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Hormone-independent Transcriptional Activation and Coactivator Binding by Novel Orphan Nuclear Receptor ERR3*

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Orphan nuclear receptors share sequence homology with members of the nuclear receptor superfamily, but ligands are unknown or unnecessary. A novel orphan receptor, estrogen receptor-related protein 3 (ERR3), was identified by yeast two-hybrid screening, using the transcriptional coactivator glucocorticoid receptor interacting protein 1 (GRIP1) as bait. The putative full-length mouse ERR3 contains 458 amino acids and is closely related to two known orphan receptors ERR1 and ERR2. All the ERR family members share an almost identical DNA-binding domain, which has 68% amino acid identity with that of estrogen receptor. ERR3 bound specifically to an estrogen response element and activated reporter genes controlled by estrogen response elements, both in yeast and in mammalian cells, in the absence of any added ligand. A conserved AF-2 activation domain located in the hormone-binding domain of ERR3 was primarily responsible for transcriptional activation. The ERR3 AF-2 domain bound GRIP1 in a ligand-independent manner both *in vitro* and *in vivo*, through the L*XX*LL motifs of GRIP1, and GRIP1 functioned as a transcriptional coactivator for ERR3 in both yeast and mammalian cells. Expression of ERR3 in adult mouse was restricted; highest expression was observed in heart, kidney, and brain. In the mouse embryo no expression was observed at day 7, and highest expression occurred around the 11–15 day stages. Although ERR3 is much more closely related to ERR2 than to ERR1, the expression pattern for ERR3 was similar to that of ERR1 and distinct from that for ERR2, suggesting a unique role for ERR3 in development.

The nuclear receptor (NR)[1] superfamily is composed of both constitutive and ligand-inducible transcription factors that regulate pivotal gene networks important for eukaryotic cell growth, development, and homeostasis (1–4). The most well studied members of the NR superfamily are receptors for steroid hormones, thyroid hormone, vitamin D, and retinoid acid, but the family also includes a group called orphan receptors whose cognate ligands are unknown or unnecessary. At present orphan receptors are by far the largest subclass of the family, although some orphan receptors later may be removed from this subclass after the identification of their ligands (5, 6). Most orphan receptors are composed of three structural domains which are characteristic of NRs (7–9). The N-terminal domain, which often contains a transcriptional activation function, is usually not conserved among the family members, and is absent in some NRs. The C-terminal domains of NRs share 10–60% homology among the family members; this domain is responsible for binding ligands in the hormonally regulated NRs, and it also contributes to transcriptional activation. The centrally located DNA-binding domain (DBD) is typically composed of two four-cystine zinc fingers and is responsible for specific recognition of enhancer or hormone response elements in the promoter region of target genes. The DBD is the region which shares the highest homology among the NR family members (40–90%); its sequence is often used to classify NRs into subfamilies.

Although the biological functions of most orphan NRs are largely unknown, they are likely to play some important roles, since their sequences are usually highly conserved among mammals and even other taxa, and their expression in either embryonic or adult tissues often occurs in very restricted spatial and temporal patterns (5). For example, steroidogenic factor 1 regulates many of the enzymes that synthesize steroids and plays a key role in the development and differentiated function of the adrenal gland and gonads (10–12). Hepatic nuclear factor 4 is preferentially found in the liver where it controls the expression of liver-specific genes (13). Orphan receptors may act by different mechanisms (5). Some act as enhancer element-bound transcriptional activators for specific genes, in a manner similar to the well documented mechanism of hormone-binding NRs. Other orphan receptors may regulate the function of other NRs, by competing for the same enhancer element or forming active or inactive heterodimers with the other NRs.

NRs use one or more activation domains to mediate their functions (8, 9, 14). In some NRs, the N-terminal AF-1 activation domain makes the major contribution to transcriptional activation. However, there is generally little if any sequence conservation among the N-terminal domains of different NRs, and some NRs lack an N-terminal activation function. The AF-2 activation domain within the C-terminal hormone-binding domain (HBD) of NRs is also important for transcriptional activation and is highly conserved among essentially all NRs that activate transcription (15–17). Recently, a new class of proteins called transcriptional coactivators have been found to play key roles in mediating transcriptional activation by NRs (18, 19). While transcriptional coactivators for the AF-1 do-

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[1] The abbreviations used are: NR, nuclear receptor; AD, activation domain; DBD, DNA-binding domain; HBD, hormone-binding domain; GR, glucocorticoid receptor; AR, androgen receptor; ER, estrogen receptor; TR, thyroid hormone receptor; ERR, estrogen receptor-related protein; SRC-1, steroid receptor coactivator-1; GRIP1, glucocorticoid receptor interacting protein 1; ACTR, activator of the thyroid and retinoic acid receptors; HA, hemagglutinin; PCR, polymerase chain reaction; TRE, thyroid hormone response element; GST, gluathione *S*-transferase; ERE, estrogen response element; GRE, glucocorticoid response elements; MMTV, mouse mammary tumor virus.

mains are mostly unknown, quite a few putative coactivators for the AF-2 domain have been reported, and some were shown to enhance or be required for transcriptional activation by NRs, including some orphan receptors. The best characterized coactivators for NRs are three genetically distinct but structurally and functionally related proteins of approximately 160 kDa known as NR coactivators or p160 coactivators: SRC-1 (20, 21), GRIP1 (22, 23) (also known as transcriptional intermediary factor 2 (24)), and p300/CBP interacting protein (25) (also known as ACTR (26), receptor-associated coactivator 3 (27), amplified in breast cancer 1 (28), and thyroid hormone receptor activator molecule-1 (29)). The physical interactions between NRs and these transcriptional coactivators are quite conserved: the conserved AF-2 domains of apparently all NRs that function as transcriptional activators form a conserved hydrophobic groove that accommodates an L*XX*LL motif (where L is leucine and *X* can be any amino acid) found in several copies in the p160 coactivators (30–35). Classical NRs require binding of ligand before they can interact with coactivators and activate transcription (21, 23, 24). However, at least some orphan receptors have been found to bind coactivators and activate transcription in the absence of any added ligand. For example, orphan receptor CARβ apparently functions as a transcriptional activator in the absence of ligand, but the binding of its ligand results in the dissociation of bound coactivators, thus providing a negative regulatory mechanism for the ligand (36).

Since the first two orphan NRs, estrogen receptor related proteins 1 and 2 (ERR1 and ERR2), were identified 10 years ago based on their sequence homology with the DBD of estrogen receptor (ER) (37), a large number of new orphan NRs have been identified (5, 6). Orphan NRs have recently been one of the most rapidly growing families of proteins, and characterization of their functions has contributed to our understanding of important biological processes. This paper reports the identification of mouse ERR3, a novel member of the ERR subfamily of orphan NRs, by a new approach which is based on the interaction of the receptor with its transcriptional coactivator GRIP1. We tested the ability of ERR3 to act as a transcriptional activator, both in mammalian cells and in yeast, identified a functional domain responsible for transcriptional activation, and explored the role of coactivators in ERR3 function. A comparison of the sequence homology and expression patterns for the three ERR proteins suggests distinct developmental and/or regulatory roles for all three.

MATERIALS AND METHODS

Plasmids—Mammalian expression vectors: pSG5.HA was constructed by inserting AATTACCGCCGCCATGGGCTACCCATAC-GATGTTCCTGACTATGCGGAATTCTCGA, containing a translation start signal and codons for hemagglutinin (HA) epitope tag YPYDVP-DYA (38) (both underlined), into the *Eco*RI/*Bam*HI sites of pSG5 (Stratagene); the original *Eco*RI site was thus inactivated, and a new *Eco*RI site and an *Xho*I site were introduced after the HA coding sequence. The multiple cloning site following the HA tag contains *Eco*RI, *Xho*I, *Bam*HI, and *Bgl*II sites. The following ERR3 expression vectors were constructed by inserting the indicated PCR amplified fragment into pSG5.HA: pSG5.HA-ERR3$_{AF-1/DBD}$, an *Xho*I/*Bgl*II fragment encoding ERR3$_{1-216}$; pSG5.HA-ERR3$_{DBD/HBD}$ and pSG5.HA-ERR3$_{\Delta AF-2}$, *Xho*I/*Bam*HI fragments encoding ERR3$_{119-458}$ and ERR3$_{119-448}$, respectively. Expression vectors for ERR3$_{DBD/HBD}$ containing L449A/F450A or M453A/L454A amino acid substitutions were made by including relevant point mutations in PCR primers, and then inserting the PCR amplified fragments into the *Xho*I/*Bam*HI sites of pSG5.HA. pSG5.HA-ERR3, encoding full-length ERR3, was constructed by inserting the PCR amplified C-terminal region of ERR3 into the *Bam*HI/*Bgl*II sites of pSG5.HA-ERR3$_{AF-1/DBD}$. Reporter genes EREII-LUC GL450 (39), MMTV-LUC, MTV(ERE)-LUC, and MTV(TRE)-LUC (40), and expression vectors pSG5.GRIP1 for full-length GRIP1 (30), pCMX.ACTR for ACTR (26), pHE0 for ER (41), pSV40.AR for androgen receptor (AR) (42), and pCMX.hTRβ1 for thyroid hormone receptor β1 (TRβ1) (43)

were described previously. Expression vector pSG5.HA-SRC-1a for full-length SRC-1a was made by inserting *Sma*I/*Sal*I fragment at the *Eco*RI site (which was blunted by filling with Klenow polymerase) and the *Xho*I site of pSG5.HA.

Yeast expression vectors: pGBT9.GRIP1$_{5-765}$ encoding a Gal4DBD/GRIP1 fusion protein was made by inserting a GRIP1 *Eco*RI/*Xho*I fragment into *Eco*RI/*Sal*I sites of pGBT9 (CLONTECH). pGAD10.ERR3 encoding the fusion protein of the Gal4 activation domain (Gal4AD) and mouse ERR3 amino acids 28–458 were isolated by yeast two-hybrid screening (see below). pGBT9.ERR3 encoding the Gal4DBD/ERR3 fusion protein was made by subcloning an *Eco*RI fragment encoding the ERR3$_{28-458}$ from pGAD10.ERR3 (derived by partial digestion with *Eco*RI) into pGBT9. Vectors for fusion proteins of Gal4 DBD and ERR3 AF-2 mutations were made by a strategy similar to that described above for the corresponding mammalian vectors containing ERR3 AF-2 mutations; the resultant PCR amplified fragments coding for ERR3 HBD (amino acids 214–458) were inserted into the *Bam*HI site of pGBT9. Vectors for full-length GRIP1 (pGRIP1/fl) and for fusion proteins of Gal4AD with wild type GRIP1 and its NR Box mutants were described previously (23, 30). Yeast expression vector pGBT9.p300 for the fusion protein of Gal4DBD and p300 (amino acids 1856–2414) was described preciously (44). Yeast expression vectors pRS314-GR N795 encoding full-length rat glucocorticoid receptor (GR) and pRS314-hER encoding full-length human ER were constructed by first inserting a *Cla*I/*Bam*HI DNA fragment containing the yeast *gpd* promoter into pRS314 (*trp1*, cen-ars) (45). A *Bam*HI fragment encoding rat GR or a *Bam*HI/*Sst*I fragment encoding human ER was then inserted.[2] YEp46.hTRβ1 encoding full-length human TRβ1 was described previously (46), as were yeast reporter genes ERE1-CYC-LacZ (47), GRE3-CYC-LacZ (48), and F2-CYC-LacZ (containing a thyroid hormone response element (TRE)) (46).

Bacterial expression vector for GST/GRIP1 fusion protein, pGEX.2TK.GRIP1$_{563-1121}$, was made by inserting a PCR-amplified GRIP1 fragment into *Bam*HI/*Eco*RI sites of pGEX.2TK (Amersham Pharmacia Biotech). Bacterial expression vector for GST/ERR3 fusion protein, pGEX.4T-1.ERR3, was made by subcloning the insert of pGAD10.ERR3 (a partially digested *Eco*RI fragment) into the *Eco*RI site of pGEX.4T-1 (Amersham Pharmacia Biotech).

Isolation of ERR3 cDNA by Yeast Two-hybrid Screening—Yeast two-hybrid system screening (49) was performed as described before by using a CLONTECH MATCHMAKER system and a 17-day mouse embryo cDNA library (22). GRIP1$_{5-765}$ was fused to Gal4DBD and used as bait in the screening, which was performed without any added hormone but in the presence of 20 mM 3-aminotriazole because of the high background caused by the bait alone. Of the 84 colonies selected in the screening, three cDNA clones contained the same 1.5-kilobase pairs insert, which encoded ERR3$_{28-458}$. The cDNA of ERR3 was sequenced in both directions with an ABI automatic sequencer in the University of Southern California Norris Comprehensive Cancer Center Microchemical Core Facility.

Northern Blotting—Multiple Tissue Northern blots (CLONTECH) for mouse adult tissues and whole mouse embryo were hybridized with an ERR3 cDNA fragment encoding amino acids 28–110 by using ExpressHyb Hybridization Solution (CLONTECH) according to the manufacturer's protocols.

Cell Culture and Transient Transfection—CV-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% charcoal/dextran-treated fetal bovine serum (Gemini Bio-Products, Inc.). Transient transfection was performed in 6-well plates (3.3-cm diameter wells) by using SuperFect Transfection Reagent (Qiagen) according to the manufacturer's protocols. Total DNA used in each transfection was adjusted to 2.0 μg by adding the appropriate amount of pSG5.HA vector. Cell extracts were prepared 48 h after transfection and tested with the Promega Luciferase Assay kit. Data shown represents the mean and S.D. for three transfected cultures.

Yeast Transformation and β-Galactosidase Assays—These were performed as described previously (30). β-Galactosidase activities shown are the mean and S.D. from three independent yeast transformants.

In Vitro Transcription and Translation of Proteins and GST Pulldown Assays—These assays were performed as described previously (44).

RESULTS

Identification of Mouse ERR3—To identify proteins that interact with transcriptional coactivator GRIP1, a fragment of

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[2] S. P. Bohen, personal communication.

A

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mERR1    1   ...........................MSS...QVVGIEPLYIKAEPASP.........DSPKGS
                                        |||           || || ||            || ||
mERR2    1   ....................MSSEDRHLGSSCGSFIKTEPSSPSSGIDALSHHSPSGS
                                 ||   |||   |||  ||||||||||| |   |    |||| ||
mERR3    1   MDSVELCLPESFSLHYEEELLCRMSNKDRHIDSSCSSFIKTEPSSPASLTDSVNHHSPGGS


mERR1   27   S..........ETETEPPVTLASGPAPARCLPGHKEEEDGEGAGSG....EQGSGKLVLSS
             |              |            | |            |    ||            |
mERR2   39   SDASGGFGIALSTHANGLDSPP.MFAGAGLGG.NPCRKSYEDCTSGIMEDSAIKCEYMLNA
             |||||   |  ||||| | |||||||| |||   ||| |||||||
mERR3   62   SDASGSYSSTMNGHQNGLDSPPLYPSAPILGGSGPVRKLYDDCSSTIVEDPQTKCEYMLNS


mERR1   74   LPKRLCLVCGDVASGYHYGVASCEACKAFFKRTIQGSIEYSCPASNECEITKRRRKACQAC
             |||||||||||  |||||||||||||||||||||||  ||| ||| |||||||||| ||||
mERR2   98   IPKRLCLVCGDIASGYHYGVASCEACKAFFKRTIQGNIEYNCPATNECEITKRRRKSCQAC
             ||||||||||||||||||||||||||||||||||||  |||||||  |||||||||||||
mERR3  123   MPKRLCLVCGDIASGYHYGVASCEACKAFFKRTIQGNIEYSCPATNECEITKRRRKSCQAC


mERR1  135   RFTKCLRVGMLKEGVRLDRVRGGRQKYKRRPEVDPLPFPGPFPAGPLAVAGGPRKTAPVNA
             ||  |||  ||||||||||||||||||||||         |              |   |   |
mERR2  159   RFMKCLKVGMLKEGVRLDRVRGGRQKYKRR........LDSENSPYLNLPISPPAKKPLTK
             ||||||||||||||||||||||||||||||        | |||||||     ||||| |
mERR3  184   RFMKCLKVGMLKEGVRLDRVRGGRQKYKRR........IDAENSPYLNPQLVQPAKKPYNK


mERR1  196   LVSHLLVVEPEKLYAMPDPASPDGHLPAVATLCDLFDREIVVTISWAKSIPGFSSLSLSDQ
             || || ||  |   |     |     |  || |      ||||| || ||||| |    |
mERR2  212   IVSNLLGVEQDKLYAMPPNDIPEGDIKALTTLCELADRELVFLINWAKHIPGFPSLTLGDQ
             ||| ||   |  || ||||    |    |||||||| ||||||| |  | ||||||| | | |
mERR3  237   IVSHLLVAEPEKIYAMPDPTVPDSDIKALTTLCDLADRELVVIIGWAKHIPGFSTLSLADQ


mERR1  257   MSVLQSVWMEVLVLGVAQGSLPLQDELAFAEDLVLDEEGARAAGLGDL.GAALQLVRRLQA
             || || || ||| ||    |  ||  ||| |   |||   |||   ||| |      ||||||
mERR2  273   MSLLQSAWMEILILGIVYRSLPYDDKLAYAEDYIMDEEHSRLVGLLDLYRAILQLVRRYKK
             ||||||||||||||||| |||||     | | || |||||   | | ||||   ||||||  ||
mERR3  298   MSLLQSAWMEILILGVVYRSLSFEDELVYADDYIMDEDQSKLAGLLDLNNAILQLVKKYKS


mERR1  317   LRLEREEYVLLKALALANSDSVHIEDAEAVEQLREALHEALLEYEAGRAGPGGGAERRRAG
             | | |||  |||||||||||| ||  ||| |     |||||  ||            | ||||
mERR2  334   LKVEKEEFMILKALALANSDSMYIENLEAVQKLQDLLHEALQDYE....LSQRHEEPRRAG
             |  |||||  ||||||||||| | ||||   ||||||| ||||||||        | ||||
mERR3  359   MKLEKEEFVTLKAIALANSDSMHIEDVEAVQKLQDVLHEALQDYE....AGQHMEDPRRAG


mERR1  378   RLLLTLPLLRQTAGKVLAHFYGVKLEGKVHA.QVFLEMLEAMMD
             || |||||||| |    ||| ||| ||| |||        |||||||
mERR2  391   KLLWTLPLLRQTAAKAVQHFYSVKLQGKVPMHKLFLEMLEAKV
             | |  |||||| |||||    | |||||||||||||||||||||
mERR3  416   KMLMTLPLLRQTSTKAVQHFYNIKLEGKVPMHKLFLEMLEAKV
```

B

FIG. 1. **A novel member of the ERR orphan NR family.** *A,* protein sequence alignment of three ERR orphan receptors. The predicted amino acid sequence of mouse ERR3 (GenBank accession number AF117254) was aligned with those of mouse ERR1 and ERR2 (GenBank accession numbers U85259 and X89594, respectively) by using the GENALIGN program of IntelliGenetics software. *Vertical bars* (|) indicate identical amino acids between two proteins, and *dots* (. . .) indicate gaps introduced between amino acids for optimum alignment. *B,* major regions of sequence homology among the ERR orphan receptors. The putative N-terminal activation domains (*AF-1*), DBDs, and HBDs of the ERR proteins are shown. The *percentage* of amino acid identities in each relevant region between ERR3 and the other proteins was determined by the GAP program of Genetic Computer Group (University of Wisconsin) software. The *numbers above* each protein refer to the amino acid sequences predicted from the cDNA sequences.

GRIP1 (amino acids 5–765) was used as bait to screen a mouse 17-day embryo cDNA library by the yeast two-hybrid system. The screening was performed in the absence of any hormone in order to avoid isolating classical hormone-regulated NRs, which interact with GRIP1 in a strictly hormone-dependent manner (21, 23, 24, 30). As a result of the screening, a 1.5-kilobase cDNA clone was identified. A BLAST search (50) of GenBank using the predicted amino acid sequence indicated that the known proteins with the highest sequence homology were two orphan NRs, ERR1 and ERR2 (37) (Fig. 1, *A* and *B*). Therefore, the new protein was named estrogen receptor-related protein 3 (ERR3). Like other orphan NRs, ERR3 exhib-

ited typical structural features of the NR family (Fig. 1*B*): a putative DBD, which is almost identical to that of ERR1 and ERR2; a putative HBD, sharing 57% amino acid identity with ERR1 and 73% with ERR2; and an N-terminal domain which shares very little homology with ERR1 but 58% amino acid identity with ERR2. The regions of sequence similarity and divergence between ERR3 and the other two ERR proteins were finely dispersed throughout the entire lengths of the proteins (Fig. 1*A*), indicating that ERR3 is not a splicing variant of either ERR1 or ERR2, and thus is a novel member of the ERR subfamily of orphan NRs.

Since the original ERR3 cDNA sequence isolated from the

yeast two-hybrid screening was presumably only a partial sequence, it was used to perform a BLAST search of GenBank EST Data base. A human cDNA sequence (GenBank Accession Number W26274) overlapped with the 5′-end of our partial mouse ERR3 cDNA clone, and the overlapping portions shared 93% identity; PCR primers designed from this presumed human ERR3 sequence were then used to amplify a DNA fragment from the mouse 17-day embryo cDNA library by PCR. The amplified DNA fragment was identical to the original mouse ERR3 sequence in their overlapping region and had a 5′-end extension relative to the original mouse cDNA clone. The combination of both sequences resulted in a cDNA sequence of 1567 nucleotides (GenBank Accession Number AF117254), which contains an open reading frame of 458 amino acids and is proposed to encode the full-length mouse ERR3 (Fig. 1*A*). The putative start codon was designated because of an upstream stop codon in the same reading frame and because the sequence around the putative start codon resembled the consensus for a translation start signal (51). The same PCR primers were also used to amplify a DNA fragment from a human brain cDNA library. The resulting cDNA sequence (not shown, GenBank accession number AF117255) corrected several likely sequencing mistakes from the original human EST cDNA, and encoded the N-terminal 129 amino acids of a human ERR3 protein which is identical to that part of mouse ERR3. During our characterization of mouse ERR3, Eudy *et al.* (52) reported the isolation of a human cDNA sequence ESRRG, which encoded a protein sequence sharing 98% amino acid identity with mouse ERR3 codons 24–458; no functional analysis of ESRRG was reported. Our mouse and human ERR3 cDNA sequences are totally unrelated to that of ESRRG before our ERR3 amino acid 24 (amino acid 1 in ESRRG), indicating that ESRRG and ERR3 are probably splicing variants produced from the same gene.

RNA blot analysis of adult mouse tissues, using the N-terminal region of ERR3 as a probe, indicated the presence of a 5.7-kilobase transcript of ERR3 in selected tissues, with the highest expression in the heart, brain, and kidney, much lower expression in the liver, and no detectable expression in other tested tissues (Fig. 2*A*). In mouse embryo, ERR3 was not expressed at the 7-day stage; highest expression was detected during the 11–15-day period, and decreased expression at 17 days (Fig. 2*B*). Eudy *et al.* (52) reported a different distribution of ERR3 transcripts in adult mouse and human tissues. However, we have observed the same expression pattern (Fig. 2*A*) in two independent adult mouse tissue RNA blots.

Activation of ERE-controlled Reporter Genes by ERR3 Without Any Added Ligand—Since ERR3 contains a putative DBD which shares 68% amino acid identity with the DBD of ER, we tested whether ERR3 could bind a functional estrogen response element (ERE). In yeast, while Gal4AD by itself did not produce detectable β-galactosidase activity from an ERE-controlled reporter gene, a Gal4AD/ERR3 fusion protein activated the same reporter gene (Table I). Since Gal4AD cannot bind the ERE, the activation of the reporter gene must result from the binding of ERR3 to the ERE and the subsequent activation by the tethered Gal4AD. Co-expression of the Gal4AD/ERR3 fusion protein with reporter genes controlled by glucocorticoid response elements (GRE) or a TRE caused only a very weak activation of the reporter gene, indicating that the binding of ERR3 with ERE is DNA sequence-specific (Table I). All those reporter genes were functional in yeast in the presence of the appropriate NRs and hormones.

When ERR3 was transiently expressed in mammalian cells, it activated a reporter gene controlled by a basal herpesvirus thymidine kinase promoter and two EREs, and the activity increased with the amount of ERR3 expression vector trans-
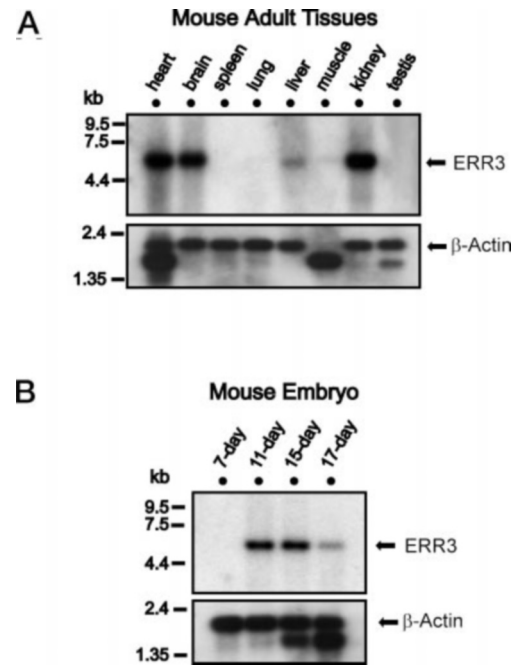

FIG. 2. **Expression of ERR3 in mouse adult tissues and different embryo stages.** RNA blot analyses of mouse ERR3 and β-actin (control) expression in adult mouse tissues or embryos were performed by successive hybridization with cDNA probes for the N-terminal part (codons 28–110) of ERR3 and, after stripping, for human β-actin. Sizes of RNA markers (CLONTECH) are indicated (*kb*, kilobases).

TABLE I
Specific binding of ERR3 to ERE DNA sequence

Gal4AD or the Gal4AD/ERR3$_{28-458}$ fusion protein was expressed in yeast strain w303a, together with a β-galactosidase reporter gene controlled by the indicated enhancer element (ERE, GRE, or TRE). Four colonies of each yeast transformant were tested for β-galactosidase activity by transferring them to nitrocellulose filters and incubating the lysed colonies with a chromogenic substrate.

	β-Galactosidase activity		
	ERE	GRE	TRE
Gal4 AD	−[a]	−	−
Gal4 AD/ERR3	++[b]	±[c]	±
NR[d]	++	++	++

[a] −, white color, no β-galactosidase activity.

[b] ++, strong blue color appeared within 1–2 h.

[c] ±, faint blue color indicating β-galactosidase activity appeared after 6–12 h incubation.

[d] NRs and hormones used as positive controls for the reporter genes: for ERE, ER with 100 nM estradiol; for GRE, glucocorticoid receptor with 10 μM deoxycorticosterone, for TRE, thyroid hormone receptor β1 with 10 μM triiodothyronine.

fected (Fig. 3*A*). ERR3 also activated a MTV(ERE)-LUC reporter gene controlled by a modified mouse mammary tumor virus (MMTV) promoter with an ERE substituted for the original GREs (Fig. 3*B*). ERR3 increased the activity of this reporter gene 100-fold but only caused a very weak activation of reporter genes with a native MMTV promoter (containing GREs) or a modified MMTV promoter with a TRE substituted for the GREs. Thus ERR3 selectively activated reporter genes regulated by EREs, and the ERE-dependent activation effect was observed with different types of basal promoters. Note that activation of the reporter genes by ERR3 occurred in the absence of any exogenously added hormone. For these experiments the serum used in the growth medium was pretreated by charcoal/dextran adsorption, which removes many potential small ligands including steroids. Adding some known hormones, estradiol or dihydrotestosterone, to the growth medium at a concentration of 100 nM did not change the activity of
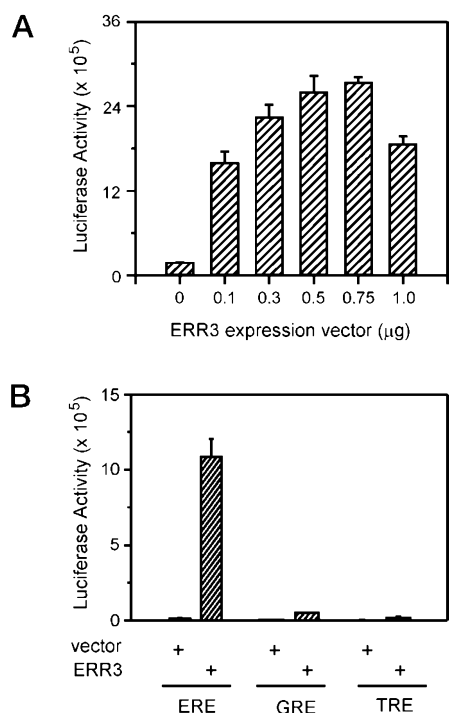
FIG. 3. **Activation of reporter genes by ERR3 in mammalian cells.** *A,* different amounts of pSG5.HA-ERR3 were transiently transfected into CV-1 cells with 0.5 µg of luciferase reporter gene EREII-LUC. CV-1 cells were grown in charcoal/dextran-treated serum with no exogenously added hormone. *B,* CV-1 cells were transiently transfected with 0.5 µg of pSG5.HA-ERR3 (*ERR3*) or empty pSG5.HA vector (*vector*), together with 0.5 µg of a luciferase reporter gene controlled by either MTV(ERE), native MMTV (containing GREs) or MTV(TRE) promoter.

ERR3 (data not shown). These results suggested that ERR3 was able to function in a ligand-independent manner.

To localize the transcriptional activation domain(s) of ERR3, deletion mutants of ERR3 lacking the N-terminal domain or the C-terminal HBD were tested in mammalian cells for their ability to activate reporter genes. When the C-terminal region homologous to NR HBDs was deleted, the N-terminal region (the putative AF-1 domain) of ERR3 showed only very weak transcriptional activation activity compared with the control of reporter gene alone (Fig. 4*B*). In contrast, after deletion of the N-terminal region, the remaining DBD/HBD fragment of ERR3 was still very active. Sequence analysis indicated that all three ERR family members share a highly conserved region near their C termini, which is also highly homologous with the typical AF-2 activation domain found in the analogous location in essentially all NRs that function as transcriptional activators (Fig. 4*A*). In order to test whether the putative AF-2 domain in ERR3 is also responsible for the transcriptional activation activity in its HBD, we altered that region by making alanine substitutions in pairs of conserved hydrophobic residues (L449A/F450A and M453A/L454A) or by deleting amino acids 449–458 (ΔAF-2). Similar mutations in ER as well as other NRs eliminated transcriptional activation activity but did not disrupt other functions of the HBD such as hormone binding (9, 15). Either the alanine substitutions or the deletion essentially eliminated the transcriptional activation function of the ERR3 HBD (Fig. 4*B*), indicating that this highly conserved region in ERR3 also represents a functional AF-2 activation domain. Immunoblot analyses indicated that the mutant ERR3 proteins were expressed at levels about one-third to one-half that of wild type ERR3 (data not shown). The almost complete loss of activity observed cannot be explained by this moderate reduction in expression and indicates that the mutations
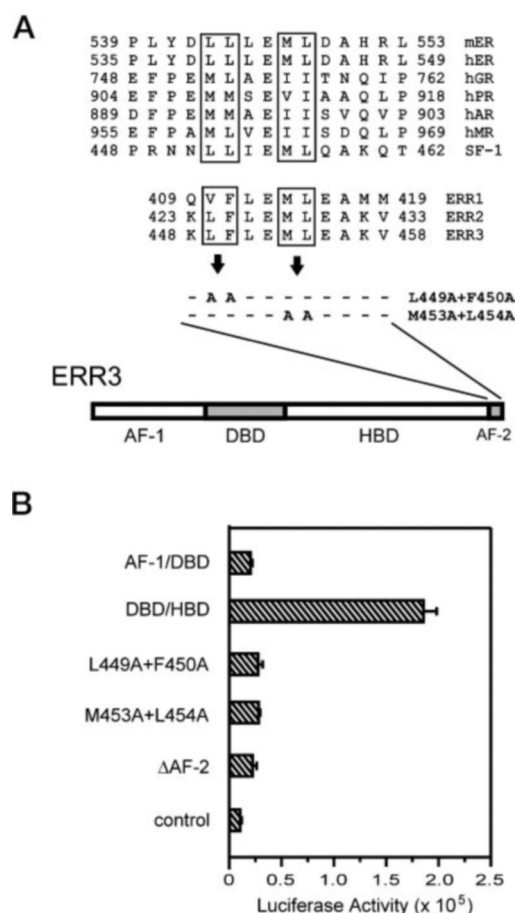
FIG. 4. **Locating the major ERR3 activation domain.** *A,* the highly conserved AF-2 regions located near the C termini of several previously identified NRs are aligned with putative AF-2 regions from the ERR proteins. *Boxes* indicate highly conserved pairs of hydrophobic amino acids within this region. Point mutations changing the hydrophobic amino acid pairs to alanines in ERR3s putative AF-2 domain are shown. *B,* CV-1 cells were transiently transfected with 0.5 µg of pSG5.HA vectors expressing different ERR3 fragments or mutants (described under "Materials and Methods"), together with 0.5 µg of the MTV(ERE)-LUC reporter gene. AF-2 mutations L449A/F450A, M453A/L454A, and ΔAF-2 were made in the context of the ERR3 DBD/HBD fragment. *Control* indicates a transfection performed with reporter gene but no ERR3 expression vector.

caused severe loss of protein function.

Ligand-independent and AF-2 Domain-dependent Binding of Transcriptional Coactivators by ERR3—Transcriptional coactivators, including GRIP1 and other p160 coactivators, play key roles in the normal function of NRs, and such roles are based on the physical interaction between NRs and coactivators (18, 19, 23, 30). ERR3 was isolated through its ability to interact with GRIP1. To confirm the specificity of this interaction, the isolated Gal4AD/ERR3 clone was coexpressed with Gal4DBD/GRIP1$_{5–765}$ and other control proteins in yeast two-hybrid assays. Gal4AD/ERR3 interacted with Gal4 DBD/GRIP1$_{5–765}$, but not with the Gal4DBD itself or its fusion protein with an irrelevant protein, p53 (Fig. 5*A*). The interaction between ERR3 and GRIP1 in the yeast two-hybrid assays occurred in the absence of any exogenously added hormones, whereas classical nuclear hormone receptors interact with GRIP1 in a strictly hormone-dependent manner (21, 23, 24, 30). The ligand-independent interaction between GRIP1 and ERR3 was also observed *in vitro* by GST pull-down assays. While bead-bound GST did not bind ERR3 synthesized *in vitro*, the bead-bound GST-GRIP1$_{563–1121}$ bound ERR3 (Fig. 5*B*). No hormone was added either in the synthesis of the proteins or in the
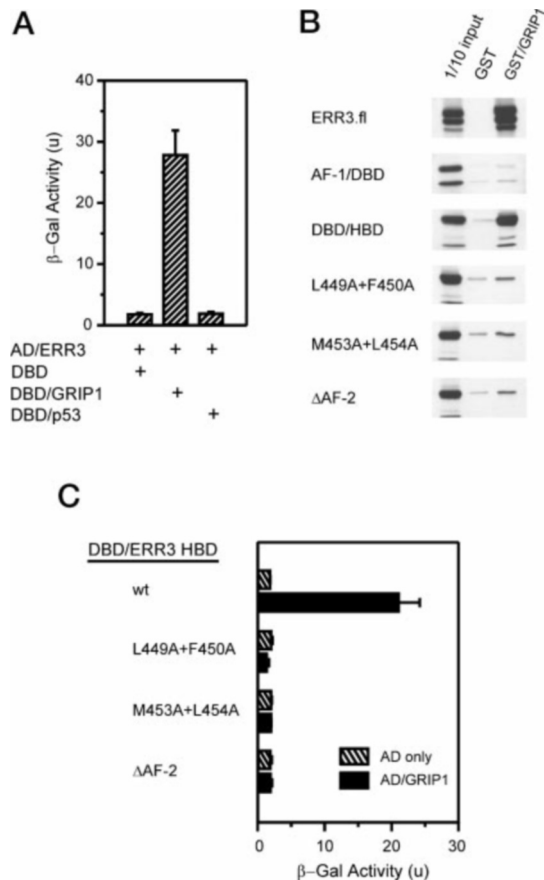
FIG. 5. **AF-2-dependent interaction of ERR3 with GRIP1.** *A,* yeast strain SFY526, which contains an integrated β-galactosidase reporter gene controlled by Gal4-binding sites, was transformed with pGAD10.ERR3 encoding Gal4AD/ERR3$_{28-458}$ and pGBT9 plasmids encoding Gal4DBD or Gal4DBD fused with GRIP1$_{5-765}$ or p53$_{72-390}$. Yeast two-hybrid assays were performed with no added ligand, and β-galactosidase activity from the yeast cell extracts indicated protein-protein interactions. *DBD,* Gal4DBD; *AD,* Gal4AD; *u,* units. *B,* full-length ERR3 (*ERR3.fl*) or its fragments or mutants (as described in Fig. 4*B*) were synthesized *in vitro* with [³⁵S]methionine and then incubated with Sepharose bead-bound GST or GST/GRIP1$_{563-1121}$, in the absence of any added hormone. After washing, the bead-bound ERR3 proteins were eluted and analyzed by SDS-PAGE and autoradiography. For reference a sample equivalent to 1/10 of the labeled protein in the binding assay is shown along with the total amount of labeled protein bound to the beads. *C,* Gal4DBD fused with wild type or mutant ERR3 HBD (amino acids 214–458) and Gal4AD or Gal4AD fused to full-length GRIP1 were co-expressed in yeast two-hybrid assays as in *A.*

binding reactions. GST pull-down assays also indicated that the HBD of ERR3 was responsible for the interaction with GRIP1, while its N-terminal domain (AF-1) did not interact with GRIP1 (Fig. 5*B*). The same mutations in the AF-2 domain of ERR3 that eliminated its ability to activate a reporter gene also eliminated the interaction between ERR3 and GRIP1 *in vitro* (Fig. 5*B*) and in yeast two-hybrid assays (Fig. 5*C*). These AF-2 mutation studies established a strong correlation between the ERR3s abilities to bind coactivator and activate transcription.

Three L*XX*LL motifs (called NR boxes) located in the central region of GRIP1 (Fig. 6*A*) are essential for its interaction with the HBD of classical hormone-regulated NRs (25, 30, 53, 54). To test the importance of those L*XX*LL motifs for GRIP1s interaction with ERR3, yeast two-hybrid assays were used to test the interaction between ERR3 and full-length GRIP1, with various leucine to alanine substitutions in its L*XX*LL motifs. Mutations in either NR Box II or III resulted in partial loss of ERR3 binding to GRIP1 (Fig. 6*B*); however, the combined mu-
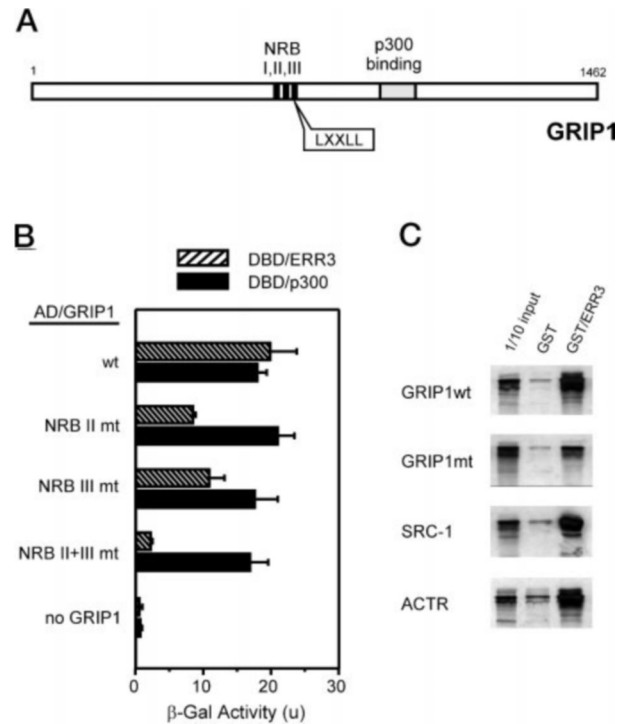

FIG. 6. **Role of NR boxes of GRIP1 for interaction with ERR3.** *A,* two key functional domains of coactivator GRIP1 are indicated. *Vertical black bars* represent NR boxes (*NRB*) I, II, and III, each composed of an L*XX*LL motif. The *shaded box* represents the CBP/p300 interaction domain of GRIP1. *B,* interactions of ERR3 with wild type GRIP1 or its NR Box mutants in yeast two-hybrid assays. Gal4DBD fused to ERR3$_{28-458}$ or p300$_{1856-2414}$ was tested in yeast two-hybrid assays (as in Fig. 5*A*) for binding to Gal4AD (*no GRIP1*) or Gal4AD fused to full-length wild type (*wt*) GRIP1 or GRIP1 containing mutations in NR box II (*NRB II mt*), NR box III (*NRB III mt*), or both NR boxes (*NRB II+III mt*). Mutants: *NRB II mt,* L693A/L694A; *NRB III mt,* L748A/L749A. *C,* full-length wild type (*wt*) GRIP1, GRIP1 with mutations in NR boxes II and III (*GRIP1mt*), SRC-1a, or ACTR were synthesized with [³⁵S]methionine *in vitro* and then incubated with Sepharose bead-bound GST or GST/ERR3$_{28-458}$ in the absence of any added hormone. After washing, the bead-bound ERR3 proteins were eluted and analyzed by SDS-PAGE and autoradiography.

tations in both NR Boxes II and III almost totally eliminated the ERR3/GRIP1 interaction. In control assays, the wild type and mutant GRIP1 fusion proteins were all able to interact with p300. The combined NR Box II and III mutations of GRIP1 also drastically reduced binding of GRIP1 to GST-ERR3 *in vitro* (Fig. 6*C*). ERR3 bound the other two p160 coactivator family members, SRC-1 and ACTR, *in vitro* in addition to GRIP1 (Fig. 6*C*), thus indicating a conserved interaction of ERR3 with all three p160 coactivator family members.

GRIP1 Functions as a Transcriptional Coactivator for ERR3 in Both Yeast and Mammalian Cells—GRIP1 can function as a transcriptional coactivator for a wide group of NRs, both in yeast and mammalian cells (23, 30, 54). Since ERR3 can interact with GRIP1 and this interaction closely correlates with the transcriptional activation activity of ERR3, we predicted that GRIP1 would also serve as a transcriptional coactivator for ERR3. In transiently transfected CV-1 cells the activation of an ERE controlled reporter gene by ERR3 was enhanced by wild type GRIP1, but not by GRIP1 containing mutations in NR Boxes II and III, and the enhancement was approximately proportional to the amount of GRIP1 expression vector used (Fig. 7*A*). GRIP1 in the absence of ERR3 did not activate the reporter gene (data not shown). Thus the orphan receptor ERR3, like the classical nuclear hormone receptors, can utilize GRIP1 as a transcriptional coactivator.

In yeast a reporter gene controlled by Gal4-binding sites was
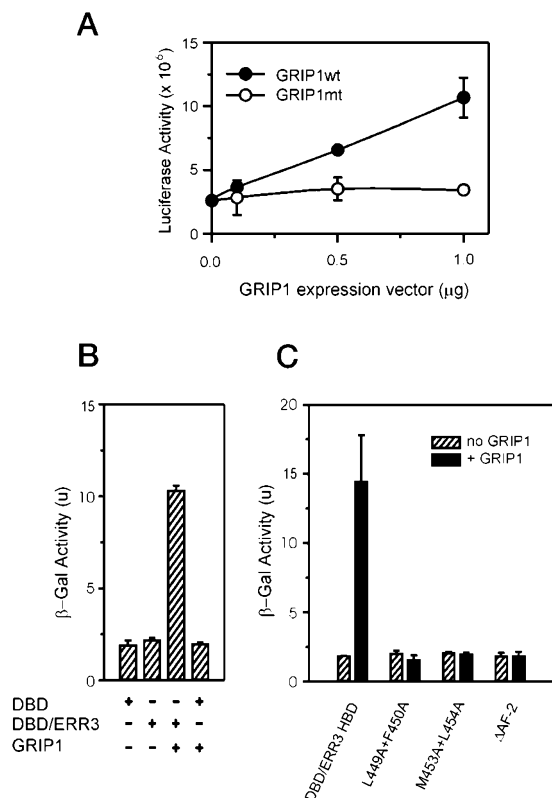
FIG. 7. **GRIP1 functioned as a coactivator for ERR3 in mammalian cells and yeast.** *A,* CV-1 cells were transiently transfected with MTV(ERE)-LUC reporter gene (0.5 μg), pSG5.HA-ERR3 (0.5 μg), and the indicated amount of pSG5.GRIP1/fl (*GRIP1wt*) or the same vector containing mutations in NR boxes II and III (*GRIP1mt*). Cells were grown in charcoal/dextran-treated serum with no added hormone. *B,* the indicated proteins were expressed in yeast SFY526 cells in the absence of hormone; the activity of the integrated β-galactosidase reporter gene controlled by Gal4-binding sites was measured in cell extracts. *DBD*, Gal4DBD; *ERR3*, ERR3$_{28-458}$; *GRIP1*, full-length GRIP1. *C*, ERR3 HBD (wild-type or AF-2 mutants) fused to Gal4DBD (as in Fig. 5*C*) was expressed in yeast SFY526 cells in the presence or absence of full-length GRIP1. Cell extracts were tested for β-galactosidase activity.

not activated by a Gal4DBD/ERR3$_{28-458}$ fusion protein above the level achieved with Gal4DBD alone (Fig. 7*B*), although ERR3 did show transcriptional activation activity in CV-1 cells (Fig. 3). The discrepancy between the activities of ERR3 in yeast and mammalian cells was consistent with our earlier findings that yeast cells do not have transcriptional coactivators to support the NR AF-2 activation functions (22, 23). Since ERR3 has a very weak AF-1 domain, its transcriptional activation activity is almost entirely due to its AF-2 domain (Fig. 4*B*). Co-expression of full-length GRIP1 in yeast with Gal4DBD/ERR3$_{28-458}$ enhanced the ability of the fusion protein to activate an integrated reporter gene controlled by Gal 4-binding sites (Fig. 7*B*). A fusion protein of Gal4DBD and ERR3 HBD also exhibited a GRIP1 dependent activity in yeast (Fig. 7*C*); however, even in the presence of GRIP1, the Gal4DBD/ERR3HBD fusion protein with AF-2 mutations still did not activate the reporter gene (Fig. 7*C*), consistent with our finding that the AF-2 domain is essential for binding GRIP1 (Fig. 5). The similar behaviors of ERR3 and other NRs in yeast and mammalian cells indicated that ERR3 and other NRs utilize similar mechanisms for transcriptional activation.

DISCUSSION

Orphan NRs have recently been a very fast growing family of proteins; during the last 10 years 19 subfamilies of orphan receptors have been identified, compared with only 10 subfamilies of NRs with known ligands (5, 6). Given the well studied

paradigm of the classical nuclear hormone receptors as a guide, the large number of currently known orphan NRs represent a valuable reservoir of research tools that can be used to elucidate new aspects of developmental and regulatory biology. Here we report the identification of a novel mouse orphan receptor ERR3, which we have assigned to the ERR family, because of its extensive sequence homology with the two earlier members of the family, ERR1 and ERR2 (37). ERR1 and ERR2 were originally isolated by low-stringency screening of cDNA libraries with the human ER DBD as a probe, and were the first two orphan receptors identified. The high degree of homology between the N-terminal domains of ERR2 and ERR3 is unusual among non-allelic NRs, even within the same subfamily, and suggests that these two genes may be the result of a recent gene duplication event or that their respective physiological roles require a conserved AF-1 function.

In many respects ERR3 functions like a classical NR. While its natural target binding sites on DNA remain to be determined, ERR3 selectively recognized EREs and selectively activated transcription of ERE controlled reporter genes in yeast and mammalian cells. ERR3 bound GRIP1 and other p160 coactivator proteins, and these transcriptional coactivators enhanced ERR3 function in yeast and mammalian cells. Both coactivator binding and transcriptional activation by ERR3 required an intact AF-2 domain, which is structurally and functionally homologous to those in classical hormonally regulated NRs. While the AF-2 activation domain and coactivator binding capabilities of ERR1 and ERR2 have not yet been defined, the degree of sequence homology among these three proteins (Fig. 1) and their similar abilities to exhibit transcriptional activation activity (55) suggest that the results reported here for ERR3 may be representative of the other two ERRs too.

In contrast to the actions of the classical nuclear hormone receptors, all of the activities of ERR3 were observed in the absence of any added ligand. The transcriptional activation by ERR3 in yeast and mammalian cells and the coactivator binding to ERR3 in yeast and *in vitro* were all observed in the absence of any added ligand. We propose two models to explain the apparently ligand independent function of ERR3 in the various studies presented here. Model 1, in the absence of any ligand ERR3 is a constitutive transcriptional activator protein. Along with the ligand independent basal activity, this model allows three possible scenarios for an effect of ligand: (*a*) there is no ligand; (*b*) binding of a currently unknown ligand further stimulates transcriptional activation by ERR3; (*c*) binding of a currently unknown ligand inhibits transcriptional activation. Model 2, ERR3 is a ligand-dependent transcriptional activator, and the ligand is ubiquitous in the mammalian, yeast, and *in vitro* experimental systems used in our studies. Model 2 seems somewhat unlikely, since the regulatory purpose of a ubiquitous ligand is difficult to envision. It is also possible that ERR3 is activated by a post-translational modification such as phosphorylation. However, when ERR3 was synthesized in a reticulocyte lysate in the presence of [γ-^{32}P]ATP, we detected no phosphorylation of ERR3 (data not shown). The fact that ERR3 synthesized in bacteria (Fig. 6), yeast (Fig. 5*A*), mammalian cells (Fig. 7*A*), or reticulocyte lysates (Fig. 5*B*) bound GRIP1 efficiently suggests that this binding does not require ligands or post-translational modifications. Nevertheless, the possibility that the basal level of GRIP1 binding and transcriptional activation by ERR3 could be further regulated by post-translational modifications of ERR3 remains to be tested.

No easy formula has been developed for finding ligands for orphan receptors or excluding the possibility that a ligand exists. Steroidogenic factor 1 and hepatic nuclear factor-4,

which activate transcription of target genes in the absence of any added ligand, were originally thought to be ligand independent, but recent reports of putative ligands have re-opened this question (56, 57). CARβ is an example of Model 1c (36). We propose the simplest model, Model 1a, as a default model for ERR3 until evidence for the existence of a ligand is found. Classical NRs undergo a conformational change after binding hormone, and the resulting conformation allows them to interact with their transcriptional coactivators (30, 31, 34, 58). If ERR3 is indeed active in the absence of ligand, it will be interesting in the future to see what kind of structural features of ERR3 allow the constitutive interaction with coactivators.

The existence of all three ERRs in humans as well as mice implies distinct and important roles for each ERR protein. Although we have demonstrated that ERR3 is a functional transcriptional activator, its true physiological roles remain to be determined. The extensive structural homology between ERR2 and ERR3, and their similar abilities to activate ERE-controlled genes, suggest the possibility that they could have redundant functions. However, the genes for these two proteins have different temporal and spatial patterns of expression, indicating different developmental and/or regulatory roles. In adult mouse tissues, ERR2 is expressed at low levels and in a restricted pattern (37). In mouse embryo, ERR2 expression is restricted to trophoblast progenitor cells between days 6.5 and 7.5 post-coitum, and its role was found to be in the early stages of chorion formation during mouse embryogenesis (59). Homozygous mutant embryos generated by targeted disruption of the ERR2 gene have severely impaired placental formation and die at 10.5 days post-coitum (60). In contrast, the highest expression of ERR3 occurs around days 11–15 of mouse embryonic development, a period of very active organogenesis. ERR3 was also expressed in selected adult tissues. Thus ERR3's roles in mouse development are temporally distinct from that of ERR2. From our studies on ERR3 and the previously published studies of ERR1, it appears that ERR1 and ERR3 have roughly similar temporal patterns of expression in mouse embryos and somewhat similar although not identical distributions in adult tissues (37, 61, 62). ERR1 was widely distributed in later stages of mouse embryos, with a more abundant expression in the heart, skeletal muscles, and nervous system (61). While more extensive and more direct comparisons are needed, the lower degree of homology that ERR3 shares with ERR1, compared with ERR2, suggests that ERR1 and ERR3 have different physiological roles.

The fact that ERR3 can activate ERE-controlled genes raises an interesting question about its relative roles with ER in gene regulation. Do ERRs activate the same genes as ERs *in vivo*? If so, what are the respective roles of the ERs and the ERRs in regulating these genes? If not, what are the true target genes for the ERRs and what mechanisms direct ERs and ERRs to activate different target genes despite their overlapping DNA binding specificities? While ERRs have been found to activate ERE-controlled genes experimentally, if ERRs produce substantially lower levels of activity than ERs on the same target gene, then it is possible that ERRs could regulate ER function by competing for common DNA-binding sites. Another possible regulatory mechanism could involve formation of ER-ERR heterodimers which may have a different level of activity than ER homodimers. In fact, it was recently reported that ERR1 modulated the activating effect of estrogen on the lactoferrin promoter and suggested that ERR1 may interact with ER through protein-protein interactions (63).

ERR3 is the first orphan receptor identified because of its interaction with transcriptional coactivators. Most orphan receptors, including the first identified orphan receptors, ERR1 and ERR2, were identified by screening cDNA libraries for clones with homology to known NRs. The continuing rapid rate of identification of new orphan NRs suggests that more members of this subclass of NRs remain to be discovered. Our findings on the interaction of ERR3 with GRIP1 extends the paradigm established from studies with classical nuclear hormone receptors, that the interaction between the NR and p160 coactivator is highly conserved. The current study demonstrates that this apparent universality of interaction between the AF-2 domains of classical hormone binding and orphan NRs and the L*XX*LL motifs of p160 coactivators provides a novel approach for the identification of more new orphan NRs.

REFERENCES

1. Evans, R. M. (1988) *Science* **240,** 889–895
2. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486
3. Beato, M., Herrlich, P., and Schütz, G. (1995) *Cell* **83,** 851–857
4. Mangelsdorf, D. J., and Evans, R. M. (1995) *Cell* **83,** 841–850
5. Enmark, E., and Gustafsson, J. A. (1996) *Mol. Endocrinol.* **10,** 1293–1307
6. Blumberg, B., and Evans, R. M. (1998) *Genes Dev.* **12,** 3149–3155
7. Kumar, V., Green, S., Stack, G., Berry, M., Jin, J.-R., and Chambon, P. (1987) *Cell* **51,** 941–951
8. Hollenberg, S. M., and Evans, R. M. (1988) *Cell* **55,** 899–906
9. Durand, B., Saunders, M., Gaudon, C., Roy, B., Losson, R., and Chambon, P. (1994) *EMBO J.* **13,** 5370–5382
10. Parker, K. L. (1998) *Mol. Cell. Endocrinol.* **140,** 59–63
11. Wong, M., Ikeda, Y., Luo, X., Caron, K. M., Weber, T. J., Swain, A., Schimmer, B. P., and Parker, K. L. (1997) *Recent Prog. Horm. Res.* **52,** 167–182
12. Ingraham, H. A., Lala, D. S., Ikeda, Y., Luo, X., Shen, W. H., Nachtigal, M. W., Abbud, R., Nilson, J. H., and Parker, K. L. (1994) *Genes Dev.* **8,** 2302–2312
13. Sladek, F. M., Zhong, W. M., Lai, E., and Darnell, J. E., Jr. (1990) *Genes Dev.* **4,** 2353–2365
14. Lees, J. A., Fawell, S. E., and Parker, M. G. (1989) *Nucleic Acids Res.* **17,** 5477–5489
15. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) *EMBO J.* **11,** 1025–1033
16. Ito, M., Yu, R. N., and Jameson, J. L. (1998) *Mol. Endocrinol.* **12,** 290–301
17. Crawford, P. A., Polish, J. A., Ganpule, G., and Sadovsky, Y. (1997) *Mol. Endocrinol.* **11,** 1626–1635
18. Torchia, J., Glass, C., and Rosenfeld, M. G. (1998) *Curr. Opin. Cell Biol.* **10,** 373–383
19. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) *Mol. Endocrinol.* **10,** 1167–1177
20. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1996) *Cell* **85,** 403–414
21. Oñate, S. A., Tsai, S. Y., Tsai, M.-J., and O'Malley, B. W. (1995) *Science* **270,** 1354–1357
22. Hong, H., Kohli, K., Trivedi, A., Johnson, D. L., and Stallcup, M. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4948–4952
23. Hong, H., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997) *Mol. Cell. Biol.* **17,** 2735–2744
24. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) *EMBO J.* **15,** 3667–3675
25. Torchia, J., Rose, D. W., Inostroza, J., Kamei, Y., Westin, S., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387,** 677–684
26. Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90,** 569–580
27. Li, H., Gomes, P. J., and Chen, J. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8479–8484
28. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X.-Y., Sauter, G., Kallioniemi, O.-P., Trent, J. M., and Meltzer, P. S. (1997) *Science* **277,** 965–968
29. Takeshita, A., Cardona, G. R., Koibuchi, N., Suen, C.-S., and Chin, W. W. (1997) *J. Biol. Chem.* **272,** 27629–27634
30. Ding, X. F., Anderson, C. M., Ma, H., Hong, H., Uht, R. M., Kushner, P. J., and Stallcup, M. R. (1998) *Mol. Endocrinol.* **12,** 302–313
31. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev.* **12,** 3343–3356
32. McInerney, E. M., Rose, D. W., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchia, J., Nolte, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) *Genes Dev.* **12,** 3357–3368

33. Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G., and Glass, C. K. (1998) *Nature* **395,** 199–202

34. Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395,** 137–143

35. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95,** 927–937

36. Forman, B. M., Tzameli, I., Choi, H. S., Chen, J., Simha, D., Seol, W., Evans, R. M., and Moore, D. D. (1998) *Nature* **395,** 612–615

37. Giguere, V., Yang, N., Segui, P., and Evans, R. M. (1988) *Nature* **331,** 91–94

38. Wilson, I. A., Niman, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) *Cell* **37,** 767–778

39. Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J.-A., Kushner, P. J., and Scanlan, T. S. (1997) *Science* **277,** 1508–1510

40. Umesono, K., and Evans, R. M. (1989) *Cell* **57,** 1139–1146

41. Green, S., Issemann, I., and Sheer, E. (1988) *Nucleic Acids Res.* **16,** 369

42. Brinkmann, A. O., Faber, P. W., van Rooij, H. C. J., Kuiper, G. G. J. M., Ris, C., Klaassen, P., van der Korput, J. A. G. M., Voorhorst, M. M., van Laar, J. H., Mulder, E., and Trapman, J. (1989) *J. Steroid Biochem. Mol. Biol.* **34,** 307–310

43. Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M. P., Chen, D., Huang, S. M., Subramanian, S., McKinerney, E., Katzenellenbogen, B. S., Stallcup, M. R., and Kushner, P. J. (1998) *Mol. Endocrinol.* **12,** 1605–1618

44. Hong, H., Darimont, B. D., Ma, H., Yang, L., Yamamoto, K. R., and Stallcup, M. R. (1999) *J. Biol. Chem.* **274,** 3496–3502

45. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27

46. Walfish, P. G., Yoganathan, T., Yang, Y.-F., Hong, H., Butt, T. R., and Stallcup, M. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3697–3702

47. Picard, D., Khursheed, B., Garabedian, M. J., Fortin, M. G., Lindquist, S., and Yamamoto, K. R. (1990) *Nature* **348,** 166–168

48. Garabedian, M. J., and Yamamoto, K. R. (1992) *Mol. Biol. Cell* **3,** 1245–1257

49. Fields, S., and Song, O.-k. (1989) *Nature* **340,** 245–246

50. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402

51. Kozak, M. (1996) *Mamm. Genome* **7,** 563–574

52. Eudy, J. D., Yao, S., Weston, M. D., Ma-Edmonds, M., Talmadge, C. B., Cheng, J. J., Kimberling, W. J., and Sumegi, J. (1998) *Genomics* **50,** 382–384

53. Heery, D. M., Kalkhoven, E., Hoare, S., and Parker, M. G. (1997) *Nature* **387,** 733–736

54. Voegel, J. J., Heine, M. J. S., Tini, M., Vivat, V., Chambon, P., and Gronemeyer, H. (1998) *EMBO J.* **17,** 507–519

55. Lydon, J. P., Power, R. F., and Conneely, O. M. (1992) *Gene Expr.* **2,** 273–283

56. Lala, D. S., Syka, P. M., Lazarchik, S. B., Mangelsdorf, D. J., Parker, K. L., and Heyman, R. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4895–4900

57. Hertz, R., Magenheim, J., Berman, I., and Bar-Tana, J. (1998) *Nature* **392,** 512–516

58. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389,** 753–758

59. Pettersson, K., Svensson, K., Mattsson, R., Carlsson, B., Ohlsson, R., and Berkenstam, A. (1996) *Mech. Dev.* **54,** 211–223

60. Luo, J., Sladek, R., Bader, J. A., Matthyssen, A., Rossant, J., and Giguere, V. (1997) *Nature* **388,** 778–782

61. Bonnelye, E., Vanacker, J. M., Spruyt, N., Alric, S., Fournier, B., Desbiens, X., and Laudet, V. (1997) *Mech. Dev.* **65,** 71–85

62. Sladek, R., Bader, J. A., and Giguere, V. (1997) *Mol. Cell. Biol.* **17,** 5400–5409

63. Yang, N., Shigeta, H., Shi, H., and Teng, C. T. (1996) *J. Biol. Chem.* **271,** 5795–5804