MOLECULAR AND CELLULAR BIOLOGY, Jan. 2003, p. 238–249 Vol. 23, No. 1
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.1.238–249.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

LXXLL-Related Motifs in Dax-1 Have Target Specificity for the Orphan Nuclear Receptors Ad4BP/SF-1 and LRH-1

Taiga Suzuki,[1,2] Megumi Kasahara,[3] Hidefumi Yoshioka,[3] Ken-ichirou Morohashi,[1,4]* and Kazuhiko Umesono[2,5]

Department of Developmental Biology, National Institute for Basic Biology, Okazaki 444-8585,[1] Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507,[2] Department of Natural Sciences, Hyogo University of Teacher Education, Hyogo 673-1494,[3] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Kawaguchi 332-0012,[4] and Graduate School for Biostudies, Kyoto University, Sakyo-ku, Kyoto 606-8502,[5] Japan

Received 5 April 2002/Returned for modification 25 June 2002/Accepted 24 September 2002

The orphan receptor Ad4BP/SF-1 (NR5A1) is a constitutive activator, and its activity is repressed by another orphan receptor, Dax-1 (NR0B1). In the present study, we investigated the molecular mechanisms underlying this repression by Dax-1. Yeast two-hybrid and transient-transfection assays confirmed the necessity of three LXXLL-related motifs in Dax-1 for interaction with and repression of Ad4BP/SF-1. In vitro pull-down experiments confirmed that Dax-1 interacts with Ad4BP/SF-1 and also with LRH-1 (NR5A2). The target specificity of the LXXLL-related motifs was indicated by the observations that Ad4BP/SF-1, ERα (NR3A1), LRH-1, ERR2 (NR3B2), and fly FTZ-F1 (NR5A3) interacted through their ligand binding domains with all the LXXLL-related motifs in Dax-1 whereas HNF4 (NR2A1) and RORα (NR1F1) did not. Transcriptional activities of the receptors whose DNA binding domains (DBDs) were replaced by the GAL4 DBD were repressed by Dax-1 to various levels, which correlated with the strength of interaction. Amino acid substitutions revealed that Ad4BP/SF-1 and LRH-1 preferentially interact with L(+1)XXLL-related motifs containing serine, tyrosine, serine, and threonine at positions −2, +2, +3, and +6, respectively. Taken together, our results indicate that the specificities of LXXLL-related motifs in Dax-1 based on their amino acid sequences play an important role in regulation of orphan receptors.

More than 70 molecular species of the nuclear receptor superfamily have been identified to date in animals ranging from hydras to humans (47). These transcription factors are generally characterized by the presence of two conserved structural features, a DNA binding domain (DBD) composed of two zinc fingers and a ligand binding domain (LBD) located at the C-terminal region. Various lipophilic ligands interact with the cognate LBDs in apo-type receptors, converting them into an active holo-type conformation that can dynamically regulate transcription (14, 38).

The general structure of the LBD is composed predominantly of 12 helices. Interaction with ligand induces allosteric changes in conformation, especially in the configuration of helix 12 at the C terminus of the LBD, leading to transcriptional activation (3, 9) or repression (4, 57). Helix 12 is often referred to as the AF-2 core, which in some receptors is a conserved domain essential for ligand-dependent transcriptional activation (14, 37). Transcriptional coactivators mediate activating signals by binding to nuclear receptors in a ligand-dependent manner (reference 67 and references therein). For this receptor-coactivator interaction, conserved sequences containing a short signature motif of LXXLL (where L is leucine and X can be any amino acid) have been implicated (18, 31, 60). The conserved leucines in these so-called LXXLL motifs, or NR-boxes, appear to be indispensable for hydrophobic interaction with nuclear receptors (10, 46, 57). In addition, amino acid residues flanking the core LXXLL are known to influence the binding specificity (8, 17, 29, 36, 39, 65). In the absence of ligand, transcriptional corepressors are recruited to nuclear receptors and mediate repression (67). This receptor-corepressor interaction requires conserved sequences containing a signature such as ΦXXΦΦ (where Φ is leucine or isoleucine and X can be any amino acid), the so-called ΦXXΦΦ motif (45), LXX I/H IXXX I/L helix (51), or CoRNR motif (19), in corepressors. Biochemical and stereochemical approaches have demonstrated that the coactivators and corepressors resemble each other in the molecular basis of interaction with nuclear receptors. The most remarkable difference between the two types of interaction is that the coactivators require the AF-2 core for interaction (10, 46, 57), whereas corepressors do not (19, 45, 51).

The nuclear receptor superfamily includes, however, a number of "orphan receptors," for which cognate ligands have not yet been identified (14). Ad4BP/SF-1 is an orphan receptor essential for the formation of steroidogenic tissues and the control of steroidogenesis (35, 54, 58). At the molecular level, Ad4BP/SF-1 is known as a constitutive activator (43, 50), while it also interacts with several transcription factors regulating its downstream genes (7, 22, 23, 33, 42, 44, 61). One such accompanying factor is Dax-1/DAX-1 (Dax-1 is the conventional nomenclature of the gene and product in mice), which was originally identified as the product of a human X-linked gene in a region critical for the congenital diseases dosage-sensitive sex reversal and adrenal hypoplasia congenita (69). Dax-1 expression overlaps with that of Ad4BP/SF-1 (20, 21, 28), and Dax-1 acts as a repressor against transcription activated by Ad4BP/SF-1 (22) and estrogen receptors (ERs) (70) through direct interaction. Structurally, Dax-1 is an atypical

* Corresponding author. Mailing address: Department of Developmental Biology, National Institute for Basic Biology, Myodaiji-cho, Okazaki 444-8585, Japan. Phone: 81-564-55-7560. Fax: 81-564-55-7561. E-mail: moro@nibb.ac.jp.

orphan receptor containing the conserved LBD but not the canonical DBD. Instead, the N-terminal half of Dax-1 contains a unique region composed mainly of three repeats, each 65 to 67 amino acids in length. Zhang et al. (70) pointed out that this region contains LXXLL motif-like sequences possessing a signature LXX(L/M)L (where L and M are leucine and methionine, respectively, and X can be any amino acid) and showed that these sequences are necessary for interaction with ERs in the presence of ligand. Since the C-terminal half of Dax-1 containing a putative LBD functions as a repressor domain (22) through interaction with corepressors (1, 6), two mechanisms have been proposed to act cooperatively for Dax-1 to repress transcription. In such a model, Dax-1 would block the binding of coactivators to ERs with its LXXLL-related motifs and then recruit corepressors with its C-terminal half.

In the present study, we analyzed the interaction between Ad4BP/SF-1 and Dax-1 to illustrate an example of a mechanism by which an orphan receptor is regulated. We found that a subset of orphan receptors, including Ad4BP/SF-1, interact with the LXXLL-related motifs in Dax-1 in a manner similar to the interaction between ligand-bound receptors and LXXLL motifs and that particular amino acid residues in the LXXLL-related motifs are critically required for interaction with these receptors. Our results provide evidence that the specificity of LXXLL motifs in cofactors plays an important role in controlling the activity of orphan receptors as well as ligand-bound receptors.

MATERIALS AND METHODS

Plasmids. pCMX (62) expression constructs were used to express proteins in cultured cells. pCMX-Dax-1[WT] was constructed by inserting the mouse Dax-1 (NR0B1) cDNA derived from pSP65-Dax-1 (28) into *Eco*RI and *Xho*I cloning sites of pCMX. pCMX-Ad4BP was constructed by inserting the mouse Ad4BP/SF-1 (NR5A1) cDNA into pCMX at the *Eco*RI site. pCMX-VP16-Ad4BP-LBD, pCMX-VP16-ERα-LBD (amino acids 241 to 595), pCMX-VP16-LRH-1-LBD (amino acids 168 to 560), pCMX-VP16-ERR2-LBD (amino acids 171 to 433), pCMX-VP16-HNF4-LBD (amino acids 125 to 455), and pCMX-VP16-RORα-LBD (amino acids 165 to 548) were constructed by inserting the corresponding cDNAs of mouse Ad4BP/SF-1, human ERα (NR3A1), mouse LRH-1 (NR5A2), human ERR2 (NR3B2), rat HNF4 (NR2A1), and human RORα (NR1F1), respectively, into the multiple-cloning site of pCMX-VP16 (53) that encodes the VP16 transactivation domain (VP16 AD; amino acids 1 to 78). pCMX-GAL4 (52) encodes the GAL4 DBD (amino acids 1 to 147). pCMX-GAL4-DAXpep7–23, pCMX-GAL4-DAXpep74–90, pCMX-GAL4-DAXpep140–156, pCMX-GAL4-TIFpep684–700, and related constructs containing an amino acid substitution(s) were constructed by inserting double-stranded synthetic oligonucleotides encoding the corresponding amino acid sequences into pCMX-GAL4 cleaved with *Asp*718 and *Bam*HI. pCMX-GAL4-Ad4BP-LBD was constructed by inserting a cDNA fragment encoding amino acids 64 to 462 of mouse Ad4BP/SF-1 into pCMX-GAL4 cleaved with *Sma*I and *Bam*HI. pCMX-GAL4-ERα-LBD, pCMX-GAL4-LRH-1-LBD, pCMX-GAL4-ERR2-LBD, pCMX-GAL4-HNF4-LBD, and pCMX-GAL4-RORα-LBD were constructed by inserting corresponding cDNA fragments derived from pCMX-VP16-ERα-LBD, pCMX-VP16-LRH-1-LBD, pCMX-VP16-ERR2-LBD, pCMX-VP16-HNF4-LBD, and pCMX-VP16-RORα-LBD, respectively, into the multiple cloning site of pCMX-GAL4. Mutations in pCMX-Dax-1[M1], pCMX-Dax-1[M2], pCMX-Dax-1[M3], and pCMX-Dax-1[M123] and truncations in pCMX-VP16-Ad4BP-LBDΔAF2C and pCMX-VP16-ERα-LBDΔAF2C were introduced by PCR-mediated mutagenesis using primers containing the mutations and were verified by DNA sequencing. The reporter plasmid pS2.3H-LUC was constructed by inserting a 2.3-kb *Hin*dIII-*Pvu*II fragment containing the transcriptional initiation site and its 5′-upstream region of the human *CYP11A1* gene into pGL3-Basic (Promega, Madison, Wis.) at the blunt-ended *Hin*dIII site. MT-CEVα (40) was a kind gift from G. Stanley McKnight (University of Washington, Seattle, Wash.). The expression plasmids, pCMX-SAH/Y145F (48), pCMX-GAL4-FTZ-F1-LBD (59), pCMX-VP16-FTZ-F1-LBD (59), UAS_G-TK-LUC (12), and pCMX-βGAL (62), have been described previously. For expression of Dax-1 proteins in reticulocyte lysate, pCMX expression constructs were used. pCMX-GFP-Dax-1[WT] and pCMX-GFP-Dax-1[M123] were constructed by inserting the cDNAs excised from pCMX-Dax-1[WT] and pCMX-Dax-1[M123] into pCMX-SAH/Y145F at the *Eco*RV site. For yeast two-hybrid interaction assays, the bait plasmids, pGBT9-Dax-1[WT], pGBT9-Dax-1[N], pGBT9-Dax-1[C], pGBT9-Dax-1[M1], pGBT9-Dax-1[M2], pGBT9-Dax-1[M3], and pGBT9-Dax-1[M123], were constructed by inserting cDNA fragments encoding corresponding Dax-1 proteins at the *Sma*I site of pGBT9 (Clontech Laboratories, Palo Alto, Calif.) that encodes the GAL4 DBD. The prey plasmid pGAD424-Ad4BP was constructed by inserting mouse Ad4BP/SF-1 cDNA into pGAD424 (Clontech), which encodes a GAL4 AD, cleaved with *Eco*RI and *Bam*HI. For bacterial expression of maltose binding protein (MBP) fusions, pMal-Ad4BP and pMal-LRH-1 were constructed by inserting cDNA fragments encoding amino acids 5 to 462 of Ad4BP/SF-1 and amino acids 83 to 560 of LRH-1 into the pMal-cRI expression plasmid (New England Biolabs, Beverly, Mass.) at the multiple-cloning site. The cDNA used for all expression plasmids encoding mouse Ad4BP/SF-1 proteins was obtained from Keith L. Parker (University of Texas, Dallas, Tex.), except for pGAD424-Ad4BP, which contains cDNA obtained from Ohtsura Niwa (Kyoto University, Kyoto, Japan). Mouse LRH-1 cDNA was isolated from BALB/c mouse adult liver by reverse transcription-PCR amplification and verified by DNA sequencing. Human ERα and RORα cDNAs were obtained from Vincent Giguère (McGill University, Toronto, Canada). The cDNA of human ERR2 was obtained from Ronald M. Evans (The Salk Institute for Biological Studies, La Jolla, Calif.). Rat HNF4 cDNA was obtained from Frances M. Sladek (University of California, Riverside, Calif.). The unified nomenclature system used here for nuclear receptors was established by the Nuclear Receptors Nomenclature Committee (47).

Yeast two-hybrid interaction assays. PJ69-4A yeast cells (24) were transformed by a standard technique using polyethylene glycol and lithium acetate with pGBT9 plasmids carrying the *TRP1* gene and pGAD424 plasmids carrying the *LEU2* gene. Yeast was then plated onto Sabouraud dextrose (SD) agar plates without leucine and tryptophan and incubated for 3 days until the appearance of colonies. The colonies were replicated onto SD agar plates without leucine, tryptophan, and histidine but containing 3-aminotriazole (3-AT) at 2, 5, 10, 20, 50, and 100 mM. The plates were incubated for 4 days, and the growth of colonies was observed to indicate expression of the *HIS3* reporter gene.

Cell culture and cotransfection assays. CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo.) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. EPC cells (11) were grown in DMEM-FBS at 30°C in 5% CO_2. The cells were plated at 10 to 20% confluence 1 day prior to transfection. Unless otherwise indicated, the following procedures were performed: transfections were conducted in 24-well plates in triplicate either by the calcium phosphate-DNA precipitation method (see Fig. 5C) or by the lipofection technique using LipofectAMINE PLUS (Gibco BRL, Gaithersburg, Md.) (see Fig. 2, 4B and C, 5B, and 6B to G) as specified by the manufacturer. Each well received 75 ng of the indicated expression plasmid, 325 ng of reporter plasmid, and 350 ng of reference pCMX-βGAL. pCMX-SAH/Y145F containing the humanized green fluorescent protein (GFP) gene was added to equalize amounts of transfected plasmid DNA concentrations. Cells treated for 4 to 6 h with calcium phosphate-DNA precipitate were washed and then incubated in DMEM-FBS alone or with 1 μM 17β-estradiol (β-E_2; Sigma) for 36 to 48 h. Cells treated for 3 h with the lipofection reagent in DMEM without serum were then incubated for 36 to 48 h in DMEM supplemented with 1× insulin-transferrin-sodium selenite supplement (Roche, Mannheim, Germany) and 0.1% lipid-free bovine serum albumin (BSA) (Sigma) alone or with 1 μM β-E_2. The cells were harvested and assayed for luciferase and β-galactosidase activity. All luciferase activities were normalized with β-galactosidase activity. The data presented in each panel represent one of at least two independent transfection experiments with similar results. Bars and vertical lines represent the mean and standard deviation, respectively, of values obtained in triplicate for each data point, and the value obtained without effector was set as 1 in each experiment.

Production of MBP fusion proteins. The expression and purification of MBP fusion proteins using Amylose Resin (New England Biolabs) were performed essentially as specified by the manufacturer, with several modifications. Briefly, overnight cultures of *Escherichia coli* expressing the recombinant MBP proteins were diluted 1:50 and continuously cultured for 4 h (MBP-Ad4BP) or 9 h (MBP-LRH-1) until the absorbance at 600 nm reached 1.0. Cultures were then continued at 25°C in the presence of 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h. Bacteria were collected by centrifugation, resuspended at 1:10 in homogenization buffer (20 mM Tris, 1 mM EDTA, 1 mM dithiothreitol [DTT], 0.25 M sucrose) containing 1× Complete protease inhibitor cocktail (Roche), sonicated, and centrifuged. Fusion proteins in 5 ml of supernatant were loaded onto columns with 1 ml of amylose resin that was equilibrated with 5 volumes of

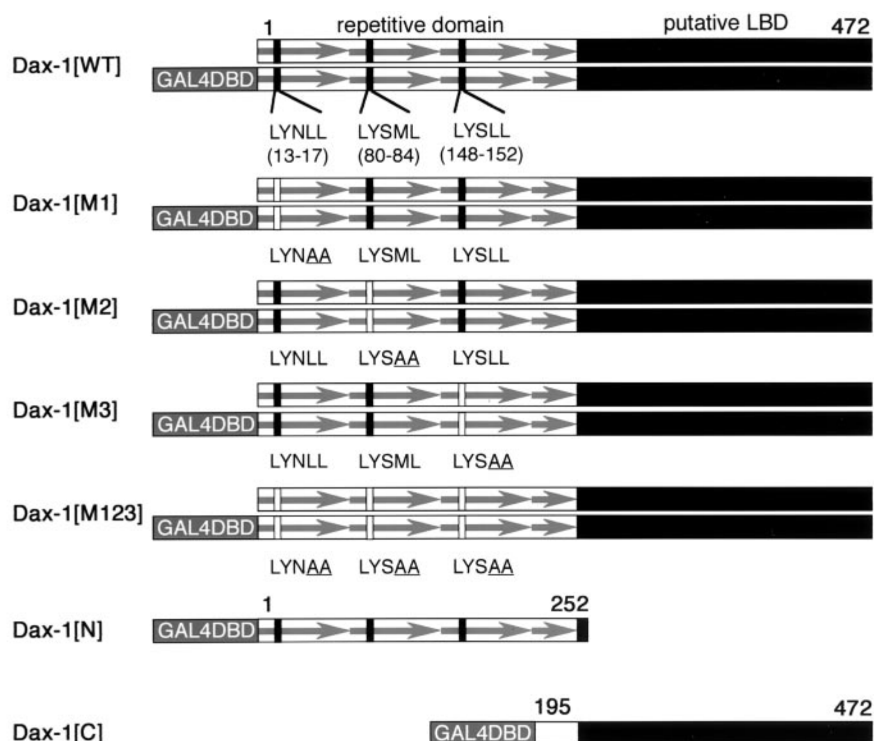

FIG. 1. Structure of Dax-1 constructs used in repression assays (Fig. 2 and 5C) and yeast two-hybrid assays (Fig. 3). For repression assays, full-length proteins were expressed using the pCMX vector. To be used as bait in yeast two-hybrid assays, proteins were fused at their N termini to GAL4 DBD in the pGBT9 vector. Numbers represent amino acid positions in Dax-1, reading from the N terminus. The sequences of three LXXLL-related motifs in these constructs are shown together with their position numbers (in brackets), and substituted amino acid residues are underlined. Black and white bars represent intact and altered LXXLL-related motifs, respectively.

TED (20 mM Tris, 1 mM EDTA, 1 mM DTT). After being given washes with 5 ml of TED and 5 ml of TED containing 0.2 mM sodium chloride, proteins were repeatedly eluted with 0.25 ml of TED containing 20 mM maltose and then several fractions containing high concentrations of protein were collected. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with SYPRO Orange (Molecular Probes, Eugene, Oreg.) as specified by the supplier. The concentration of intact proteins was estimated by comparison with a BSA standard on the stained gel, because this revealed that the eluates contained several undesired proteins with different molecular weights.

MBP pull-down assays. Recombinant cDNAs in pCMX-GFP-Dax-1[WT] and pCMX-GFP-Dax-1[M123] were transcribed and translated in vitro in a reticulocyte lysate using the TNT T7 quick coupled transcription-translation system (Promega) in the presence of Fluorotect-Green$_{Lys}$-tRNA (Promega) by the method recommended by the manufacturer. By this protocol, the proteins were labeled at lysine residues with a green-fluorescent fluorophore. Amylose resin loaded with 1 μg of MBP fusions was incubated for 1 h at 4°C with 5 μl of reticulocyte lysate containing fluorescently labeled proteins in a total volume of 100 μl of binding buffer (20 mM Tris, 1 mM EDTA, 1 mM DTT, 10% glycerol, 150 mM sodium chloride, 0.5 mg of BSA per ml) containing 1× Complete protease inhibitor cocktail. Beads were washed twice with binding buffer and three times with buffer without BSA. The beads were then incubated with 100 mM maltose to elute the proteins and centrifuged. Supernatants were mixed with loading buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Signals were directly scanned and visualized by laser fluoroimager (FMBIOII Multi-View; Hitachi Software, Yokohama, Japan).

RESULTS

Three LXXLL-related motifs in the repetitive region of Dax-1 are required for its repressor activity against Ad4BP/SF-1-dependent transcription. It is accepted that Dax-1 functions as a transcriptional repressor against Ad4BP/SF-1 (22). Since the LXXLL-related motifs in the three repeats of the N-terminal half of Dax-1 were reported to interact with ERs and thereby repress the activator function of ERs in the presence of ligand (70), we reasoned that the LXXLL-related motifs might be required for repression of Ad4BP/SF-1. To test this assumption, we constructed expression plasmids of Dax-1

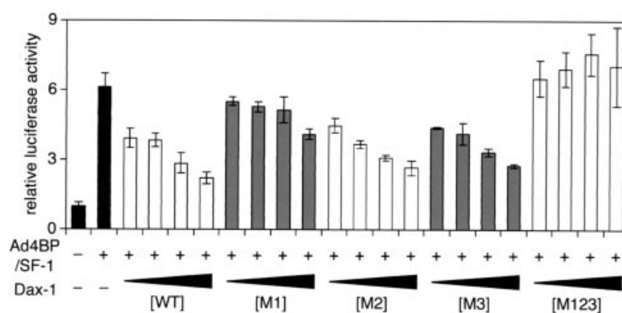

FIG. 2. LXXLL-related motifs are necessary for the repressor function of Dax-1. Repressor activities of the Dax-1 constructs were examined against *CYP11A1* gene promoter transcription. CV-1 cells were transfected as described in Materials and Methods, except that the cells in each well received 200 ng of the reporter plasmid pS2.3H-LUC, 130 ng of pCMX-βGAL for reference, and 300 ng of MT-CEVα (an expression plasmid of the catalytic subunit of protein kinase A) with or without 40 ng of pCMX-Ad4BP and with increasing amounts (0, 10, 20, 40, and 80 ng) of pCMX-Dax-1 plasmids as indicated. Data are mean and SD.

bait (pGBT9)	prey (pGAD424)	2 mM	5 mM	10 mM	20 mM	50 mM	100 mM
Dax-1[WT]	GAL4 AD	+	+	−	−	−	−
	GAD-Ad4BP	+	+	+	+	+	+
Dax-1[M1]	GAL4 AD	+	−	−	−	−	−
	GAD-Ad4BP	+	+	+	+	+	−
Dax-1[M2]	GAL4 AD	+	−	−	−	−	−
	GAD-Ad4BP	+	+	+	+	−	−
Dax-1[M3]	GAL4 AD	+	−	−	−	−	−
	GAD-Ad4BP	+	+	+	+	+	−
Dax-1[M123]	GAL4 AD	+	−	−	−	−	−
	GAD-Ad4BP	+	−	−	−	−	−
Dax-1[N]	GAL4 AD	+	+	+	−	−	−
	GAD-Ad4BP	+	+	+	+	+	+
Dax-1[C]	GAL4 AD	+	+	+	+	+	−
	GAD-Ad4BP	+	+	+	+	+	−
GAL4 DBD	GAL4 AD	+	−	−	−	−	−
	GAD-Ad4BP	+	+	−	−	−	−

FIG. 3. LXXLL-related motifs are necessary for interaction of Dax-1 in yeast. This table shows a summary of the results of yeast two-hybrid assays. The bait and prey plasmids of indicated combinations were transfected into PJ69-4A yeast cells as described in Materials and Methods. + and − indicate yeast that was resistant and sensitive, respectively, to 3-AT.

containing amino acid substitutions in a single LXXLL-related motif (Dax-1[M1], Dax-1[M2], and Dax-1[M3] [Fig. 1]) or all these motifs (Dax-1[M123] [Fig. 1]) and then compared their repressor activities with that of intact Dax-1 (Dax-1[WT] [Fig. 1]) by transient-transfection assays. When Dax-1[M1], Dax-1[M2], and Dax-1[M3] were transfected, their repressor activities were lower but retained in comparison to that of Dax-1[WT]. A greater effect was observed in Dax-1[M1] than Dax-1[M2] and Dax-1[M3]. When all LXXLL-related motifs were disrupted (Dax-1[M123]), repression was completely abrogated (Fig. 2). These results indicated that the LXXLL-related motifs in Dax-1 are essential for its repressor function.

Mode of interaction between Ad4BP/SF-1 and Dax-1. The interaction between Dax-1 and Ad4BP/SF-1 was investigated using yeast two-hybrid assays. Expression plasmids were constructed to encode Dax-1 proteins fused to GAL4 DBD as the bait and full-length Ad4BP/SF-1 protein fused to the GAL4 AD (GAD-Ad4BP) as a prey. In the assays, interactions were observed as acquisition of resistance to the competitive inhibitor 3-aminotriazole (3-AT) by expression of the *HIS3* reporter gene. As shown in Fig. 3, yeast containing the plasmids encoding Dax-1[WT] (Fig. 1) and GAD-Ad4BP grew on a plate containing the reagent at 100 mM, whereas yeast containing GAL4 AD alone instead of GAD-Ad4BP were sensitive to the reagent at 10 mM. Yeast with plasmids encoding Dax-1 mutants with amino acid substitutions in one of the LXXLL-related motifs (Dax-1[M1], Dax-1[M2], and Dax-1[M3] [Fig. 1]) and GAD-Ad4BP were resistant to 3-AT at concentrations up to 50 mM (Dax-1[M1] and Dax-1[M3]) or 20 mM (Dax-1[M2]), whereas yeast with GAL4 AD alone were sensitive at 5 mM. These results indicated that the substitutions reduced but did not completely abolish the interaction between Ad4BP/SF-1 and Dax-1. When all the LXXLL-related motifs were disrupted (Dax-1[M123] [Fig. 1]), no resistant colonies were observed in the presence of the reagent at concentrations of more than 2 mM. Taken together, these results lead to the conclusion that the LXXLL-related motifs in Dax-1 are essential for interaction with Ad4BP/SF-1.

To investigate the possibility that Ad4BP/SF-1 and Dax-1 heterodimerize with each other through their LBDs, two expression plasmids encoding GAL4 DBD fused to an N-terminal portion of Dax-1 (Dax-1[N] [Fig. 1]) and a C-terminal region containing the putative LBD (Dax-1[C] [Fig. 1]) were constructed and used as bait. Yeast containing the plasmids encoding Dax-1[N] and GAD-Ad4BP grew in the presence of 3-AT at 100 mM, whereas yeast containing GAL4 AD alone were sensitive to the reagent at 20 mM. In contrast, yeast with plasmids encoding Dax-1[C] and either GAD-Ad4BP or GAL4 AD were resistant to 3-AT at concentrations up to 50 mM. This relatively high resistance is probably due to intrinsic transcriptional activity of Dax-1[C], although it is not certain why it functions as an activator in yeast. It should be noted that the observation that GAD-Ad4BP did not increase the resistance of yeast carrying Dax-1[C] does not support its heterodimerization thorough this region and is consistent with the results obtained with Dax-1[M123], although it is possible that weak interactions could not be observed due to the high background. Taking these results together, it was concluded that the interaction between Dax-1 and Ad4BP/SF-1 does not require heterodimerization of their LBDs.

Interaction of the LXXLL-related motifs in Dax-1 with Ad4BP/SF-1 and ERα. Mammalian two-hybrid assays were used to characterize interaction through the LXXLL-related motifs. Expression plasmids were constructed that encoded 17 amino acids containing each LXXLL-related motif fused to GAL4 DBD (GAL4-DAXpep7–23, GAL4-DAXpep74–90, and GAL4-DAXpep140–156) to use as bait, while the LBDs of Ad4BP/SF-1 and ERα fused to VP16 AD (VP16-Ad4BP-LBD and VP16-ERα-LBD) were constructed for use as prey (Fig. 4A). In the mammalian two-hybrid assays, an increase in lu-

FIG. 4. The interaction of Ad4BP/SF-1 and ERα requires the AF-2 core for interaction with LXXLL-related motifs in Dax-1. (A) Schematic structures of bait and prey constructs for the mammalian two-hybrid interaction assays. Numbers represent amino acid positions reading from the N terminus. (B and C) Interaction of LXXLL-related motifs in Dax-1 with ERα (B) and Ad4BP/SF-1 (C) on their LBDs. CV-1 cells were transfected as described in Materials and Methods with 25 ng of bait plasmids per well and 50 ng of prey plasmids per well as effectors, as indicated. The reporter plasmid was UAS_G-TK-LUC. Data are mean and SD.

ciferase activity in the presence of prey as a result of the expression of a reporter gene (UAS_G×4-TK-LUC) indicates direct or indirect interaction between bait and prey molecules. The interaction of ERα and the LXXLL-related motifs of Dax-1 was examined in the absence and presence (1 μM) of β-E_2, a major endogenous ligand for ERs. Consistent with the previous report by Zhang et al. (70), VP16-ERα-LBD interacted with all of the LXXLL-related motifs in a ligand-dependent manner whereas no interaction was observed when the AF-2 core of ERα was deleted (VP16-ERα-LBDΔAF2C [Fig. 4A]) (Fig. 4B). The requirement for ligand and AF-2 core suggested that the interaction between the LXXLL-related motifs in Dax-1 and ERα occurs in a manner similar to the interaction between ligand-bound nuclear receptors and LXXLL motifs in coactivators (10, 46, 57).

Likewise, the interaction between VP16-Ad4BP-LBD and each of the LXXLL-related motifs was investigated. As shown in Fig. 4C, transcriptional activation was observed by using each motif, indicating that the LXXLL-related motifs in Dax-1 are able to interact with Ad4BP/SF-1 as well as ERα. It is notable, however, that the interaction between the LXXLL-related motifs and Ad4BP/SF-1 was observed in serum-free media without addition of any putative ligand. This suggested that the LBD of Ad4BP/SF-1 would adopt a structure similar to that of nonliganded receptors that interact with corepressors and that the LXXLL-related motifs in Dax-1 interact with this LBD as ΦXXΦΦ motifs generally do. To test this, we investigated whether the LXXLL-related motifs require the AF-2 core of Ad4BP/SF-1 for interaction, since the AF-2 core is thought to be dispensable for the interaction of ΦXXΦΦ motifs (19, 45, 51). For this purpose, an expression plasmid was constructed that encoded VP16 AD fused to the LBD of Ad4BP/SF-1 without the AF-2 core (VP16-Ad4BP-LBDΔAF2C [Fig. 4A]). As shown in Fig. 4C, GAL4-DAXpep74–90 and GAL4-DAXpep140–156 did not interact with this truncated protein whereas interaction was observed with GAL4-DAXpep7–23, although the luciferase activity was weaker than when a construct containing an intact C terminus (VP16-Ad4BP-LBD) was used.

Interaction of LXXLL-related motifs in Dax-1 with LRH-1 and a subset of nuclear receptors. To confirm the direct interaction between Ad4BP/SF-1 and Dax-1, in vitro pull-down assays were performed using a recombinant Ad4BP/SF-1 (amino acids 5 to 462) fused to MBP (MBP-Ad4BP) and in vitro-translated Dax-1 with a GFP tag (GFP-Dax-1[WT]). For comparison, interaction with a fragment encoding liver receptor homolog 1 (LRH-1; NR5A2) (amino acids 79 to 560) was also examined by using the MBP fusion (MBP–LRH-1). As shown in Fig. 5A, GFP-Dax-1[WT] was coprecipitated by MBP-Ad4BP but more efficiently by MBP–LRH-1 (Fig. 5A, left). In contrast, both MBP-Ad4BP and MBP–LRH-1 failed to coprecipitate the in vitro product when all the LXXLL-related motifs in GFP-Dax-1[WT] were disrupted (GFP-Dax-1[M123]) (Fig. 5A, right). Similar results were obtained using recombinant Dax-1[WT] and Dax-1[M123] fused to MBP with in vitro-synthesized Ad4BP/SF-1 and LRH-1 (data not shown). It should be noted that the strength of interaction was considerably different between these orphan receptors. The recovery of GFP-Dax-1[WT] by MBP-Ad4BP was less than 1% of the input, although it was reproducibly more than the recovery of GFP-Dax-1[M123]. These results indicated that Ad4BP/SF-1 and LRH-1 could interact directly with Dax-1 through its LXXLL-related motifs with different affinities. The difference between these receptors is presumed to reflect particular properties of Ad4BP/SF-1 (see Discussion).

Interactions of LRH-1 and, for comparison, ERR2 (NR3B2), FTZ-F1 (NR5A3; the fly homolog of Ad4BP/SF-1 and LRH-1), HNF4 (NR2A1), and RORα (NR1F1) with the LXXLL-related motifs in Dax-1 were examined by mammalian two-hybrid interaction assays using GAL4-DAXpep7–23, GAL4-DAXpep74–90, and GAL4-DAXpep140–156 as bait. Expression plasmids encoding the LBDs of these receptors

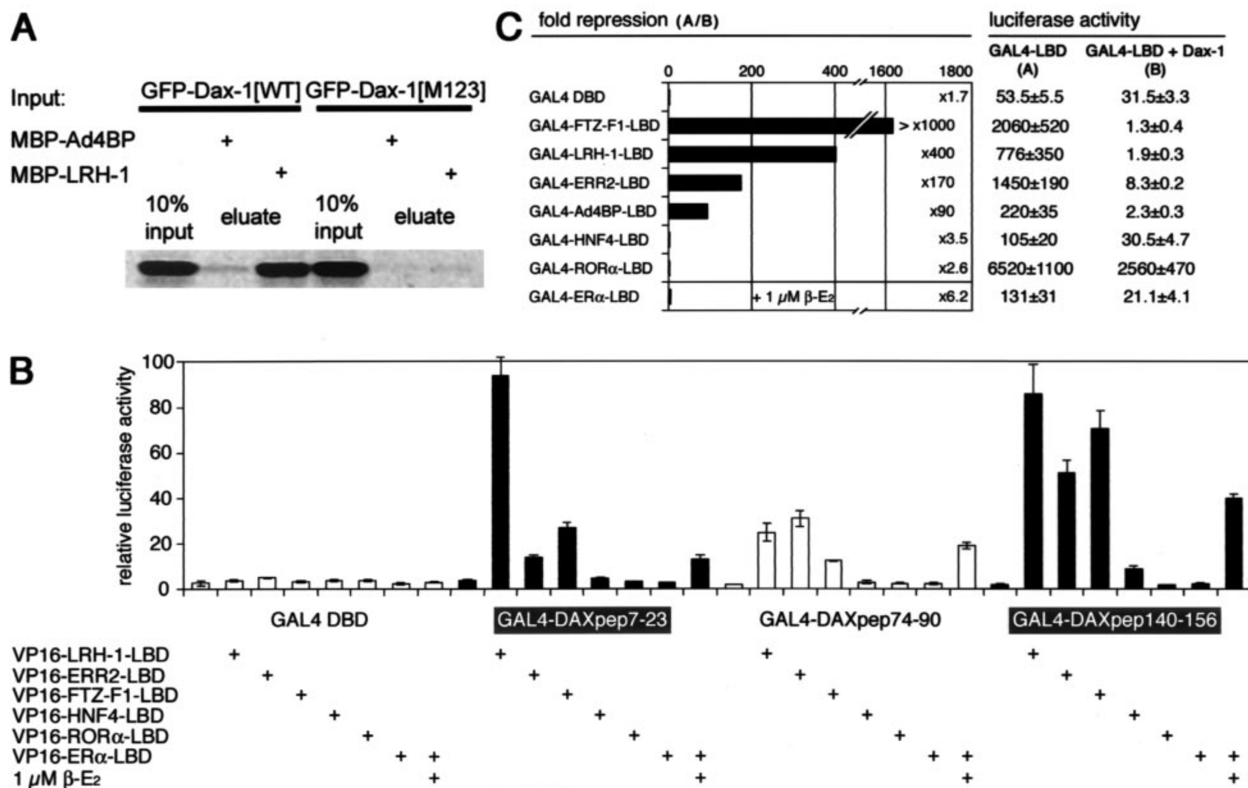

FIG. 5. LXXLL-related motifs in Dax-1 interact with LRH-1, ERR2, and fly FTZ-F1. (A) In vitro interaction of MBP-Ad4BP and MBP-LRH-1 with fluorescence-labeled GFP-Dax-1[WT] and GFP-Dax-1[M123]. GFP was used not as a source of fluorescence but as a sequence that allows efficient expression (see Materials and Methods), because for unknown reasons, pCMX-Dax-1[WT] and pCMX-Dax-1[M123] failed to express the proteins in vitro. "10% input" represents one-tenth of the amount of labeled protein in a reaction mixture. (B) Interaction of LXXLL-related motifs in Dax-1 with orphan receptors on their LBDs. CV-1 cells were transfected as described in Materials and Methods with 25 ng of bait plasmids per well and 50 ng of prey plasmids per well as effectors as indicated. Data are mean and SD. (C) Repression by Dax-1 against transcription activated by orphan receptors whose DBDs were replaced by GAL4 DBD. EPC cells were transfected as described in Materials and Methods with 25 ng of pCMX-GAL4-LBD plasmids per well, with or without 50 ng of pCMX-Dax-1[WT] per well as effectors in the presence or absence of β-E_2 as indicated. Bars represent fold repression by Dax-1. Relative luciferase activities are expressed numerically. In these experiments, the reporter plasmid was UAS_G-TK-LUC.

fused to VP16 AD (VP16-LRH-1-LBD, VP16-ERR2-LBD, VP16-FTZ-F1-LBD, VP16-HNF4-LBD, and VP16-RORα-LBD) were prepared and used as prey. As shown in Fig. 5B, VP16-LRH-1-LBD, VP16-ERR2-LBD, and VP16-FTZ-F1-LBD activated transcription of the reporter gene $UAS_G\times4$-TK-LUC in conjunction with all the bait constructs. VP16-HNF4-LBD exhibited a relatively weak level of activation with GAL4-DAXpep140–156 but not with GAL4-DAXpep7–23 or GAL4-DAXpep74–90. No significant activation by VP16-RORα-LBD was observed with any of these constructs.

Correlation between interaction with LXXLL-related motifs and transcriptional repression by Dax-1. We investigated whether the repressor activity of Dax-1 correlates with the interaction of LXXLL-related motifs with the receptors described above. Transcriptional repression by Dax-1 was examined against the activities of GAL4 DBD fused to the LBDs of the nuclear receptors (GAL4-Ad4BP-LBD, GAL4-LRH-1-LBD, GAL4-ERR2-LBD, GAL4-FTZ-F1-LBD, GAL4-HNF4-LBD, and GAL4-RORα-LBD). As shown numerically in Fig. 5C, transcription of the reporter gene $UAS_G\times4$-TK-LUC was activated by GAL4-Ad4BP-LBD, GAL4-LRH-1-LBD, GAL4-ERR2-LBD, GAL4-FTZ-F1-LBD, GAL4-HNF4-LBD, and GAL4-RORα-LBD, as well as by GAL4-ERα-LBD in the presence of 1 μM β-E_2 (compare with the transcriptional activity when only GAL4 DBD was expressed [column A]). When Dax-1[WT] was cotransfected, it exerted efficient repression on transcription of the reporter gene activated by GAL4-Ad4BP-LBD, GAL4-LRH-1-LBD, GAL4-ERR2-LBD, and GAL4-FTZ-F1-LBD (left panel in Fig. 5C). For these fusion constructs, the addition of Dax-1 decreased the levels of transcription by a factor of dozens or even a thousand, resulting in transcriptional activities that were lower than that of GAL4 DBD in the presence of Dax-1 (column B). In contrast, only weak repression was observed when the reporter gene was activated by GAL4-HNF4-LBD and GAL4-RORα-LBD. Consistent with the report by Zhang et al. (70), transcription driven by GAL4-ERα-LBD in the presence of 1 μM β-E_2 was repressed by Dax-1, although this repression was less efficient than when the reporter gene was activated by GAL4-Ad4BP-LBD, GAL4-LRH-1-LBD, GAL4-ERR2-LBD, and GAL4-FTZ-F1-LBD. These observations were essentially consistent with the results of the interaction assays for all the

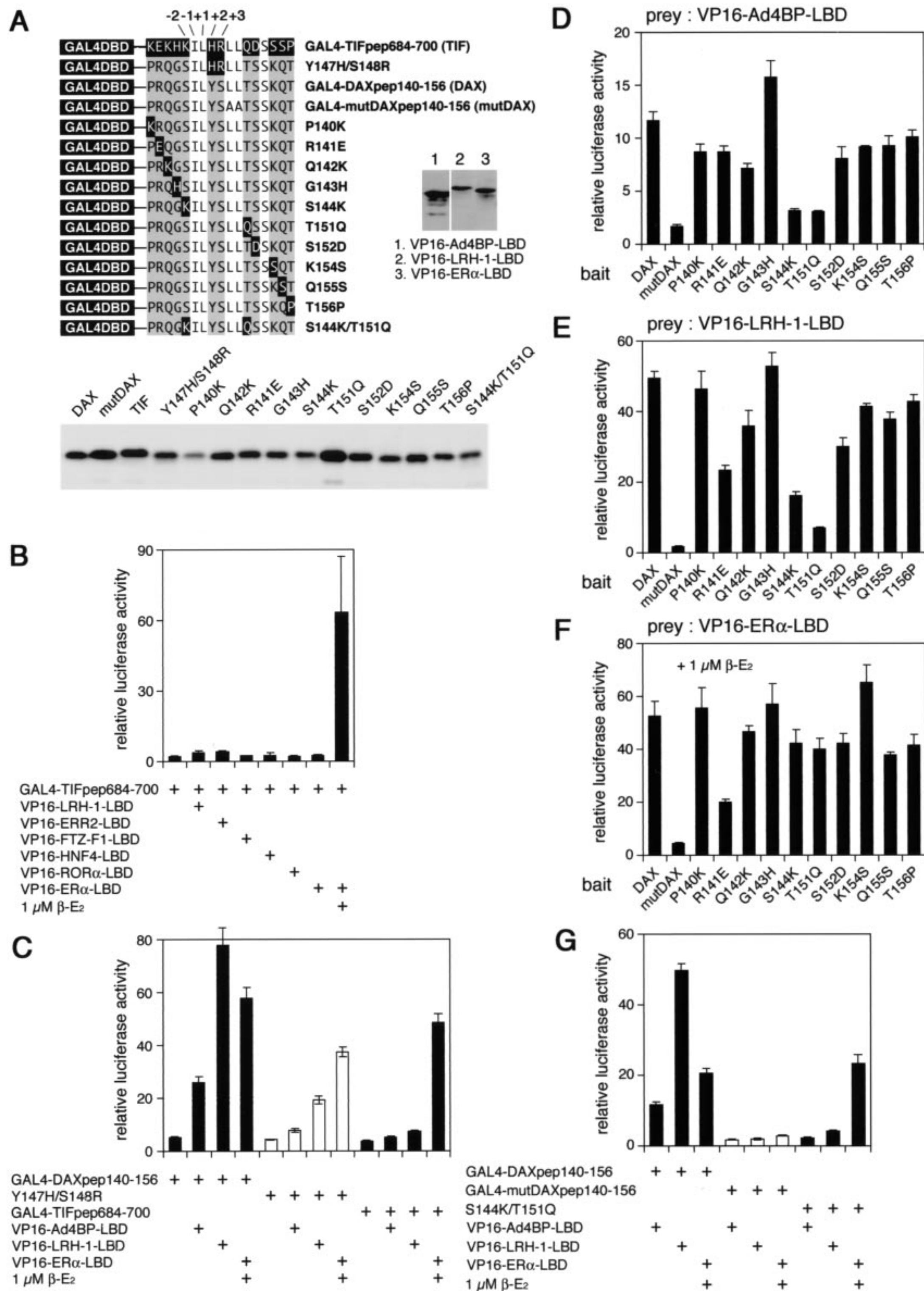

FIG. 6. Critical residues on DAXpep140–156 for interaction with Ad4BP/SF-1 and LRH-1. (A) Amino acid sequences of GAL4-TIFpep684–700, GAL4-DAXpep140–156, and related constructs that contain an amino acid substitution(s). Residues characteristic of GAL4-TIFpep684–700 and GAL4-DAXpep140–156 are indicated by black and gray boxes, respectively. For reference, the first leucine in the core sequence was defined as position +1. Protein expression in these bait constructs was confirmed by Western blot analysis with anti-GAL4 DBD monoclonal antibody RK5C1 (Santa Cruz Biotech, Santa Cruz, Calif.) as shown together with the result of the prey constructs of Ad4BP/SF-1, LRH-1, and ERα probed with anti-VP16 AD monoclonal antibody 1–21 (Santa Cruz Biotech). (B) Interaction of GAL4-TIFpep684–700 with orphan receptors and ERα on their LBDs. (C) Interaction of Y147H/S148R with Ad4BP/SF-1, LRH-1, and ERα on their LBDs. (D to F) Effects of amino acid substitutions on interaction with Ad4BP/SF-1 (D), LRH-1 (E), and ERα (F) on their LBDs. In these panels, GAL4-DAXpep140–156 and GAL4-mutDAX-pep140–156 are abbreviated as "DAX" and "mutDAX," respectively. (G) Effect of substitutions in S144K/T151Q on interaction with Ad4BP/SF-1, LRH-1, and ERα. In these experiments, CV-1 cells were transfected as described in Materials and Methods with 25 ng of bait plasmids and 50 ng of prey plasmids as effectors as indicated. The reporter plasmid was UAS_G-TK-LUC. Data shown in panels B to G are mean and SD.

receptors except ERα and indicated that interaction through LXXLL motifs is critical for the repressor function of Dax-1.

Requirement of amino acids in an LXXLL-related motif in Dax-1 for specific interaction. These observations strongly suggested that the LXXLL-related motifs in Dax-1 have a preference for interaction with a group of nuclear receptors such as Ad4BP/SF-1, LRH-1, ERR2, and FTZ-F1, because HNF4 and RORα are also postulated to interact with cofactors through LXXLL or LXXLL-related motifs (16, 27). Therefore, we decided to characterize the specificity for interaction by LXXLL-related motifs of Dax-1 by comparison with an LXXLL motif that does not interact with these receptors, such as that found in a p160 family coactivator, TIF2 (63). We constructed an expression plasmid that encodes GAL4 DBD fused with one of the three LXXLL motifs in human TIF2 (GAL4-TIFpep684–700 [Fig. 6A]). Interaction of this LXXLL motif was examined with VP16-Ad4BP-LBD, VP16-LRH-1-LBD, VP16-ERR2-LBD, VP16-FTZ-F1-LBD, VP16-HNF4-LBD, VP16-RORα-LBD, and VP16-ERα-LBD. The LXXLL motif interacted with none of the LBDs (Fig. 6B and C for VP16-Ad4BP-LBD) except VP16-ERα-LBD, which exhibited interaction in the presence of 1 μM β-E_2 (Fig. 6B). This observation suggested that the amino acid sequence of LXXLL or LXXLL-related motifs is critical for interaction with these receptors. Since LXXLL-related motifs in Dax-1 required the hydrophobic residues in the core sequence (leucine and methionine) for interaction, similar to LXXLL motifs (Fig. 3 and 6G), the amino acids responsible for the specific interaction were expected to be in the remaining positions in LXXLL or LXXLL-related motifs. Based on this assumption, we investigated which amino acid residues are required for interaction with Ad4BP/SF-1 and LRH-1 by comparing the LXXLL motif in TIF2 (GAL4-TIFpep684–700) and the third motif in Dax-1 (GAL4-DAX-pep140–156) as a representative of the three motifs. The expression plasmid Y147H/S148R (Fig. 6A) was constructed to examine the importance of hydrophilic amino acids (i.e., the "XX") in the core LXXLL. This plasmid encodes GAL4-DAXpep140–156 in which the amino acids at positions +2 (tyrosine) and +3 (serine) (see Fig. 6A for position assignment) are replaced by the corresponding amino acids (histidine and arginine) of GAL4-TIFpep684–700. Interaction of this protein with VP16-Ad4BP-LBD, VP16-LRH-1-LBD, and VP16-ERα-LBD was then examined. Compared with the interaction of GAL4-DAXpep140–156, these substitutions considerably reduced the interaction with VP16-Ad4BP-LBD and VP16-LRH-1-LBD but not with VP16-ERα-LBD (Fig. 6C). These results indicated that the two hydrophilic amino acids in the core sequence contribute to the specific interaction with Ad4BP/SF-1 and LRH-1.

These substitutions, therefore, did not completely abolish the interaction; the remaining capability for interaction strongly suggested that amino acids adjacent to the core sequence, as well as the internal "XX," are involved in the interaction. To identify the important amino acid residues, expression plasmids were constructed to encode GAL4-DAX-pep140–156 in which individual amino acids were replaced by corresponding residues of GAL4-TIFpep684–700 (Fig. 6A) and interactions were examined using the same set of prey constructs. Among these constructs, S144K and T151Q containing a substitution at either position −2 or +6 (Fig. 6A) exhibited the most significant reductions in interaction with VP16-Ad4BP-LBD (Fig. 6D) and VP16-LRH-1-LBD (Fig. 6E). In contrast, such reduction did not occur for VP16-ERα-LBD (Fig. 6F). The substitution in R141E at position −5 (Fig. 6A) reduced the interaction with VP16-LRH-1-LBD. This amino acid residue was, however, unlikely to be associated with specificity since the interaction with VP16-ERα-LBD was also impaired by this substitution. The substitutions in P140K, Q142K, G143H, and K154S (Fig. 6A) had marginal effects on the interaction with VP16-Ad4BP-LBD and/or VP16-LRH-1-LBD, and the other substitutions exerted no specific effect on the interaction. When substitutions were simultaneously introduced at positions −2 and +6 (S144K/T151Q), the interaction with VP16-Ad4BP-LBD and VP16-LRH-1-LBD was severely impaired. The luciferase activity was almost reduced to the level of GAL4-mutDAXpep140–156, which is no longer able to function as an LXXLL motif due to the lack of the tandem leucines in the core LXXLL. In contrast, the substitutions exerted no effect on the interaction with VP16-ERα-LBD (Fig. 6G). Taken together, these findings allow us to conclude that the amino acids at positions −2, +2, +3, and +6 on GAL4-DAXpep140–156 are especially important for its specific interaction with VP16-Ad4BP-LBD and VP16-LRH-1-LBD.

DISCUSSION

Specificity of LXXLL-related motifs in Dax-1. In this study, we investigated the interaction between Ad4BP/SF-1 and Dax-1 through three LXXLL-related motifs in Dax-1 and showed that these motifs can interact specifically with LRH-1, ERR2, and FTZ-F1 as well as with Ad4BP/SF-1 and ERα. Under the conditions used in our study, RORα and HNF4 showed no significant interaction with the LXXLL-related motifs in Dax-1 except for a weak interaction of HNF4 with one LXXLL-related motif (GAL4-DAXpep140–156). These findings suggested that the features of the LBD necessary for the specific interaction are shared among Ad4BP/SF-1, LRH-1, ERR2, and FTZ-F1. Taking these results together with the finding that none of these receptors interacted with an LXXLL motif in TIF2 (GAL4-TIFpep684–700), we postulated that the ability to distinguish differences in the amino acid sequence of LXXLL or LXXLL-related motifs was a shared feature. Since the LXXLL-related motifs in Dax-1 were expected to satisfy the sequence requirement for interaction with these receptors, we attempted to identify the prerequisite amino acids for the specific interaction by comparison with the LXXLL motif in TIF2. Interaction assays using constructs containing the GAL4-DAXpep140–156 sequence with single and multiple amino acid substitutions revealed that amino acids critical for interaction with Ad4BP/SF-1 and LRH-1 are located at positions from −2 to +6 of the LXXLL or LXXLL-related motif (the first leucine of the core sequence was set to +1 [Fig. 6A]).

Indeed, with a few exceptions, the amino acids at these positions are conserved among mammalian species, and similar sequences have been found in other species (Fig. 7). Remarkable evidence of the importance of the amino acids at these positions was also provided by previous studies of other cofactors containing the LXXLL or LXXLL-related motif. As shown in Fig. 7, an LXXLL motif containing serine at position −2 and threonine at +6 is present in the fly transcription factor

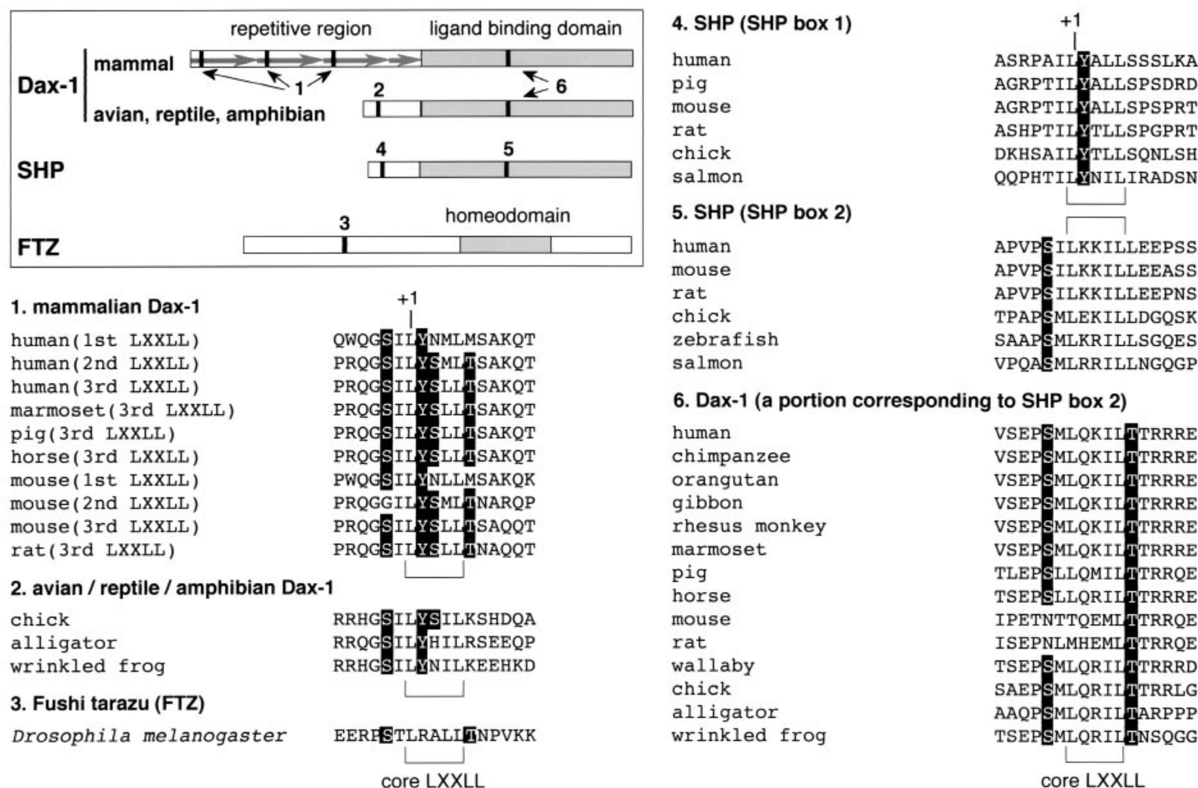

FIG. 7. Cofactors for Ad4BP/SF-1, LRH-1, and FTZ-F1 contain LXXLL or LXXLL-related motifs with S, Y, S, and/or T at positions −2, +2, +3, and +6, respectively. Six amino acid alignments show sequences of the LXXLL or LXXLL-related motifs (many of these are still putative) in Dax-1, FTZ, and SHP. Black boxes in the alignments highlight serine, tyrosine, serine, and threonine at positions −2, +2, +3, and +6, respectively. Solid bars in the schematic diagram indicate the positions of the LXXLL or LXXLL-related motifs, and numbers indicate which alignment (1 to 6) corresponds to the bar. Sequence comparison of Dax-1 and SHP suggested that the positions of the LXXLL-related motifs in the LBD shown in alignments 5 and 6 are essentially identical (data not shown). All sequences were retrieved from the GenBank database. The sequences of pig, chicken, zebrafish, and salmon SHP have putatively been obtained by conceptual translation from expressed sequence tag sequences retrieved by homology. Accession numbers are as follows: for Dax-1, 5016090 (human), AAK01643 (chimpanzee), AAK01644 (orangutan), AAK01645 (gibbon), AAK01647 (rhesus monkey), AAK01646 (marmoset), 1870692 (pig), AAF89613 (horse), 1345127 (mouse), 1620449 (rat), 4100303 (wallaby), AAF19395 (chicken), AAD55095 (alligator), and BAB85864 (wrinkled frog); for FTZ, 123366 (*Drosophila melanogaster*); for SHP, BAB68530 (human), AW325605 (pig expressed sequence tag), 1374949 (mouse), 1871465 (rat), BG712451 (chicken expressed sequence tag), AA495245 (zebrafish expressed sequence tag), and BG935659 (salmon expressed sequence tag).

Fushi tarazu (FTZ), which interacts with FTZ-F1 in an LXXLL motif-dependent manner, as reported by our group and other investigators (55, 59, 68). Similar examples are found in SHP (NR0B2), a member of the nuclear receptor superfamily that has structural features similar to Dax-1 and functions as a repressor of nuclear receptors CAR (NR1I4), RARα (NR1B1) (56), ERs (26), LRH-1 (15), HNF4 (32), and the fusion proteins GAL4-Ad4BP-LBD, GAL4-LRH-1-LBD, GAL4-ERR2-LBD, GAL4-HNF4-LBD, and GAL4-FTZ-F1-LBD (data not shown). SHP contains two LXXLL-related motifs, which are considered to be implicated in interaction with ERα and ERβ (25). One of the LXXLL-related motifs (SHP box 1) has a tyrosine conserved at position +2, and the other (SHP box 2) contains a serine at −2. Intriguingly, Dax-1 contains a sequence similar to SHP box 2 at the same position in the LBD. This sequence, which contains threonine at +6 as well as serine at −2, is conserved among animal species including frog, alligator, chicken, and mammals, except that those of mouse and rat seem highly varied. Although we have not been able to confirm that this motif interacts with Ad4BP/SF-1, LRH-1, and ERα by mammalian two-hybrid experiments using a strategy similar to that adopted in the present study (data not shown), this motif might play a role in the function of Dax-1, especially of nonmammalian species that also have structural features similar to SHP. Together with our results with a construct simultaneously mutated at these positions (S144K/T151Q), these examples indicate the importance of these two amino acids for the specificity. The amino acid at position −1 is probably also important, because we observed that replacement of isoleucine by threonine at this position abolished the interaction of a peptide related to GAL4-DAX-pep140–156 (data not shown).

It should be noted that Chang et al. (5) obtained a series of LXXLL motifs interacting with ERα by means of phage display screening and classified them into three classes: class I, II, and III NR-boxes (LXXLL motifs). According to the classification based on the sequences flanking the core LXXLL, the motifs in Dax-1 belong to class III, which is characterized by serine at position −2 and isoleucine at −1. Our finding that none of the LXXLL-related motifs in Dax-1 interacted with

RORα is consistent with a report that RORα preferentially interacts with class II motifs, which contain proline at −2 (27). We therefore concluded that Ad4BP/SF-1 and LRH-1 prefer class III-type sequences and that the particular sequence SILYSLLT may stand as a typical binding sequence for Ad4BP/SF-1 and LRH-1.

Underlying mechanisms of transcriptional repression by Dax-1. The interaction between Ad4BP/SF-1 and Dax-1 has previously been investigated by Ito et al. (22), who showed the importance of the N-terminal repetitive region of Dax-1 for interaction. Later, Zhang et al. (70) pointed out that the repeats contain sequences similar to the LXXLL motif and proved that the sequences are necessary for interaction with ERs in the presence of ligand. This information suggested that Dax-1 interacts with Ad4BP/SF-1 through these LXXLL-related motifs, which was confirmed by our observations in this study. The interaction through the LXXLL-related motifs also indicated that the mechanism of repression proposed by Zhang et al. (70) might also be applicable to the repression of Ad4BP/SF-1. In this model, transcription activated by Ad4BP/SF-1 may be repressed by both competition with coactivators such as SRC-1 (7, 23) and recruitment of corepressors such as N-CoR (6) and Alien (1) through the C-terminal half of Dax-1.

Our conclusion that Dax-1 interacts with Ad4BP/SF-1 principally via its N-terminal half is consistent with the results of in vitro pull-down assays by Ito et al. (22). However, it has recently been reported by Lopez et al. (34) that an intact C terminus of mouse Dax-1 is required for its interaction with Ad4BP/SF-1 in mammalian two-hybrid assays, which is apparently inconsistent with the present results. Although the two lines of evidence might reflect different aspects of Dax-1 interaction and this inconsistency might be attributable to differences in the assay systems, it is difficult to find a reasonable explanation that accounts for both observations. These observations might be consistent with each other because our results do not exclude the possibility that the C-terminal half could partly contribute to the interaction with Ad4BP/SF-1 as a secondary interaction domain.

The sequence requirement of Ad4BP/SF-1 for interaction with Dax-1 has previously been investigated by Crawford et al. (6). Their results showed that a fragment of Ad4BP/SF-1 (amino acids 120 to 447) is sufficient for interaction with Dax-1. Since the AF-2 core of Ad4BP/SF-1 is located at amino acids 452 to 457, their observations indicated that the AF-2 core is not essential for the interaction, which is apparently inconsistent with the notion that the interaction motifs in Dax-1 are similar to the LXXLL motif. This contradiction was explained by the results of mammalian two-hybrid experiments with individual LXXLL-related motifs as bait. With respect to ERα, the interaction with these motifs was AF-2 core dependent, which confirmed that they indeed act in a way as LXXLL motifs interact with the receptor. This was found to be true of Ad4BP/SF-1, except that the LXXLL-related motif in the first repeat (GAL4-DAXpep7–23) unexpectedly interacted with Ad4BP/SF-1 truncated at the AF-2 core (VP16-Ad4BP-LBDΔAF2C). Based on these results, we inferred that the LXXLL-related motif in the first repeat has a special property that enables Dax-1 to interact with Ad4BP/SF-1 lacking the AF-2 core. Further analyses are necessary to clarify the structural basis of this interaction. Crawford et al. (6) also observed that the Ad4BP/SF-1 fragment comprising amino acids 226 to 462 efficiently interacted with Dax-1 but that comprising amino acids 230 to 462 did not. Since amino acids 226 to 230 are at the N-terminal end of the conserved LBD, these observations strongly suggested that the integrity of the LBD structure is important for interaction with the LXXLL-related motifs in Dax-1. Furthermore, deletion of the C terminus up to amino acid 437 markedly decreased the interaction. This observation could be interpreted as showing that amino acids 437 to 442 represent a component of the binding surface for the first LXXLL-related motif in Dax-1. However, we think this is unlikely, since if it were true, the LXXLL-related motif should interact with Ad4BP/SF-1 in a manner distinct from that of interaction of LXXLL and ΦXXΦΦ motifs with nuclear receptors, since biochemical and structural studies (8, 10, 19, 39, 45, 46, 51, 57) have suggested that the corresponding portion in LBD had no direct association with cofactor binding through these motifs.

Conformation of Ad4BP/SF-1 and its ligand. The interaction of LXXLL-related motifs in Dax-1, which interact with the holo ERα, suggested that Ad4BP/SF-1, LRH-1, ERR2, and FTZ-F1 have transcriptionally active conformations, and in fact they have consistently been regarded as constitutive activators (13, 43, 49, 50, 64, 66). Our results suggested that the binding specificity of LXXLL or LXXLL-related motifs might play a critical role, similar to that of the ligand, in regulating the activity of these orphan receptors. However, it would be necessary to further consider the issue of conformational regulation of one of these, Ad4BP/SF-1.

There is controversy about whether Ad4BP/SF-1 has a cognate ligand (30, 41). Although it is generally accepted that binding of ligands to nuclear receptors reversibly changes their allosteric state from the apo type (inactive form) to the holo type (active form) so that they can activate transcription, the constitutive activity of Ad4BP/SF-1 by itself does not prove that it has a structure like the holo type. In this respect, suggestive evidence for its structural peculiarity was obtained by the pull-down assays, in which Dax-1 coprecipitated Ad4BP/SF-1 in a much smaller amount compared to LRH-1. Since Dax-1 was able to repress the activity of FTZ-F1, the fly homolog of Ad4BP/SF-1 and LRH-1, it is presumed that Dax-1 recognizes structural features conserved among all these receptors, and thus the weak interaction of Ad4BP/SF-1 observed in the pull-down experiments would be attributed to properties that might have been acquired during the evolution of the molecule. The behavior of Ad4BP/SF-1 would be accounted for by one of the following four explanations. (i) Ad4BP/SF-1 might need to interact with Dax-1 at a certain strength, because weaker or stronger interactions would cause loss-of-function or gain-of-function phenotypes of Dax-1, respectively, similar to those observed in patients with the congenital diseases adrenal hypoplasia congenita and dosage-sensitive sex reversal (69). In this case, Ad4BP/SF-1 might be constitutively in a structural state similar to the holo type but unfavorable for strong interaction. (ii) Ad4BP/SF-1 might require a ligand or intracellular factors that were absent in the binding reaction, in order to maintain the structure preferable for efficient interaction with Dax-1. (iii) Ad4BP/SF-1 might need posttranslational modification or the assistance of intracellular factors such as molecular chaperones for adopting

proper conformations that do not take place in the expression systems used in our studies. (iv) Interaction might be abrogated by events such as posttranslational modification taking place in the experimental systems. Since LXXLL-related motifs in Dax-1 interacted with Ad4BP/SF-1 in a manner similar to already-known LXXLL motifs in mammalian two-hybrid assays (Fig. 4B and C), it is feasible that Ad4BP/SF-1 adopts a structural state like the holo type rather than the apo type in cultured cells, which might contain molecules that could function as a ligand for Ad4BP/SF-1.

LRH-1 as a novel target of Dax-1. The LXXLL-related motifs in Dax-1 efficiently interacted with LRH-1 in both mammalian two-hybrid and in vitro pull-down assays, which correlated well with the strong repression by Dax-1. Recently, two groups have independently reported that LRH-1 is expressed in steroidogenic tissues (2, 64) where both Ad4BP/SF-1 and Dax-1 are also expressed (20, 21, 28). Among the members of the nuclear receptor superfamily, LRH-1 structurally resembles Ad4BP/SF-1 most closely (47). Since their DBDs are quite similar and can recognize the same sequences on promoters (13), these two nuclear receptors may share the target specificity of various steroidogenic genes such as *CYP11B1* and *CYP17* (64). Our present study has provided evidence that LRH-1 is a potential target of Dax-1. Since the regulation of steroidogenic genes has been investigated mainly in relation to Ad4BP/SF-1 and Dax-1, future studies focusing on LRH-1 would enhance our understanding of the highly elaborate regulation of steroidogenic gene expression. ERR2 has also been postulated as a potential target of Dax-1. However, to date, no evidence has emerged to support the spatiotemporal overlap of ERR2 and Dax-1.

ACKNOWLEDGMENTS

We are most grateful to Ronald M. Evans, Vincent Giguère, Masato Kinoshita, G. Stanley McKnight, Ohtsura Niwa, Yoshinori Ohsumi, Keith L. Parker, Frances M. Sladek, Hitoshi Ueda, and Ruth T. Yu for providing materials and/or valuable advice; to Kaname Kawajiri, Hirofumi Mizusaki, Hidesato Ogawa, Shiho Ogawa, and Sanae Oka for constructing plasmids and/or for helpful discussions; to Ryoichiro Kageyama and Shuh Narumiya for generous support; and to the NIBB Center for Analytical Instruments for assistance in operating the laser-excited gel scanner.

This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology; Japan Science and Technology Corporation; Japan Society for Promotion of Science; and Human Frontiers Science Program.

REFERENCES

1. **Altincicek, B., S. P. Tenbaum, U. Dressel, D. Thormeyer, R. Renkawitz, and A. Baniahmad.** 2000. Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita. J. Biol. Chem. **275:**7662–7667.
2. **Boerboom, D., N. Pilon, R. Behdjani, D. W. Silversides, and J. Sirois.** 2000. Expression and regulation of transcripts encoding two members of the NR5A nuclear receptor subfamily of orphan nuclear receptors, steroidogenic factor-1 and NR5A2, in equine ovarian cells during the ovulatory process. Endocrinology **141:**4647–4656.
3. **Bourguet, W., M. Ruff, P. Chambon, H. Gronemeyer, and D. Moras.** 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXRα. Nature **375:**377–382.
4. **Brzozowski, A. M., A. C. Pike, Z. Dauter, R. E. Hubbard, T. Bonn, O. Engstrom, L. Ohman, G. L. Greene, J. A. Gustafsson, and M. Carlquist.** 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature **389:**753–758.
5. **Chang, C., J. D. Norris, H. Gron, L. A. Paige, P. T. Hamilton, D. J. Kenan, D. Fowlkes, and D. P. McDonnell.** 1999. Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors α and β. Mol. Cell. Biol. **19:**8226–8239.
6. **Crawford, P. A., C. Dorn, Y. Sadovsky, and J. Milbrandt.** 1998. Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. Mol. Cell. Biol. **18:**2949–2956.
7. **Crawford, P. A., J. A. Polish, G. Ganpule, and Y. Sadovsky.** 1997. The activation function-2 hexamer of steroidogenic factor-1 is required, but not sufficient for potentiation by SRC-1. Mol. Endocrinol. **11:**1626–1635.
8. **Darimont, B. D., R. L. Wagner, J. W. Apriletti, M. R. Stallcup, P. J. Kushner, J. D. Baxter, R. J. Fletterick, and K. R. Yamamoto.** 1998. Structure and specificity of nuclear receptor-coactivator interactions. Genes Dev. **12:**3343–3356.
9. **Egea, P. F., A. Mitschler, N. Rochel, M. Ruff, P. Chambon, and D. Moras.** 2000. Crystal structure of the human RXRα ligand-binding domain bound to its natural ligand: 9-cis retinoic acid. EMBO J. **19:**2592–2601.
10. **Feng, W., R. C. Ribeiro, R. L. Wagner, H. Nguyen, J. W. Apriletti, R. J. Fletterick, J. D. Baxter, P. J. Kushner, and B. L. West.** 1998. Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science **280:**1747–1749.
11. **Fijan, N., D. Sulimanovic, M. Bearzotti, D. Muzinc, L. O. Zwillenberg, S. Chilmonczyk, J. F. Vautherot, and P. de Kinkelin.** 1983. Some properties of the Epithelioma papulosum cyprini (EPC) cell line from carp *Cyprinus carpio.* Ann. Virol. (Inst. Pasteur) **134E:**207–220.
12. **Forman, B. M., K. Umesono, J. Chen, and R. M. Evans.** 1995. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell **81:**541–550.
13. **Galarneau, L., J. F. Pare, D. Allard, D. Hamel, L. Levesque, J. D. Tugwood, S. Green, and L. Belanger.** 1996. The α_1-fetoprotein locus is activated by a nuclear receptor of the *Drosophila* FTZ-F1 family. Mol. Cell. Biol. **16:**3853–3865.
14. **Giguere, V.** 1999. Orphan nuclear receptors: from gene to function. Endocr. Rev. **20:**689–725.
15. **Goodwin, B., S. A. Jones, R. R. Price, M. A. Watson, D. D. McKee, L. B. Moore, C. Galardi, J. G. Wilson, M. C. Lewis, M. E. Roth, P. R. Maloney, T. M. Willson, and S. A. Kliewer.** 2000. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol. Cell **6:**517–526.
16. **Harish, S., T. Khanam, S. Mani, and P. Rangarajan.** 2001. Transcriptional activation by hepatocyte nuclear factor-4 in a cell- free system derived from rat liver nuclei. Nucleic Acids Res. **29:**1047–1053.
17. **Heery, D. M., S. Hoare, S. Hussain, M. G. Parker, and H. Sheppard.** 2001. Core LXXLL motif sequences in CREB-binding protein, SRC1, and RIP140 define affinity and selectivity for steroid and retinoid receptors. J. Biol. Chem. **276:**6695–6702.
18. **Heery, D. M., E. Kalkhoven, S. Hoare, and M. G. Parker.** 1997. A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature **387:**733–736.
19. **Hu, X., and M. A. Lazar.** 1999. The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature **402:**93–96.
20. **Ikeda, Y., A. Swain, T. J. Weber, K. E. Hentges, E. Zanaria, E. Lalli, K. T. Tamai, P. Sassone-Corsi, R. Lovell-Badge, G. Camerino, and K. L. Parker.** 1996. Steroidogenic factor 1 and Dax-1 colocalize in multiple cell lineages: potential links in endocrine development. Mol. Endocrinol. **10:**1261–1272.
21. **Ikeda, Y., Y. Takeda, T. Shikayama, T. Mukai, S. Hisano, and K. I. Morohashi.** 2001. Comparative localization of Dax-1 and Ad4BP/SF-1 during development of the hypothalamic-pituitary-gonadal axis suggests their closely related and distinct functions. Dev. Dyn. **220:**363–376.
22. **Ito, M., R. Yu, and J. L. Jameson.** 1997. DAX-1 inhibits SF-1 mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. Mol. Cell. Biol. **17:**1476–1483.
23. **Ito, M., R. N. Yu, and J. L. Jameson.** 1998. Steroidogenic factor-1 contains a carboxy-terminal transcriptional activation domain that interacts with steroid receptor coactivator-1. Mol. Endocrinol. **12:**290–301.
24. **James, P., J. Halladay, and E. A. Craig.** 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics **144:**1425–1436.
25. **Johansson, L., A. Bavner, J. S. Thomsen, M. Farnegardh, J. A. Gustafsson, and E. Treuter.** 2000. The orphan nuclear receptor SHP utilizes conserved LXXLL-related motifs for interactions with ligand-activated estrogen receptors. Mol. Cell. Biol. **20:**1124–1133.
26. **Johansson, L., J. S. Thomsen, A. E. Damdimopoulos, G. Spyrou, J. A. Gustafsson, and E. Treuter.** 1999. The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERα and ERβ. J. Biol. Chem. **274:**345–353.
27. **Kane, C. D., and A. R. Means.** 2000. Activation of orphan receptor-mediated transcription by Ca^{2+}/calmodulin-dependent protein kinase IV. EMBO J. **19:**691–701.
28. **Kawabe, K., T. Shikayama, H. Tsuboi, S. Oka, K. Oba, T. Yanase, H. Nawata, and K. Morohashi.** 1999. Dax-1 as one of the target genes of Ad4BP/SF-1. Mol. Endocrinol. **13:**1267–1284.
29. **Ko, L., G. R. Cardona, T. Iwasaki, K. S. Bramlett, T. P. Burris, and W. W.**

Chin. 2002. Ser-884 adjacent to the LXXLL motif of coactivator TRBP defines selectivity for ERs and TRs. Mol. Endocrinol. **16:**128–140.
30. **Lala, D. S., P. M. Syka, S. B. Lazarchik, D. J. Mangelsdorf, K. L. Parker, and R. A. Heyman.** 1997. Activation of the orphan nuclear receptor steroidogenic factor 1 by oxysterols. Proc. Natl. Acad. Sci. USA **94:**4895–4900.
31. **Le Douarin, B., A. L. Nielsen, J. M. Garnier, H. Ichinose, F. Jeanmougin, R. Losson, and P. Chambon.** 1996. A possible involvement of TIF1α and TIF1β in the epigenetic control of transcription by nuclear receptors. EMBO J. **15:**6701–6715.
32. **Lee, Y. K., H. Dell, D. H. Dowhan, M. Hadzopoulou-Cladaras, and D. D. Moore.** 2000. The orphan nuclear receptor SHP inhibits hepatocyte nuclear factor 4 and retinoid X receptor transactivation: two mechanisms for repression. Mol. Cell. Biol. **20:**187–195.
33. **Li, L. A., E. F. Chiang, J. C. Chen, N. C. Hsu, Y. J. Chen, and B. C. Chung.** 1999. Function of steroidogenic factor 1 domains in nuclear localization, transactivation, and interaction with transcription factor TFIIB and c-Jun. Mol. Endocrinol. **13:**1588–1598.
34. **Lopez, D., W. Shea-Eaton, M. D. Sanchez, and M. P. McLean.** 2001. DAX-1 represses the high-density lipoprotein receptor through interaction with positive regulators sterol regulatory element-binding protein-1a and steroidogenic factor-1. Endocrinology **142:**5097–5106.
35. **Luo, X., Y. Ikeda, and K. L. Parker.** 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell **77:**481–490.
36. **Mak, H. Y., S. Hoare, P. M. Henttu, and M. G. Parker.** 1999. Molecular determinants of the estrogen receptor-coactivator interface. Mol. Cell. Biol. **19:**3895–3903.
37. **Mangelsdorf, D. J., and R. M. Evans.** 1995. The RXR heterodimers and orphan receptors. Cell **83:**841–850.
38. **Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans.** 1995. The nuclear receptor superfamily: the second decade. Cell **83:**835–839.
39. **McInerney, E. M., D. W. Rose, S. E. Flynn, S. Westin, T. M. Mullen, A. Krones, J. Inostroza, J. Torchia, R. T. Nolte, N. Assa-Munt, M. V. Milburn, C. K. Glass, and M. G. Rosenfeld.** 1998. Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation. Genes Dev. **12:**3357–3368.
40. **Mellon, P. L., C. H. Clegg, L. A. Correll, and G. S. McKnight.** 1989. Regulation of transcription by cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. USA **86:**4887–4891.
41. **Mellon, S. H., and S. R. Bair.** 1998. 25-Hydroxycholesterol is not a ligand for the orphan nuclear receptor steroidogenic factor-1 (SF-1). Endocrinology **139:**3026–3029.
42. **Monte, D., F. DeWitte, and D. W. Hum.** 1998. Regulation of the human P450scc gene by steroidogenic factor 1 is mediated by CBP/p300. J. Biol. Chem. **273:**4585–4591.
43. **Morohashi, K. I., and T. Omura.** 1996. Ad4BP/SF-1, a transcription factor essential for the transcription of steroidogenic cytochrome P450 genes and for the establishment of the reproductive function. FASEB J. **10:**1569–1577.
44. **Nachtigal, M. W., Y. Hirokawa, D. L. Enyeart-VanHouten, J. N. Flanagan, G. D. Hammer, and H. A. Ingraham.** 1998. Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell **93:**445–454.
45. **Nagy, L., H. Y. Kao, J. D. Love, C. Li, E. Banayo, J. T. Gooch, V. Krishna, K. Chatterjee, R. M. Evans, and J. W. Schwabe.** 1999. Mechanism of corepressor binding and release from nuclear hormone receptors. Genes Dev. **13:**3209–3216.
46. **Nolte, R. T., G. B. Wisely, S. Westin, J. E. Cobb, M. H. Lambert, R. Kurokawa, M. G. Rosenfeld, T. M. Willson, C. K. Glass, and M. V. Milburn.** 1998. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-γ. Nature **395:**137–143.
47. **Nuclear Receptors Nomenclature Committee.** 1999. A unified nomenclature system for the nuclear receptor superfamily. Cell **97:**161–163.
48. **Ogawa, H., and K. Umesono.** 1998. Intracellular localization and transcriptional activation by the human glucocorticoid receptor-green fluorescent protein (GFP) fusion proteins. Acta Histochem. Cytochem. **31:**303–308.
49. **Ohno, C. K., H. Ueda, and M. Petkovich.** 1994. The *Drosophila* nuclear receptors FTZ-F1α and FTZ-F1β compete as monomers for binding to a site in the fushi tarazu gene. Mol. Cell. Biol. **14:**3166–3175.
50. **Parker, K. L., and B. P. Schimmer.** 1997. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr. Rev. **18:**361–377.
51. **Perissi, V., L. M. Staszewski, E. M. McInerney, R. Kurokawa, A. Krones, D. W. Rose, M. H. Lambert, M. V. Milburn, C. K. Glass, and M. G. Rosenfeld.** 1999. Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev. **13:**3198–3208.
52. **Perlmann, T., and L. Jansson.** 1995. A novel pathway for vitamin A signaling mediated by RXR heterodimerization with NGFI-B and NURR1. Genes Dev. **9:**769–782.
53. **Perlmann, T., P. N. Rangarajan, K. Umesono, and R. M. Evans.** 1993. Determinants for selective RAR and TR recognition of direct repeat HREs. Genes Dev. **7:**1411–1422.
54. **Sadovsky, Y., P. A. Crawford, K. G. Woodson, J. A. Polish, M. A. Clements, L. M. Tourtellotte, K. Simburger, and J. Milbrandt.** 1995. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proc. Natl. Acad. Sci. USA **92:**10939–10943.
55. **Schwartz, C. J., H. M. Sampson, D. Hlousek, A. Percival-Smith, J. W. Copeland, A. J. Simmonds, and H. M. Krause.** 2001. FTZ-Factor1 and Fushi tarazu interact via conserved nuclear receptor and coactivator motifs. EMBO J. **20:**510–519.
56. **Seol, W., H. S. Choi, and D. D. Moore.** 1996. An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. Science **272:**1336–1339.
57. **Shiau, A. K., D. Barstad, P. M. Loria, L. Cheng, P. J. Kushner, D. A. Agard, and G. L. Greene.** 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell **95:** 927–937.
58. **Shinoda, K., H. Lei, H. Yoshii, M. Nomura, M. Nagano, H. Shiba, H. Sasaki, Y. Osawa, Y. Ninomiya, O. Niwa, K. Morohashi, and E. Li.** 1995. Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the *Ftz-F1* disrupted mice. Dev. Dyn. **204:**22–29.
59. **Suzuki, T., H. Kawasaki, R. T. Yu, H. Ueda, and K. Umesono.** 2001. Segmentation gene product Fushi tarazu is an LXXLL motif-dependent coactivator for orphan receptor FTZ-F1. Proc. Natl. Acad. Sci. USA **98:**12403–12408.
60. **Torchia, J., D. W. Rose, J. Inostroza, Y. Kamei, S. Westin, C. K. Glass, and M. G. Rosenfeld.** 1997. The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. Nature **387:**677–684.
61. **Tremblay, J. J., and R. S. Viger.** 1999. Transcription factor GATA-4 enhances Mullerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. Mol. Endocrinol. **13:**1388–1401.
62. **Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. Cell **65:**1255–1266.
63. **Voegel, J. J., M. J. Heine, C. Zechel, P. Chambon, and H. Gronemeyer.** 1996. TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J. **15:**3667–3675.
64. **Wang, Z. N., M. Bassett, and W. E. Rainey.** 2001. Liver receptor homologue-1 is expressed in the adrenal and can regulate transcription of 11β-hydroxylase. J. Mol. Endocrinol. **27:**255–258.
65. **Warnmark, A., T. Almlof, J. Leers, J. A. Gustafsson, and E. Treuter.** 2001. Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERα and ERβ. J. Biol. Chem. **276:**23397–23404.
66. **Xie, W., H. Hong, N. N. Yang, R. J. Lin, C. M. Simon, M. R. Stallcup, and R. M. Evans.** 1999. Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. Mol. Endocrinol. **13:**2151–2162.
67. **Xu, L., C. K. Glass, and M. G. Rosenfeld.** 1999. Coactivator and corepressor complexes in nuclear receptor function. Curr. Opin. Genet. Dev. **9:**140–147.
68. **Yussa, M., U. Lohr, K. Su, and L. Pick.** 2001. The nuclear receptor Ftz-F1 and homeodomain protein Ftz interact through evolutionarily conserved protein domains. Mech. Dev. **107:**39–53.
69. **Zanaria, E., F. Muscatelli, B. Bardoni, T. M. Strom, S. Guioli, W. Guo, E. Lalli, C. Moser, A. P. Walker, E. R. McCabe, T. Meitinger, A. P. Monaco, P. Sassone-Corsi, and G. Camerino.** 1994. An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature **372:**635–641.
70. **Zhang, H., J. S. Thomsen, L. Johansson, J. A. Gustafsson, and E. Treuter.** 2000. DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. J. Biol. Chem. **275:**39855–39859.