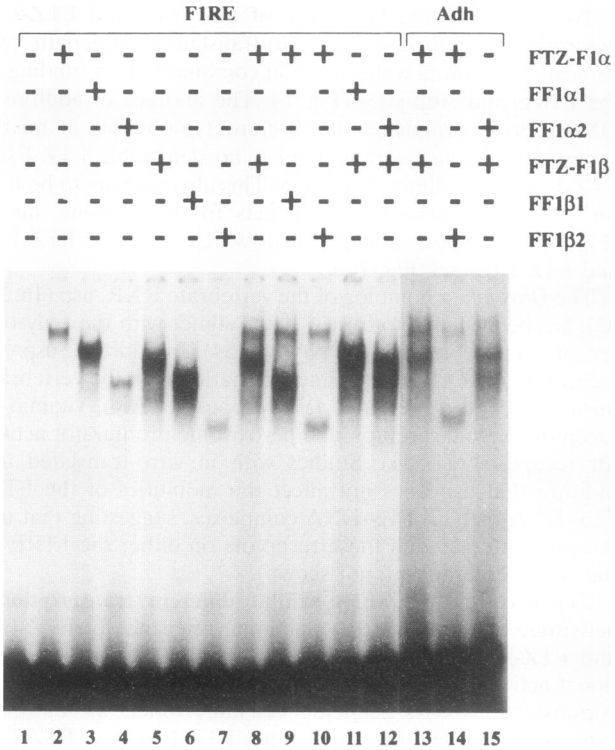


FIG. 6. FTZ-F1α and FTZ-F1β do not form dimers in vitro. Binding of in vitro-translated wild-type or N-terminally truncated FTZ-F1α and FTZ-F1β alone or in combination with ^{32}P-labeled F1RE (lanes 1 to 12) or Adh (lanes 13 to 15) binding sites was assessed by gel mobility shift assay. +, receptor present; −, receptor absent.

copies of the site I F1RE sequence upstream of the SV40 early promoter and luciferase reporter gene. The insertion of the F1RE into the reporter vector did not appear to affect the basal activities (Fig. 7A). Although we cannot explain the apparent increase in relative luciferase activity upon the insertion of a single F1RE (Fig. 7B), this difference is not observed in all similar transfection experiments (data not shown) and is likely an artifact reflecting differences in plasmid quality. Expression vectors for FTZ-F1α and FTZ-F1β were cotransfected with the F1RE reporter constructs, and efficient expression of functional receptors was verified by gel mobility shift analysis (Fig. 7C). Cotransfection of the pTL1, FTZ-F1α, or FTZ-F1β expression vector produced no effect on basal activity of the SV40 early promoter alone (Fig. 7A and data not shown). Cotransfection of increasing amounts of FTZ-F1α expression vector with 2F1RE-SV40-Luc reporter plasmid resulted in a 2- to 3-fold increase in F1RE-dependent transcription (Fig. 7A), whereas cotransfection of increasing amounts of FTZ-F1α expression vector with F1RE-SV40-Luc resulted in a 1.5- to 2-fold increase in transcription above the basal level (Fig. 7B). In contrast, cotransfection of similar amounts of FTZ-F1β expression vector with either the 2F1RE-SV40-Luc or the F1RE-SV40-Luc reporter plasmid produced transcriptional activity which was essentially at the basal level (Fig. 7A and B). FTZ-F1β exhibited little activity, even though it was expressed in these cells and had DNA binding activity comparable to that of FTZ-F1α (Fig. 7C).

We have suggested above that the two nuclear receptors likely compete for binding to a single DNA element such as the site I F1RE. Therefore, we investigated whether similar competition effects could be reflected in F1RE-dependent transcriptional activity. Cotransfection of 0.4 μg of FTZ-F1α with either 0.2 or 0.6 μg of FTZ-F1β resulted in reduced transcriptional activation in comparison with the threefold increase in

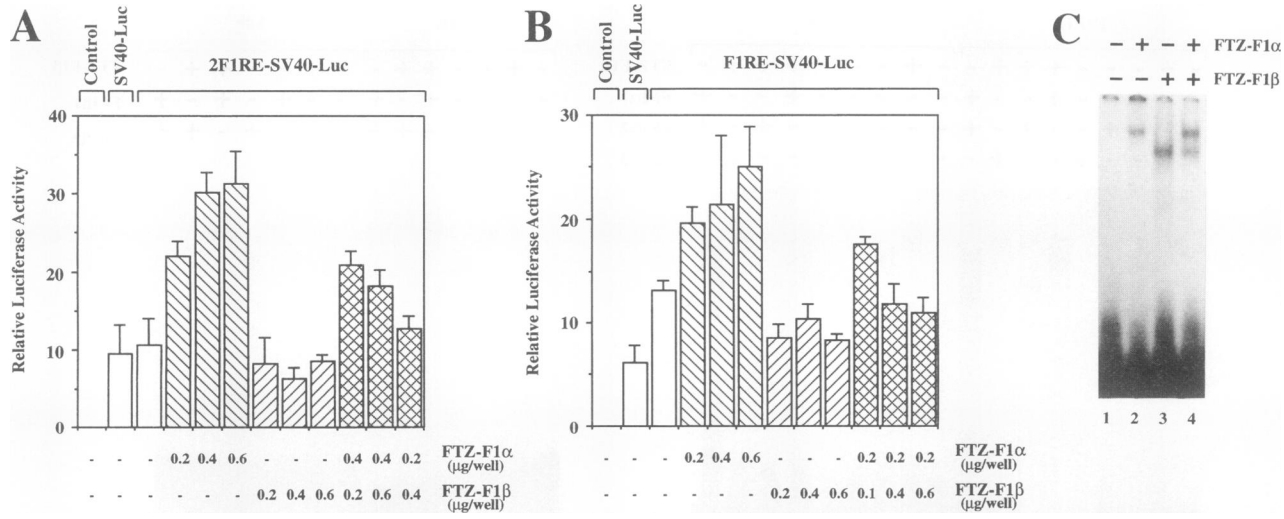


FIG. 7. FTZ-F1α and FTZ-F1β both contribute to the net transcriptional activity of a reporter gene driven by the F1RE. (A) Receptor activities were assayed in Hep3B cells cotransfected with 0.4 μg of 2F1RE-SV40-Luc reporter plasmid and 1.0 μg of expression vector (pTL1 and/or TLFTZ-F1α and/or TLFTZ-F1β). Amounts of FTZ-F1α and FTZ-F1β expression vector are indicated (a dash indicates that none of that vector was used). Control cells were mock transfected with 1.6 μg of pBluescriptSK (Control) or cotransfected with 0.4 μg of the parent pGL2-promoter plasmid (SV40-Luc) and 1.0 μg of pTL1. Relative luciferase activity values have been normalized to β-galactosidase activity (by cotransfecting 0.2 μg of LacZ expression vector, pCH110, as an internal control for differences in transfection and harvesting efficiencies) and are representative of two experiments performed in triplicate; error bars indicate the standard errors. (B) The reporter plasmid F1RE-SV40-Luc containing a single copy of the F1RE site was used in experiments parallel to those shown in panel A. For explanations and definitions, see the legend to panel A. (C) Gel mobility shift assay showing binding of FTZ-F1α and FTZ-F1β expressed in Hep3B cells to the F1RE. Cytoplasmic extracts (10 μg of protein) prepared from Hep3B cells mock-transfected (lane 1) or transfected with FTZ-F1α (lane 2), FTZ-F1β (lane 3), or both FTZ-F1α and FTZ-F1β (lane 4) expression vectors were incubated with ^{32}P-labeled F1RE probe. +, receptor present; −, receptor absent.

transcription in the presence of 0.4 μg of FTZ-F1α expression vector alone (Fig. 7A). When FTZ-F1α and FTZ-F1β were transfected together with a reporter construct containing a single copy of the F1RE in parallel experiments, the activation by FTZ-F1α appeared to be attenuated by the presence of FTZ-F1β (Fig. 7B).

DISCUSSION

The F1RE at site I in the zebra element of the segmentation gene *ftz* is important for the generation of the normal seven-stripe pattern of *ftz* expression seen in early embryos (49). Attempts to identify transcription factors binding to this site have led to the discovery of FTZ-F1α (28), which is a member of the nuclear receptor family. Two isoforms of FTZ-F1α, designated early and late, have been identified as F1RE-binding components in 1.5- to 4-h and 12- to 24-h embryonic extracts, respectively, by using antibodies specific for both isoforms (27, 49). We recently reported an additional member of this family, related to FTZ-F1α, which we have called FTZ-F1β. Like FTZ-F1α, FTZ-F1β also binds specifically to the F1RE. Using a specific antibody to FTZ-F1β, we demonstrate that this receptor is also a predominant F1RE-binding component in 0- to 12-h embryonic nuclear extracts and is distinct from the early and late forms of FTZ-F1α (Fig. 1). These findings are consistent with our previous in situ hybridization experiments showing similar distribution patterns of FTZ-F1α and FTZ-F1β mRNA transcripts in early embryos (35) and support our previous suggestion that they might coregulate the expression of *ftz* through common elements. We have used the F1RE site as a paradigm to study possible interactions between these receptors and to show that similar elements in unrelated genes may also interact with FTZ-F1α and FTZ-F1β in the same manner.

The biological importance of the site I F1RE has previously been examined in germ line transformed embryos carrying a zebra element-*lacZ* construct in which point mutations disrupt the FTZ-F1 consensus sequence (49). These embryos exhibited diminished *ftz* expression in stripes 1, 2, 3, and 6. On this basis, the *FTZ-F1α* gene products which specifically bind the F1RE were suggested to function as transcriptional activators of *ftz* (49). The discovery of FTZ-F1β, as a major F1RE-binding component, suggests that both FTZ-F1α and FTZ-F1β may influence the expression of *ftz* or other genes where this FTZ-F1 consensus sequence or closely related *cis* elements have an apparent functional role in transcriptional regulation. Additional FTZ-F1 consensus sequences are noted in the autoregulatory upstream enhancer elements of the *ftz* gene (13), as well as in the upstream regulatory regions of *hairy* (25) and *Ultrabithorax* (33) genes.

We were interested in comparing the DNA binding sequence specificities and selectivities of these two nuclear receptors implicated in transcriptional regulation of *ftz*. The P-box amino acid sequences (ESCKG) found at the base of the first zinc finger motif are conserved for FTZ-F1α and FTZ-F1β. These amino acids are believed to specify half-site recognition by directly contacting bases in the major groove of DNA (30, 31, 51). The identical P-box sequences, along with similar FTZ-F1 consensus binding properties, suggested that FTZ-F1α and FTZ-F1β probably make contacts with similar bases. In vitro DNase I footprint patterns with bacterially expressed FTZ-F1α and FTZ-F1β indicate that these factors both physically contact a 14-nucleotide region of the *ftz* promoter surrounding site I within the zebra element. The sequence specificities of DNA binding for FTZ-F1α and FTZ-F1β are similar. The F1RE consensus remains the highest-affinity binding site for both receptors. FTZ-F1α, however, appears to be more sensitive to changes at positions 1 and 2 of the consensus than does FTZ-F1β. These minor differences in base-pair preference may be attributed to amino acid differences between FTZ-F1α and FTZ-F1β outside the P-box sequence. These studies do not exclude the possibility that other DNA elements which discriminate between FTZ-F1α and FTZ-F1β exist.

Deletion and mutational analysis of a minimal FTZ-F1α DNA binding domain indicates that the 30-amino-acid FTZ-F1 box, abutting the second zinc finger, stabilizes DNA binding and confers recognition of three nucleotides at the 5′ end of the FTZ-F1 consensus sequence (50). Domain-swapping experiments with the DNA binding domains of NGFI-B, RXR (H-2RIIBP), and SF-1 have been used to demonstrate that an A box confers similar sequence recognition of three nucleotides at the 5′ end of an NGFI-B response element (52, 53). It has been proposed that the A box, which is completely conserved (seven of seven residues) between *Drosophila* FTZ-F1α and mouse ELP/SF-1, is important for the recognition of nucleotides at the 5′ end of the DNA response element; however, between FTZ-F1β and FTZ-F1α there is only 29% conservation of this region (two of seven residues), yet FTZ-F1β binds to the FTZ-F1 consensus with approximately the same affinity and specificity as does FTZ-F1α. The proximal three amino acid residues of the A box, Arg-Arg-Gly for NGFI-B and Gly-Arg-Asn for SF-1, have been implicated in making minor-groove contacts with the 5′ nucleotides of a half-site. Since in FTZ-F1α and FTZ-F1β these residues are Gly-Arg-Asn and Gly-Arg-Ser, respectively, it would seem likely that at least the conserved arginine may be the important residue that participates in the proposed minor-groove interactions.

Previous studies of protein-DNA interaction by methylation interference assays have indicated that FTZ-F1α makes similar contacts with the site I F1RE (28, 49) and one half-site of the Adh element (3). In contrast, FTZ-F1β was shown to contact both half-sites of the Adh element (3). Full-length and N-terminally truncated FTZ-F1α and FTZ-F1β receptors were analyzed by gel mobility shift assays to demonstrate that they bind to the F1RE oligonucleotide or an Adh site composed of an inverted repeat of two imperfect half-sites as monomers (Fig. 5). ELP/SF-1 and NGFI-B (53) also appear to bind to their respective target elements as monomers and represent an evolutionarily conserved subclass of nuclear receptors which can bind alone to DNA. Our demonstration that the FTZ-F1β–DNA complex is monomeric contrasts with the second model proposed by Ayer et al., suggesting that FTZ-F1β binds to the Adh site as a homodimer (4). It also seems unlikely that another factor contributes to FTZ-F1β binding to the Adh element, since the relative sizes of complexes for FTZ-F1α and FTZ-F1β in either purified preparations or nuclear extracts from whole embryos or cultured cells are inconsistent with this possibility (compare Fig. 1 in this paper with Fig. 9 in reference 3). Our analyses further indicate that FTZ-F1α and FTZ-F1β heterodimerize neither with each other nor with usp, the *Drosophila* homolog of RXR (data not shown).

Overall, the two receptors displayed markedly similar in vitro DNA binding properties with respect to DNA sequence specificity and binding affinity. These characteristics indicate that FTZ-F1α and FTZ-F1β are likely candidates as competing transregulators of *ftz* gene transcription; however, this has yet to be proven directly for both receptors. It will be of interest to generate transgenic fly lines to compare the patterns of *ftz* expression in the presence of ectopically expressed FTZ-F1α and FTZ-F1β. Recently, Wilson et al. have sug-

gested a similar mechanism of transcriptional regulation through competition of the murine orphan receptors NGFI-B and SF-1 for the same binding site (52). However, in vitro DNA binding assays show that these proteins exhibit distinct DNA sequence preferences which may be the determinants of receptor target gene specificity.

We have shown that the orphan receptors, FTZ-F1α and FTZ-F1β, exhibited differential transcriptional activities in mammalian Hep3B cells. A threefold F1RE-dependent transcriptional activation was mediated by FTZ-F1α. Transient expression of FTZ-F1β produced no increase in transcriptional activity, possibly because FTZ-F1β, at least in Hep3B cells, forms a transcriptionally inert complex upon binding DNA. The coexpression of FTZ-F1α and FTZ-F1β resulted in a suppression of the level of transcriptional activation which could be attributed to FTZ-F1α alone. We propose that this response is due to antagonism between the two receptors and the net transcriptional effect of FTZ-F1α and FTZ-F1β DNA binding to the F1RE-driven reporter gene. These results are consistent with studies showing the separate transcriptional activities of FTZ-F1α and FTZ-F1β through the *Drosophila* distal *Adh* promoter in *Drosophila* cell (1006-2) transfection experiments (4).

Receptor function in the context of mammalian versus *Drosophila* cells is probably influenced by species- and cell-specific ligands and/or other factors which participate in the pathway of nuclear receptor-mediated transcriptional activation. The activity of FTZ-F1α in Hep3B cells may be due to homologous signalling pathways that utilize related mammalian receptors.

Monomer binding and competitive antagonism represent a novel genetic switching mechanism by which nuclear receptors modulate transcription. However, the effects of this competition on transcription will depend not only on the relative concentrations of each receptor but also on their intrinsic activities. In a different context, FTZ-F1α and FTZ-F1β might have different transcriptional activation functions. For example, it is possible that posttranslational modification (e.g., phosphorylation) or binding of an as-yet-unidentified receptor-specific activating ligand or transcriptional intermediary factor may modulate the transcriptional function of these nuclear receptors at the target element. In conclusion, our data suggest that FTZ-F1α and FTZ-F1β likely coregulate other common target genes in vivo by competing for binding as monomers to a single half-site recognition element. With the possibility that the transcriptional activities of these receptors and their alternatively spliced isoforms may be modified by other factors, this type of competition could generate important diversity in transcriptional responses.

ACKNOWLEDGMENTS

We thank L. Gilbert for providing the usp cDNA clone and A. Laughon for *ftz* gene subclones. We also acknowledge M. Boffa and R. Deeley for their comments on the manuscript and H. Metz for photography.

M.P. holds a scholarship from the Medical Research Council (MRC) of Canada. This work has been funded by grants from the MRC of Canada and the National Cancer Institute of Canada.

REFERENCES

1. **Akam, M.** 1987. The molecular basis for metameric pattern in the *Drosophila* embryo. Development **101**:1–22.
2. **Amero, S. A., R. H. Kretsinger, N. D. Moncrief, K. R. Yamamoto, and W. R. Pearson.** 1992. The origin of nuclear receptor proteins: a single precursor distinct from other transcription factors. Mol. Endocrinol. **6**:3–7.
3. **Ayer, S., and C. Benyajati.** 1992. The binding site of a steroid hormone receptor-like protein within the *Drosophila Adh* adult enhancer is required for high levels of tissue-specific alcohol dehydrogenase expression. Mol. Cell. Biol. **12**:661–673.
4. **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.
5. **Brown, J. L., S. Sonoda, H. Ueda, M. P. Scott, and C. Wu.** 1991. Repression of the *Drosophila fushi tarazu* (*ftz*) segmentation gene. EMBO J. **10**:665–674.
6. **Dearolf, C. R., J. Topol, and C. S. Parker.** 1989. The *caudal* gene product is a direct activator of *fushi tarazu* transcription during *Drosophila* embryogenesis. Nature (London) **341**:340–343.
7. **Dearolf, C. R., J. Topol, and C. S. Parker.** 1989. Transcriptional control of *Drosophila fushi tarazu* zebra stripe expression. Genes Dev. **3**:384–398.
8. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**:725–737.
9. **Fawell, S. E., J. A. Lees, R. White, and M. G. Parker.** 1990. Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell **60**:953–962.
10. **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.
11. **Green, S., I. Issemann, and E. Sheer.** 1988. A versatile in vivo and in vitro eukaryotic expression vector for protein engineering. Nucleic Acids Res. **16**:369–340.
12. **Hafen, E., A. Kuroiwa, and W. J. Gehring.** 1984. Spatial distribution of transcripts from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. Cell **37**:833–841.
13. **Han, W., Y. Yu, N. Altan, and L. Pick.** 1993. Multiple proteins interact with the *fushi tarazu* proximal enhancer. Mol. Cell. Biol. **13**:5549–5559.
14. **Harrison, S. D., and A. A. Travers.** 1988. Identification of the binding sites for potential regulatory proteins in the upstream enhancer element of the *Drosophila fushi tarazu* gene. Nucleic Acids Res. **16**:11403–11416.
15. **Harrison, S. D., and A. A. Travers.** 1990. The *tramtrack* gene encodes a *Drosophila* finger protein that interacts with the *ftz* transcriptional regulatory region and shows a novel embryonic expression pattern. EMBO J. **9**:207–216.
16. **Henrich, V. C., T. J. Sliter, D. B. Lubahn, A. MacIntyre, and L. I. Gilbert.** 1990. A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive sequence similarity with a mammalian homologue. Nucleic Acids Res. **18**:4143–4148.
17. **Hiromi, Y., and W. J. Gehring.** 1987. Regulation and function of the *Drosophila* segmentation gene *fushi tarazu*. Cell **50**:963–974.
18. **Hiromi, Y., A. Kuroiwa, and W. J. Gehring.** 1985. Control elements of the Drosophila segmentation gene *fushi tarazu*. Cell **43**:603–613.
19. **Honda, S.-I., K.-I. Morohashi, M. Nomura, H. Takeya, M. Kitajima, and T. Omura.** 1993. Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. J. Biol. Chem. **268**:7497–7502.
20. **Izumo, S., and V. Mahdavi.** 1988. Thyroid hormone receptor α isoforms generated by alternative splicing differentially activate myosin HC gene transcription. Nature (London) **334**:539–542.
21. **Khoury Christianson, A. M., D. L. King, E. Hatzivassiliou, J. E. Casas, P. L. Hallenbeck, V. M. Nikodem, S. A. Mitsialis, and F. C. Kafatos.** 1992. DNA binding and heteromerization of the *Drosophila* transcription factor chorion factor 1/ultraspiracle. Proc. Natl. Acad. Sci. USA **89**:11503–11507.
22. **Knowles, B. B., C. C. Howe, and D. P. Aden.** 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigens. Science **209**:497–499.
23. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
24. **Lala, D. S., D. A. Rice, and K. L. Parker.** 1992. Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression, is the

mouse homolog of *fushi tarazu*-factor 1. Mol. Endocrinol. **6**:1249–1258.
25. **Langeland, J. A., and S. B. Carroll.** 1993. Conservation of regulatory elements controlling *hairy* pair-rule stripe formation. Development **117**:585–596.
26. **Laudet, V., C. Hanni, J. Coll, F. Catzeflis, and D. Stehelin.** 1992. Evolution of the nuclear receptor gene superfamily. EMBO J. **11**:1003–1013.
27. **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.
28. **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.
29. **Lee, M. S., S. A. Kliewer, J. Provencal, P. E. Wright, and R. M. Evans.** 1993. Structure of the retinoid X receptor α DNA binding domain: a helix required for homodimeric DNA binding. Science **260**:1117–1121.
30. **Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler.** 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature (London) **352**:497–505.
31. **Mader, S., V. Kumar, H. de Verneuil, and P. Chambon.** 1989. Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. Nature (London) **345**:224–229.
32. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
33. **Muller, J., and M. Bienz.** 1992. Sharp anterior boundary of homeotic gene expression conferred by the *fushi tarazu* protein. EMBO J. **11**:3653–3661.
34. **Nüsslein-Volhard, C., and E. Wieschaus.** 1980. Mutations affecting segment number and polarity in *Drosophila*. Nature (London) **287**:795–801.
35. **Ohno, C. K., and M. Petkovich.** 1992. *FTZ-F1*β, a novel member of the *Drosophila* nuclear receptor family. Mech. Dev. **40**:13–24.
35a. **Ohno, C. K., and M. Petkovich.** Unpublished data.
36. **Oro, A. E., M. McKeown, and R. M. Evans.** 1990. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. Nature (London) **347**:298–301.
37. **Petkovich, M.** 1992. Regulation of gene expression by vitamin A: the role of nuclear retinoic acid receptors. Annu. Rev. Nutr. **12**:443–471.
38. **Petkovich, M., M. J. Brand, A. Krust, and P. Chambon.** 1987. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature (London) **330**:444–450.
39. **Rosenthal, N.** 1987. Identification of regulatory elements of cloned genes with functional assays. Methods Enzymol. **152**:704–720.
40. **Scatchard, G.** 1949. The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51**:660–672.
41. **Scott, M. P., and S. B. Carroll.** 1987. The segmentation and homeotic gene network in early *Drosophila* development. Cell **51**:689–698.
42. **Segraves, W. A.** 1991. Something old, some things new: the steroid receptor superfamily in *Drosophila*. Cell **67**:225–228.
43. **Shea, M. J., D. L. King, M. J. Conboy, B. D. Mariani, and F. C. Kafatos.** 1990. Proteins that bind to *Drosophila* chorion *cis*-regulatory elements: a new C_2H_2 zinc finger protein and a C_2C_2 steroid receptor-like component. Genes Dev. **4**:1128–1140.
44. **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.
45. **Topol, J., C. R. Dearolf, K. Prakash, and C. S. Parker.** 1991. Synthetic oligonucleotides recreate *Drosophila fushi tarazu* zebra-stripe expression. Genes Dev. **5**:855–867.
46. **Tora, L., H. Gronemeyer, B. Turcotte, M.-P. Gaub, and P. Chambon.** 1988. The N-terminal region of the chicken progesterone receptor specifies target gene activation. Nature (London) **333**:185–188.
47. **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.
48. **Ueda, H., and S. Hirose.** 1991. Defining the sequence recognized with *BmFTZ-F1*, a sequence specific DNA binding factor in the silkworm, *Bombyx mori*, as revealed by direct sequencing of bound oligonucleotides and gel mobility shift competition analysis. Nucleic Acids Res. **19**:3689–3693.
49. **Ueda, H., S. Sonoda, J. L. Brown, M. P. Scott, and C. Wu.** 1990. A sequence-specific DNA-binding protein that activates *fushi tarazu* segmentation gene expression. Genes Dev. **4**:624–635.
50. **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.
51. **Umesono, K., and R. M. Evans.** 1989. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell **57**:1139–1146.
52. **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.
53. **Wilson, T. E., R. E. Paulsen, K. A. Padgett, and J. Milbrandt.** 1992. Participation of non-zinc finger residues in DNA binding by two nuclear orphan receptors. Science **256**:107–110.
54. **Yao, T.-P., W. A. Segraves, A. E. Oro, M. McKeown, and R. M. Evans.** 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell **71**:63–72.