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Peroxisome Proliferator–Activated Receptor Subtypes Differentially Cooperate with Other Transcription Factors in Selective Transactivation of the Perilipin/PEX11α Gene Pair

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Perilipin is an adipocyte-specific protein associated with lipid droplets that is crucial for the regulation of storage and mobilization of lipids. We earlier reported that the mouse perilipin gene is regulated by peroxisome proliferator–activated receptor (PPAR) γ through a peroxisome proliferator–response element (PPRE) positioned upstream of the perilipin promoter. Moreover, we showed that this PPRE also controls expression of the PEX11α gene, which is located further upstream. We show here that three elements, A, B, and C, in close proximity downstream of the PPRE, are essential for transactivation of the perilipin gene by PPARγ. Electrophoretic gel-mobility shift assays demonstrated that nuclear factor (NF)-1 subtypes bind specifically to element B. Furthermore, chromatin immunoprecipitation using 3T3-L1 cells revealed that NF-1A and NF-1B bind to element B in a differentiation-dependent fashion, whereas binding is constitutive with NF-1C and NF-1X. Element C is likely to be a binding motif for nuclear receptors. With PPARα, elements A–C do not appear to be required for transactivation of the PEX11α gene, so that cooperation with other transcription factors may be differentially involved in selective transactivation of the PEX11α and perilipin genes by different PPAR subtypes.

Key words: nuclear factor-1, nuclear receptor, perilipin, PPAR, transcriptional regulation.

Abbreviations: PPAR, peroxisome proliferator–activated receptor; PPRE, peroxisome proliferator–response element; RXR, retinoid X receptor; EMSA, electrophoretic gel-mobility shift assay; ChIP, chromatin immunoprecipitation; NF, nuclear factor; DR, direct repeat.

Lipid droplets are subcellular structures storing triglyceride and cholesterol esters (*1*), which are mobilized upon hormonal activation of the protein kinase A signaling pathway (*2*). Recent studies have shown that many proteins, including the PAT domain family members, perilipin, adipose differentiation–related protein (ADRP), TIP47, and S3-12, are located on the surfaces of lipid droplets (*3*). Perilipin is particularly enriched in adipose tissue (*4*) and is phosphorylated by protein kinase A upon stimulation by catecholamine, triggering translocation of hormone-sensitive lipase from the cytosol to lipid droplets (*5*). Disruption of the perilipin gene results in leanness accompanied by ablation of hormone-sensitive lipolysis in adipocytes (*6*, *7*). Thus, perilipin is a crucial regulator of hormone-inducible lipolysis, and a possible therapeutic target in the treatment of obesity.

The peroxisome proliferator–activated receptors (PPARs) are a family of nuclear receptors comprising three subtypes, α, γ, and δ (or β). PPARα was the first to be identified (*8*) and is now well established to regulate genes involved in lipid metabolism (*9*). Two isoforms of PPARγ (γ1 and γ2), generated by alternative promoter usage and splicing, are known (*10*), playing major roles in adipocyte differentiation (*11*) and PPARβ/δ is also implicated in lipid metabolism (*12*, *13*). PPARs transactivate target genes upon binding to peroxisome proliferator-response elements (PPREs) through heterodimerization with another nuclear hormone receptor, retinoid X receptor (RXR) (*14*).

Perilipin expression is highly induced during adipogenesis (*4*) and we and others have identified a functional PPRE for the mouse perilipin gene (*15*–*18*). Moreover, we showed that this also serves for activation of a neighboring gene, PEX11α (*15*). This latter gene product is involved in fission of peroxisomes, and hence its enhanced expression would cause their proliferation, leading to elevation of metabolic capacity for fatty acids in the liver. The PEX11α and perilipin genes are arranged in tandem on the genome, with the transcriptional orientation in common. The PEX11α gene occupies a 5.5-kb region, and its poly (A) addition site is separated by a 5-kb spacer from the perilipin gene cap site, with the PPRE located within this spacer region. PPARα and PPARγ bind to this common PPRE selectively in the liver and adipose tissue, respectively, the former activating the PEX11α gene, and

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the latter the perilipin gene. This represents a novel mode of gene regulation in higher animals.

Eukaryotic gene expression is often attained by cooperative actions of transcription factors, hence leading to tissue specificity (*19*). This may also be applied to the gene regulation involving nuclear receptors (*20*). We show here that other than the PPRE, three additional elements, A, B, and C, are indispensable for transactivation of the perilipin gene by PPARγ but not for PPARα upregulation of the PEX11α gene. By electrophoretic gel-mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay, we here identified nuclear factor (NF)-1 as a *trans*-acting factor of element B. Thus, NF-1 and other transcription factors may selectively activate the perilipin gene in conjunction with PPARγ, contributing to its adipose-specific expression.

EXPERIMENTAL PROCEDURES

Plasmids—Genomic DNA fragments of the perilipin and PEX11α gene regions were subcloned into a promoter-less luciferase reporter plasmid, pGVB (Toyo Ink) (*15*). When the genomic fragments were placed on the downstream side, they were inserted in a restriction site downstream of the poly (A) addition site of the luciferase gene. Site-directed mutagenesis was carried out using a QuickChange mutagenesis kit (Stratagene), according to the manufacturer's protocol. The mutant clones were tested for the presence of the desired mutation and to confirm the absence of any unexpected mutations by nucleotide sequencing.

Expression vectors for mouse PPARγ1, PPARγ2, and PPARα were as described previously (*15*). cDNAs of rat NF-1 subtypes were obtained by reverse transcription–PCR (*21*) and subcloned into an expression vector, pUCSRα.

Cell Culture and DNA Transfection—HeLa cells were cultured in 96-well plates (luminescence assay grade; Sumilon), with F-12 medium containing 10% fetal bovine serum, at 37°C under 5% CO_2. Transfection was carried out by the calcium phosphate method (*22*). To each well were added 0.175 μg of a reporter plasmid, 0.1 μg each of a PPAR and/or an NF1 expression vector, and 0.1 μg of an empty vector (pCMX, pCMVNot or pUCSRα) as necessary. After 4 h, calcium phosphate precipitates were removed, and the cells were cultured for 24 h in the same medium supplemented with ligands (1 μM BRL49,653 and 100 μM Wy14,643 for PPARγ and α, respectively) or the vehicle (dimethylsulfoxide) alone. For the experiments using 3T3-L1 cells, culture, treatment for differentiation, and transfection were performed as described previously (*15*).

Luciferase Assays—In 96-well plates, cells were solubilized with 20 μl of cell lysis buffer [5 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 1 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid, 10% glycerol, 1% Triton X-100], and luciferase activity was measured using a PicaGene reagent kit (Toyo Ink), in a Lucy2 microplate luminometer (Anthos). When transfection was performed in 12-well plates, cells were extracted with 500 μl of the cell lysis buffer, and the luciferase assay was carried out using 40 μl of cell extract with the same reagent kit in a Lumat LB9501 luminometer (Berthold). The experiments using 12-well plates were performed using a β-galactosidase expression vector, pCMVβ, as an internal control for the efficiency of transfection. All transfection experiments were carried out in triplicate, and the averages are presented as relative values, together with the SD.

EMSA—The experiments were performed as described previously (*15*), using the oligonucleotides indicated in the figures. Nuclear extracts were prepared as described (*23*), from 3T3-L1 adipocytes 5 days after initiation of differentiation treatment and rat liver nuclear extracts as described (*24*).

ChIP Assay—3T3-L1 cells were cultured in 10-cm dishes, and treated for differentiation as described (*15*). On day 5 after initiation of the treatment, approximately 1×10^7 cells were processed for ChIP assay using a reagent kit (Upstate Biotechnology), as recommended by the manufacturer. Immunoprecipitation was performed with polyclonal antibodies against NF-1s and the preimmune rabbit IgG (*21*). For PCR, the primer pair 5′-AGAATCCGTACAGAAGCAGCCA-3′ (positions –1966 to –1945; relative to the transcriptional initiation site of mouse perilipin gene) and 5′-GCTTCAAGGTTCAGGACGAGTA-3′ (–1798 to –1819) was used for amplifying a region encompassing element B, and a pair 5′-CTGTGCATGAGTGACCACTCG-3′ (–5904 to –5884) and 5′-CTAAACAGTGACTAAGGAGTCATTA-3′ (–5686 to –5710) for a region distal to element B. A 5-μl aliquot from the 50 μl of solution of DNA recovered from each immunoprecipitate was used for PCR in the presence of [α-^{32}P]dCTP, and the products were analyzed on polyacrylamide gels after 28 cycles of amplification.

Immunoblot Analysis—3T3-L1 cells cultured in 6-cm dishes were washed once with phosphate-buffered saline and immediately dissolved in heated SDS-PAGE sample buffer. Western blotting was performed as described previously (*25*). Antibodies to PPARγ and perilipin were purchased from SantaCruz and Progen, respectively.

RESULTS

The PEX11α/Perilipin PPRE Alone Is Not Sufficient for Transactivation of the Perilipin Gene—We previously reported the PEX11α/perilipin PPRE to be located 8.4 kb downstream of the transcriptional initiation site of PEX11α gene, corresponding to positions –1986 to –1974 relative to the perilipin gene cap site (*15*). This motif conferred transactivation by PPARs, when combined with the SV40 viral promoter. On the other hand, we observed that this PPRE by itself was not sufficient for activation of transcription from the natural perilipin promoter (Fig. 1A). Namely, the region –2131/–1921 containing the PPRE did not fully support transactivation by either PPARγ1 or γ2 in HeLa cells, in conjunction with the basal perilipin promoter (–944/+56). In contrast, significant ligand-dependent reporter expression was observed for a construct harboring a long stretch of the upstream region (–3694/+56). Similar results were obtained when these reporters were transfected into 3T3-L1 adipocytes (Fig. 1B), indicating that elements other than the PPRE are essential for transactivation of the perilipin gene by PPARγ.

Accordingly, we searched for such elements using reporter constructs carrying deletions in various regions around the PPRE. In an assay using HeLa cells, the region

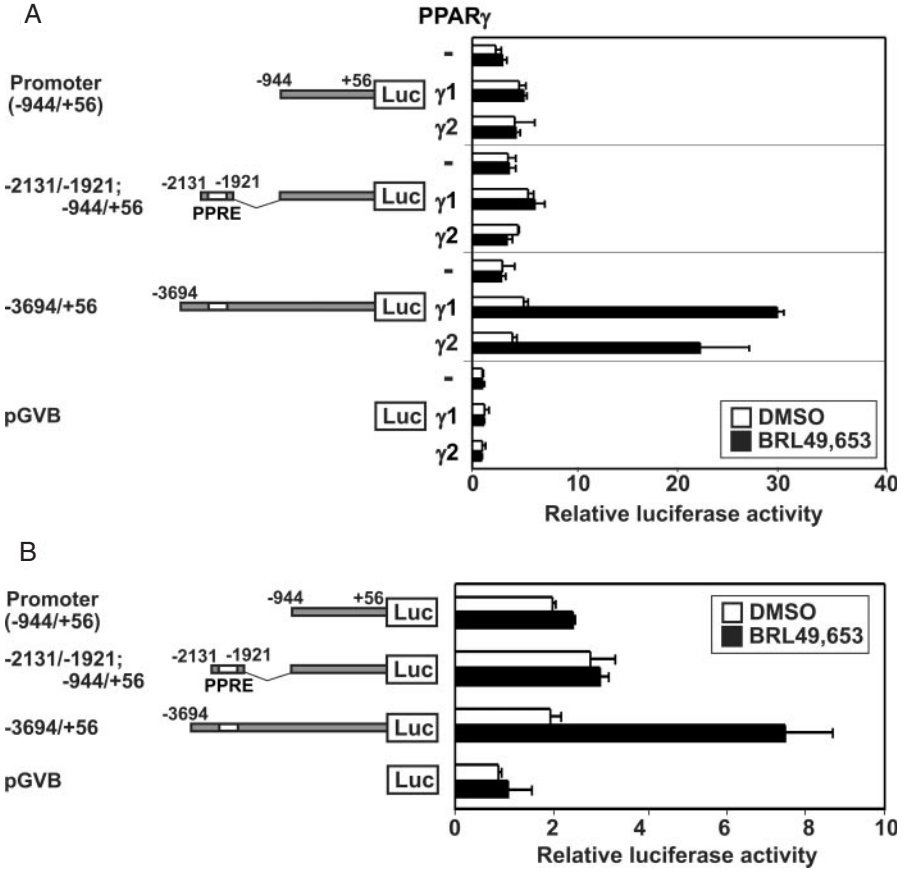

Fig. 1. **Regions other than the PPRE are essential for transactivation of the perilipin gene by PPARγ.** Maps of luciferase reporter gene constructs are depicted on the left, and results of the reporter assays on the right. Numbers in the maps indicate positions relative to the transcriptional initiation site of the mouse perilipin gene. Note that the maps are not drawn to scale. The –2131/–1921 region contains the PEX11α/perilipin PPRE (small open box). Assays were performed using HeLa cells with or without a PPARγ1 or PPARγ2 expression vector (A), or using 3T3-L1 adipocytes on day 5 of differentiation (B), as described in the "EXPERIMENTAL PROCEDURES." After transfection, cells were cultured in the presence of a PPARγ ligand, BRL49,653 (filled bar) or vehicle [dimethylsulfoxide (DMSO); open bar]. Luciferase activities are shown as relative values, taking the activity of a promoter-less reporter plasmid, pGVB, without a ligand and PPARγ expression vector, as 1.

between positions –2737 and –1409 was found important for PPARγ2-dependent transactivation of the perilipin gene (Fig. 2A). A series of deletions from downstream delimited a critical region between positions –2932 and –1709 (Fig. 2B), and by a more detailed study, the region –2127/–1709 was shown to be sufficient (Fig. 2C). We further analyzed this region by elaborate serial deletion from downstream. PPARγ2-dependent transactivation gradually decreased with stepwise deletion up to position –1820, though significant transactivation was still observed (Fig. 2D). In the region –1820/–1709, we have noted the presence of candidate binding motifs for transcription factors, possibly affecting expression of the perilipin gene (Shimizu, M. and Osumi, T., unpublished observation). Further deletion to position –1870 abolished transactivation by PPARγ2, indicating that the region –1870/–1820 is essential. This region contains two putative elements for transcription factor binding: one like a direct repeat (DR)-1 motif, a possible binding sequence for nuclear receptors such as PPAR and RXR, and the other like an octamer motif, a binding sequence for Oct-1 and related transcription factors (Fig. 2E). We examined the roles of these putative binding motifs by reporter assays employing mutated constructs and observed that mutation in the DR-1–like motif, but not the octamer-like motif, resulted in marked decrease in transactivation by PPARγ2 (Fig. 2F). We next investigated whether the PPRE and the DR-1–like motif are sufficient for transactivation by PPARγ2 and found that a small deletion between these elements (positions –1920 to –1869) also exerted a severe effect (Fig. 2F), suggesting the presence of further important elements in this region.

We searched for those elements using various deletion constructs starting from the one containing the –2131/–1820 region (Fig. 3A). A small deletion between positions –1921 and –1905 resulted in even stronger transactivation than that of the original –2131/–1820 construct, possibly due to elimination of an inhibitory sequence. Further deletion up to –1891 abolished transactivation by PPARγ2, and deletion on a downstream side between –1901 and –1868 also ablated transactivation. A more elaborate deletion study indicated the important function of the region between –1905 and –1870, both halves of this region being critical for transactivation (Fig. 3B). Accordingly, we searched for the essential elements in the –1905/–1870 region, employing linker-scanning mutations

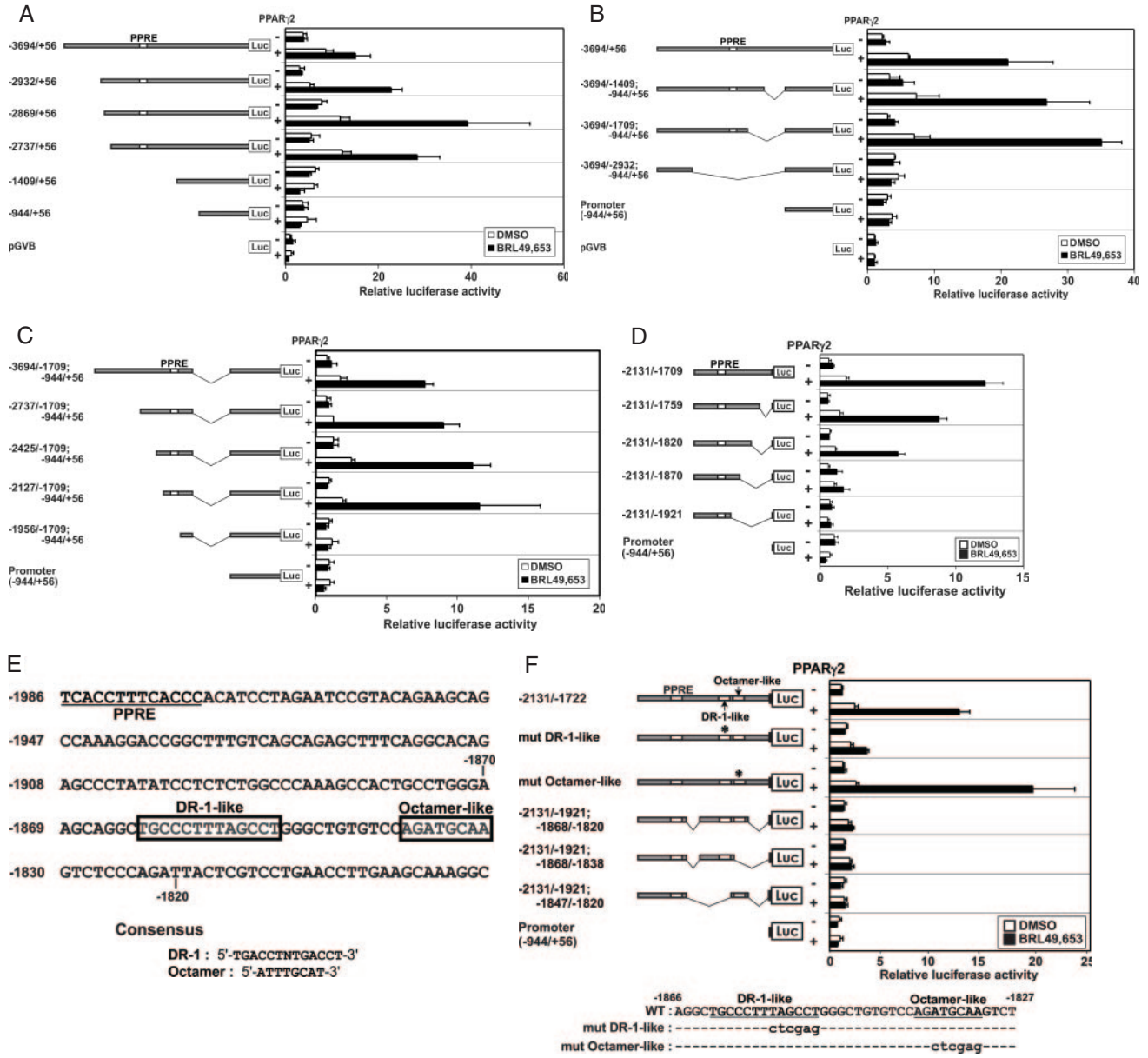

Fig. 2. **Identification of essential elements other than the PPRE.** Maps of the constructs are given as in Fig. 1. Transfection experiments were performed with or without a PPARγ2 expression vector, using HeLa cells. (A–C) Deletion analyses of the region around the PPRE. In (A) and (B), relative luciferase activities are given, taking the activity of the promoter-less reporter, pGVB, in the absence of a PPARγ2 expression vector and ligand, as 1. In (C), the activity of construct carrying only the basal promoter without PPARγ2 and ligand was taken as 1. (D) Further analysis of the region –2131/–1709 by sequential deletion from downstream. Note that the basal perilipin promoter region (–944/+56) is hereafter depicted as a small filled box. Luciferase activities are given as in (C). (E) Nucleotide sequence of the region delimited by the deletion analysis. Enclosed by squares are candidate motifs for transcription factor binding. Consensus sequences for the binding of these factors are shown at the bottom. Possible transcription factor–binding motifs were searched for with the MOTIF program (GenomeNet, Kyoto University). (F) Transactivation by PPARγ is dependent on the DR-1–like motif and a nearby region. Nucleotide sequences of this region of the wild-type and mutant constructs are shown at the bottom. Residues not involved in mutations are indicated by dashes, and the mutated residues by small letters. Asterisks indicate the positions of mutations. Luciferase activities are given as in (C).

(Fig. 3C). We observed that mutations of two regions, one corresponding to mut-1 and the other mut-3 to 5, abolished transactivation. Thus, we identified three elements, named A (positions –1905 to –1900; characterized by mut-1), B (positions –1893 to –1876; characterized by mut-3 to 5), and C (positions –1862 to –1850; renamed from DR-1–like motif), from upstream, all essential for perilipin gene expression (Fig. 3D). When mutant constructs were introduced into 3T3-L1 adipocytes, mutations in elements A, B, and C, except for mut-5, diminished the reporter activity (Fig. 3E). Hence, these elements also seemed important for perilipin gene expression in adipocytes.

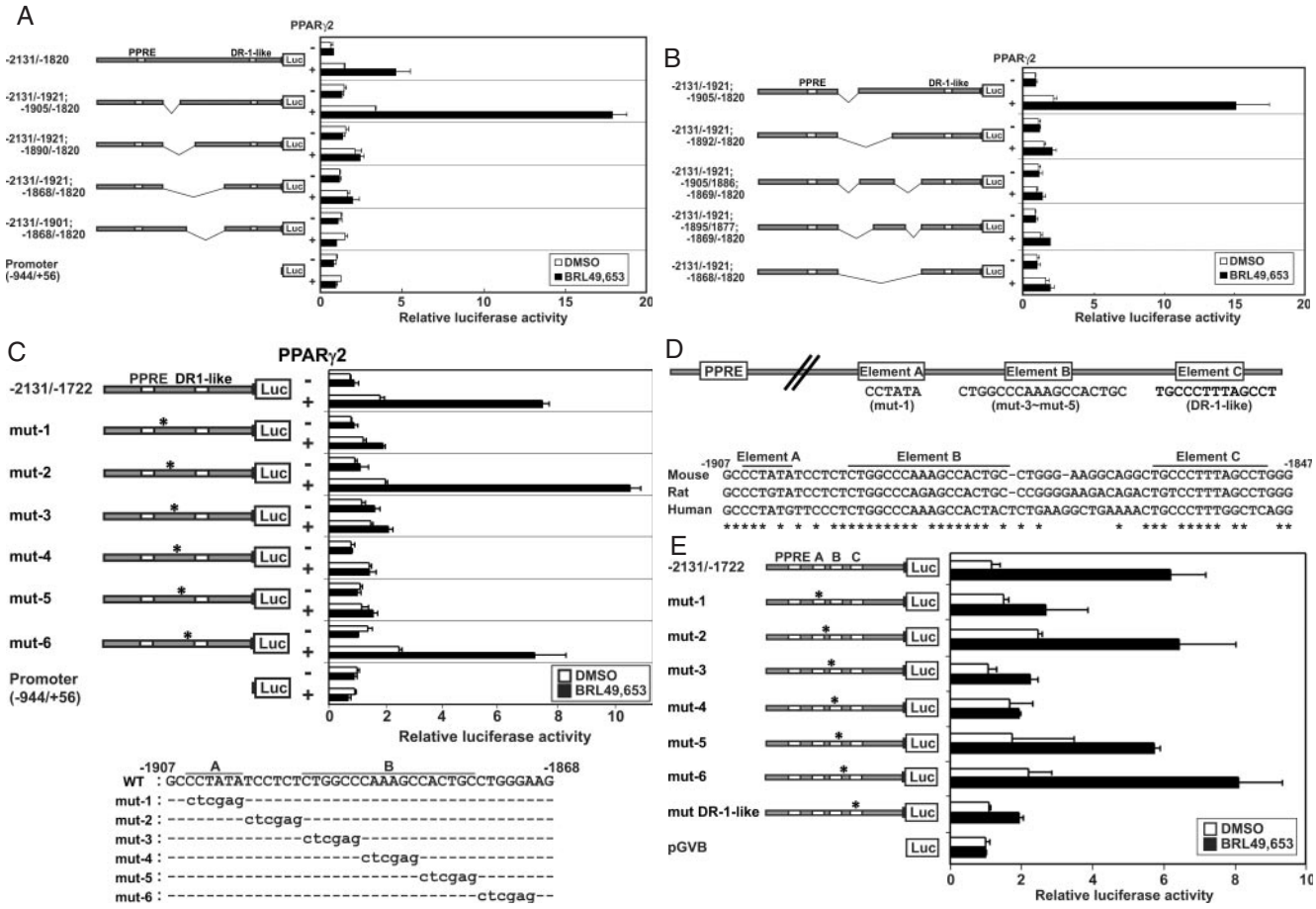

Fig. 3. **Multiple elements appear to be required for transactivation of the perilipin gene.** Maps of the constructs are given as in Fig. 1. Transfection experiments were performed with HeLa cells in the presence or absence of a PPARγ2 expression vector, except for (E). (A and B) Deletion analysis of the region between the PPRE and the DR-1–like motif. (C) Linker-scanning mutation analysis of the region –1905/–1870. The sequence of this region was sequentially replaced by the *Xho*I recognition sequence (ctcgag). The wild-type and mutant sequences are given at the bottom. In (A) and (C), luciferase activities are presented as in Fig. 2C; in (B), the activity of construct –2131/–1921; –1868/–1820 without PPARγ2 and ligand was taken as 1. (D) Sequence comparison of the regions encompassing elements A, B, and C among species. Residues conserved throughout the species are marked with asterisks. Elements A, B, and C are underlined. The alignment was made with the CLUSTAL W multiple sequence alignment program (GenomeNet, Kyoto University). (E) Transactivation of the perilipin gene depends on elements A, B, and C in adipocytes. Reporter plasmids were as used in Figs. 2F and 3C. Experiments were performed with 3T3-L1 adipocytes, as described in the "EXPERIMENTAL PROCEDURES." Luciferase activities are shown as relative values, taking the activity of pGVB without PPARγ2 and ligand, as 1

We found that their sequences, especially that of element B, are highly conserved among the mouse, rat, and human (Fig. 3D).

NF-1 Binds to Element B—We next searched for *trans*-acting factors binding to these elements by EMSA, using probes encompassing one of the corresponding sequences and nuclear extracts of differentiated 3T3-L1 adipocytes. A probe encompassing element A did not exhibit any detectable band shift (data not shown), whereas the element B probe exhibited a significant shifted band (Fig. 4A, lane 2). Because element B contained putative binding sites for transcription factors NF-1 and AP2, binding of these proteins to element B was examined and shown to be abolished by the wild-type competitor (Fig. 4A, lane 3). Mutations in the putative NF-1 site, but not the AP2 site, eliminated the capacity for competition (Fig. 4A, lanes 4 and 5). Moreover, a competitor containing a known NF-1 binding sequence (*26*), but not its mutated version, exhibited significant competition (Fig. 4A, lanes 6 and 7), while a wild-type or mutant AP2 binding sequence (*27*) did not compete for the binding (Fig. 4A, lanes 8 and 9).

Four subtypes of NF-1, NF-1A, NF-1B, NF-1C, and NF-1X, are known. They bind to the sequence TTGGCNNNNNGCCAA and related sequences, as either homodimers or heterodimers (*28*). Differential gene activation by different NF-1 subtypes has also been reported (*29*). Accordingly, we investigated which NF-1 subtypes bound to element B in adipocytes, using specific antibodies to the NF-1 subtypes in EMSA (Fig. 4B). All anti–NF-1 antibodies, but not preimmune IgG, caused a decrease in the shifted band and/or occurrence of a supershifted band. These antibodies also yielded smears of faster migrating species of probe, which were not observed with the preimmune IgG. This was possibly due to breakage of the protein-DNA complex by antibody binding during electrophoresis and the results suggest that all subtypes of NF-1 bind to element B in adipocytes. We next performed a ChIP assay to confirm that NF-1s bind to element B *in vivo*

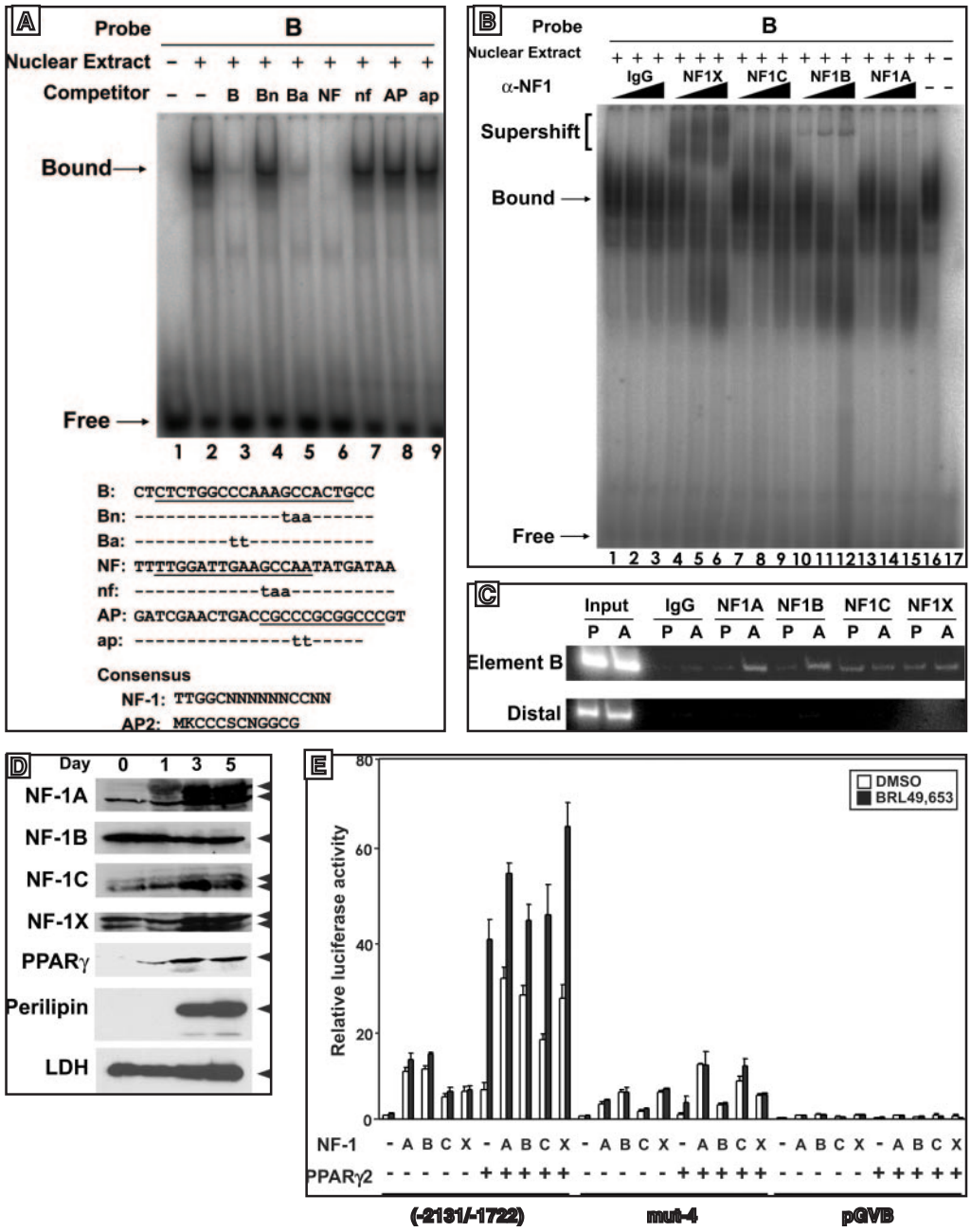

Fig. 4. **Identification of NF-1 as a *trans*-acting factor of element B.** (A) Binding of an adipocyte nuclear protein to element B. EMSA was performed using a nuclear extract of the 3T3-L1 adipocytes on day 5 of differentiation. For competition experiments, unlabeled oligonucleotides in 100-fold molar excess were applied. The bands of bound and free probes are marked. Sequences of probes and competitors are given below the panel. Competitors B, Bn, and Ba contain the element B sequences of the wild-type and mutants of the putative NF-1 and AP2 sites, respectively. Competitors NF, nf, AP, and ap have published sequences of the NF-1 binding site and its mutant (*26*), and the AP2 binding site and its mutant (*27*), respectively. Portions corresponding to element B and the consensus sites are underlined. Consensus binding sequences of NF-1 and AP2 are given at the bottom. M denotes A or C; K, G or T; S, C or G; and N, any nucleotide. (B) Identification of binding of NF-1 subtypes to element B using specific antibodies. EMSA was performed as in (A). Increasing amounts (0.1, 0.2, and 0.5 μl) of antibodies were used as indicated by filled triangles. (C) *In vivo* binding of NF-1 subtypes to element B. The ChIP assay was performed on 3T3-L1 preadipocytes (P) and adipocytes (A), using the antibodies indicated or control rabbit IgG. (D) Expression of NF-1 subtypes during adipocyte differentiation. Proteins were prepared from 3T3-L1 cells on the days indicated after induction of differentiation. Immunodetection was performed with respective antibodies. Expression of PPARγ, a presumed functional partner of NF-1, and perilipin, the target gene product, was also examined. Lactate dehydrogenase (LDH) was employed as a loading control, and the arrowhead indicates the band for the protein. (E) Enhancement of PPARγ2-dependent transactivation of the perilipin gene by NF-1s. Constructs –2131/–1722 and mut-4 of Fig. 3C were used as the wild-type and mutant element B reporters, respectively. A promoter-less reporter vector, pGVB, was used as a negative control. The reporter plasmids were transfected into HeLa cells, together with expression plasmids of PPARγ2 and rat NF-1 subtypes. A, B, C, and X indicate NF-1A, NF-1B, NF-1C, and NF-1X, respectively. Relative luciferase activities are given, taking the activity of –2131/–1722 in the absence of any expression vectors and ligand, as 1.

(Fig. 4C). NF-1A and NF-1B exhibited binding to a region encompassing element B in a differentiation-dependent manner, whereas the binding of NF-1C and NF-1X was constitutive. We examined the expression of NF-1 subtypes during adipocyte differentiation by Western blotting (Fig. 4D). All NF-1 subtypes were expressed in the differentiating adipocytes. More than two bands were detected except for NF-1B, probably due to alternative splicing. NF-1A, especially the larger isoform, was highly induced upon differentiation. On the other hand, the expression of NF-1B was observed before and remained virtually unchanged during differentiation. NF-1C and NF-1X were already expressed in the preadipocytes, and modestly induced upon differentiation. PPARγ and perilipin were significantly induced upon differentiation, as expected. The result of Western blotting was consistent with the ChIP assay results (see above), except for the differentiation-dependent *in vivo* binding of NF-1B, despite the constitutive expression. Possible reasons for this discrepancy will be discussed later (see "DISCUSSION").

Finally we investigated the effects of NF-1s on expression of the perilipin gene with a reporter assay using HeLa cells (Fig. 4E). All NF-1 subtypes significantly enhanced transactivation by PPARγ2 in the absence of added ligand, though the effects were smaller on ligand-stimulated transactivation. The effects of NF-1s were diminished when the element B sequence was mutated. We also examined transactivation by NF-1 heterodimers, but there was no significant difference among different combinations of NF-1s (data not shown).

Element C Appears to Be a Nuclear Receptor–Binding Site—We also tried to identify a *trans*-acting factor to element C. As this element has a DR-1–like sequence, we hypothesized that it might be a nuclear receptor–binding site. We examined protein binding by EMSA, using a nuclear extract from 3T3-L1 adipocytes and observed two shifted bands, both of which were abolished by competition with the wild-type but not a mutated element C sequence (Fig. 5A, lanes 2–4). The PEX11α/perilipin PPRE, a PPAR/RXR-binding site (*15*), also efficiently competed with the element C probe (lane 5), and a mutant version of this PPRE also significantly competed (lane 6), although the same mutation ablated the binding of PPAR/RXR (*15*). Neither anti-PPARα nor anti-PPARγ antibodies exhibited any supershift with the element C probe (lanes 7 and 8), though clear supershift occurred with the anti-PPARγ antibody for the binding of the 3T3-L1 proteins to the PEX11α/perilipin PPRE (data not shown). Thus, the binding activity to the element C probe is not due to the PPAR/RXR heterodimer, despite its ability to recognize the PPRE sequence. In the mutants of element C and PPRE used here, only one half-site was mutated, while the other remained unchanged. Hence, the different competition abilities of the two mutant sequences may suggest that the binding protein to element C also recognizes monovalent half-sites, if the nucleotide sequences are favorable. Hence, the mutated element C retaining a TGCCCT motif would not be sufficient for binding, while the mutated PEX11α/perilipin PPRE retaining a TCACCT motif would support binding. Consistent with this notion, sequences containing a monovalent AGGTCA half-site, the idealized sequence motif for many nuclear receptors, also competed for the binding to element C (data not shown). To examine whether both half-sites of element C are required for protein binding, we introduced various mutations into element C, to destroy one of the half-sites. Mutations of either half-site significantly affected the competition efficiency (Fig. 5B), indicating that

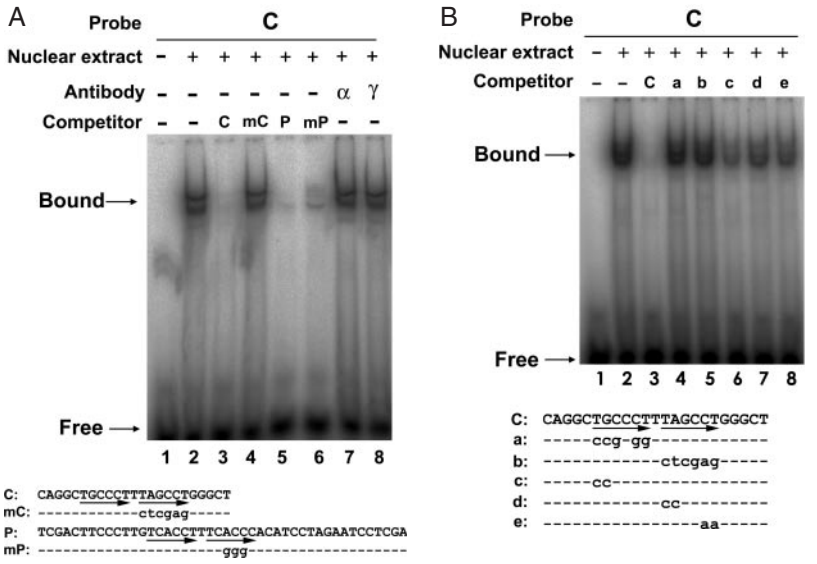

Fig. 5. **Element C probably serves for a nuclear receptor–binding site as a DR-1 motif.** EMSA was performed with the same nuclear extract as that in Fig. 4A and a probe containing the sequence of element C. (A) PPRE competes with element C for binding, but PPAR is not a major binder. Sequences of probes and competitors are given below the panel. C, mC, P, and mP are wild-type element C, mutated element C (carrying the same mutation as that of mut DR-1–like of Fig. 2F), PEX11α/perilipin PPRE, and its mutant (*15*), respectively. Sequences are shown as in Fig. 4A, except that the half-sites are indicated by horizontal arrows. Supershift assay was performed with an anti-PPARα (α) or anti-PPARγ (γ) antibody. (B) Both half-sites of element C are required for protein binding. Element C was used as a probe, and competition efficiencies of its mutants in the protein binding were examined. Residues changed in the mutants a–e are shown with small letters below the panel.

element C serves as a DR-1 motif, both half-sites being essential. We searched for this binding factor among nuclear receptors that recognize a DR-1 and/or monovalent half-site, by combined analyses using gel supershift with specific antibodies, EMSA with *in vitro* synthesized proteins, and/or gene reporter assays in the presence of expression vectors. The proteins examined included: PPARα and γ, RXRα, thyroid receptor α, hepatocyte nuclear factor (HNF)-4α and γ, Rev-erbA, NGFI-B/Nur77, estrogen receptor–related receptor (ERR) α and γ, liver receptor homolog (LRH)-1, vitamin D receptor, chicken ovalbumin upstream promoter-transcription factor (COUP-TF) 1 and 2, and retinoid-like orphan receptor (ROR) α. None of them was found likely to be the *trans*-acting factor of element C.

Transactivation of the PEX11α Gene Is Independent of Elements A, B, and C—Since the perilipin PPRE also functions in PEX11α gene activation by PPARα (*15*), we examined whether elements A, B, and C are also necessary for this process. We constructed luciferase plasmids that contained the PEX11α gene promoter and the region encompassing the PPRE and elements A, B and C, placed upstream and downstream of the luciferase gene, respectively. Reporter constructs carrying mutations in elements A, B and C were also prepared. No significant differences in transactivation by PPARα were observed between the wild-type reporter and those carrying mutations (Fig. 6A). As NF-1s are expressed ubiquitously (*28*), we examined whether transcription factors bind to element B in the liver by EMSA, using a rat liver nuclear extract. A shifted band was observed for the element B probe (Fig. 6B, lane 2), which was abolished by the wild-type competitor of element B or known NF-1 binding site, but not their mutants (Fig. 6B, lanes 3 to 6). Binding activity to element C was also found in the liver nuclear extract (Fig. 6C), which was to a large extent supershifted with an anti–HNF-4α antibody. The positions of the bands with the liver nuclear extract differed from those with the 3T3-L1 nuclear extracts, and no supershift with the antibody was observed for the latter (data not shown). Thus the binding activity to element C in 3T3-L1 cells is not due to HNF-4α.

DISCUSSION

In the present study, we identified three elements essential for transactivation of the perilipin gene, other than the PPRE. Mutation of any of them severely diminished the transactivation by PPARγ, indicating that all are essential. Thus, synergistic action between PPARγ and transcription factors recognizing these elements seems indispensable for expression of the perilipin gene. On the other hand, these elements are not required for transactivation of PEX11α gene by PPARα, at least in our reporter assay using HeLa cells.

We further identified NF-1 as a *trans*-acting factor recognizing element B. All four NF-1 subtypes bind to element B, NF-1A and NF-1B differentiation-dependently, but NF-1C and NF-1X constitutively. It is not clear why NF-1B binds more efficiently to element B in the differentiated 3T3-L1 adipocytes than the preadipocytes, despite the similar expression levels. Information is scarce on the binding specificities of NF-1 subtypes. In EMSA, all NF-1 subtypes prepared *in vitro* efficiently recognize the target DNA sequence, either as homodimers or heterodimers in all combinations (*30*). On the other hand, in the expression of murine whey acidic protein gene during mammary gland development, NF-1B is most effective in cooperative transactivation with glucocorticoid receptor and STAT5, NF-1X is the next, whereas NF-1A is a poor activator (*31*). This is possibly due to a less efficient capacity of NF-1A for specific binding to the target site. In our case, binding of NF-1B to element B may be affected *in vivo* by such factors as the local chromatin conformation and interaction with other proteins including different NF-1 subtypes, all changeable upon adipocyte differentiation. This point should be investigated in future.

Some adipogenesis-related genes such as those of aP2 and stearoyl-CoA desaturase have also been reported to be regulated by NF-1 (*32–35*), but the responsible NF-1 subtypes have yet to be clarified in these cases. For the phosphoenolpyruvate carboxykinase gene (*36*), it was reported that NF-1A and NF-1B, but not NF-1C and NF-1X, stimulate the basal promoter activity. The phosphoenolpyruvate carboxykinase gene is also a target of PPARγ, being induced during adipogenesis (*37*). Hence, regulation by NF-1 is possibly a broad characteristic of adipogenesis-related genes.

In the present study, differential expression and binding of NF-1 subtypes was apparent in relation to adipocyte differentiation. On the other hand, we did not observe significant differences in the transactivating functions among the four subtypes, all of which enhanced the transactivation by PPARγ, particularly in the absence of added ligand, in a reporter assay using HeLa cells. NF-1 is also expressed in HeLa cells, probably at the level nearly sufficient for transactivation of the perilipin gene, when PPARγ is fully activated by the ligand. Adipose-specific expression of the perilipin gene may largely be determined by PPARγ, whose expression is totally differentiation-dependent. Nevertheless, an increase in the total NF-1s during adipogenesis would also contribute to the induction of the perilipin gene. It should be noted that a mutant construct of element B (mut-5) exhibited different reporter expression in HeLa and 3T3-L1 cells (compare Fig. 3, C and E). In this mutant, only one nucleotide was changed, from A to T, in the NF-1 consensus residues within element B. This subtle change severely affected the transactivation in HeLa, but not 3T3-L1 cells, probably because of the differences in the expression level of NF-1 and/or the influences of other transcription factors between these cells.

Unfortunately, we failed to identify *trans*-acting factors for elements A and C. Reproducible protein binding was not observed for element A with the nuclear extract of 3T3-L1 adipocytes. On the other hand, element C consistently exhibited significant protein binding in EMSA with the same nuclear extract. It contains a DR-1–like sequence, and the results of EMSA suggest that the protein binding to this element is a nuclear receptor recognizing DR-1 and a monovalent half-site, depending on the sequence. However, we cannot preclude the possibility that binding to element C in 3T3-L1 adipocytes is not due to a nuclear receptor. It should also be noted that a functionally equivalent factor must be present in HeLa cells, considering the significant reporter expression in the transfection assay with these cells.

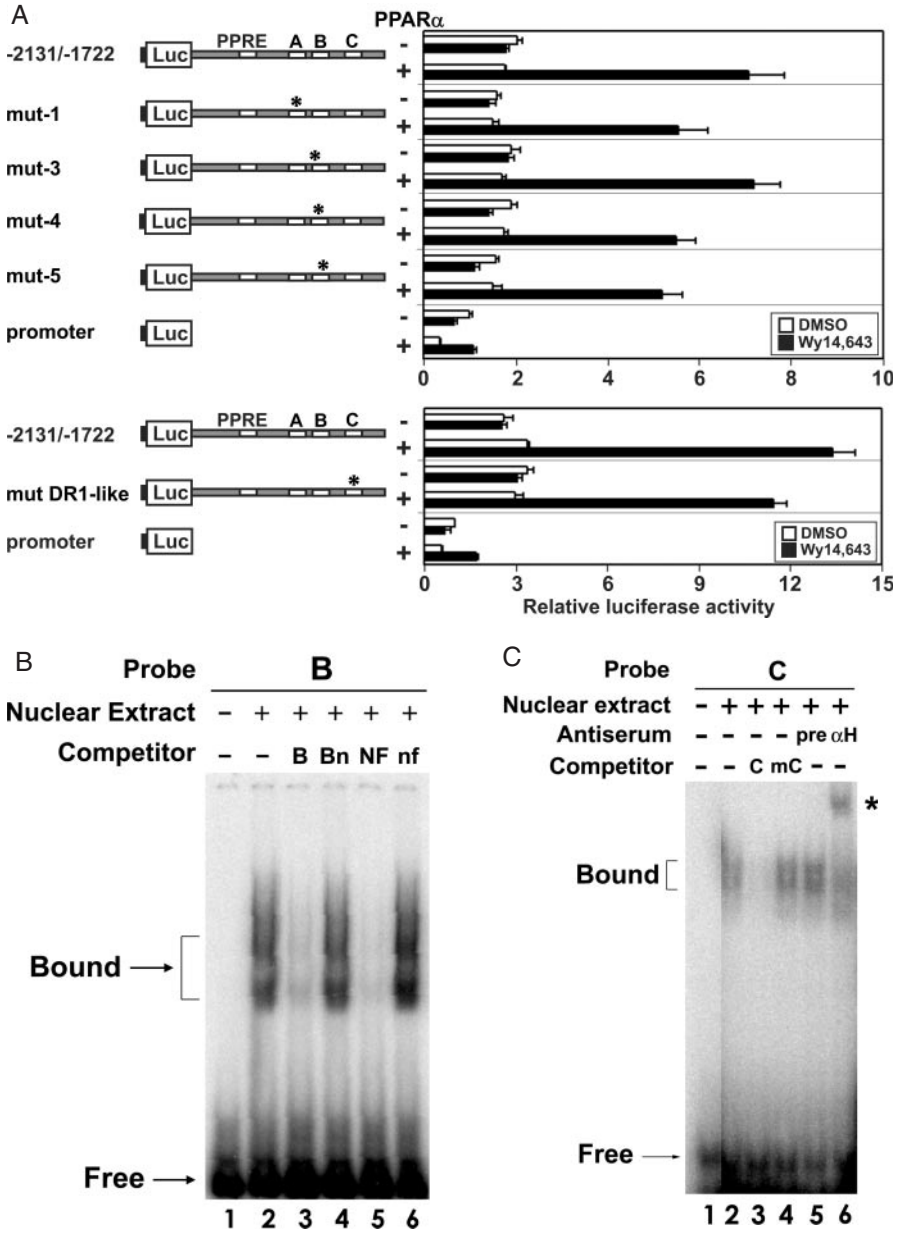

Fig. 6. **Elements A, B, and C are not required for transactivation of PEX11*α* by PPAR*α*, despite occurrence of binding in liver.** (A) Transactivation by PPARα of reporter constructs harboring the PEX11α gene promoter. The promoter region (–337/+43, relative to the PEX11α gene promoter cap site) is indicated by the small filled box on the left of the luciferase gene. The wild-type and mutant –2131/–1722 sequences used in Figs. 2F and 3C were attached downstream of the luciferase gene, and the reporter plasmids and a PPARα expression vector were co-transfected into HeLa cells. Relative luciferase activities are given, taking the activity of the construct carrying only the basal promoter without a PPARα expression vector and ligand, as 1. (B) EMSA on element B using a nuclear extract of rat liver. For each reaction, 5 μl of nuclear extract was used. The probe and competitors were as used in Fig. 4B. (C) EMSA on element C with the liver nuclear extract. The probe and competitors were as used in Fig. 5A. The asterisk indicates the band supershifted with anti–HNF-4α antiserum (αH), but not with preimmune serum (pre).

Elements A, B, and C were not required for transactivation of the PEX11α gene, at least in the reporter assays using HeLa cells. This would be due to a lack of functional synergy between PPARα and factors binding to those elements, though we cannot neglect the possibility that the factors binding to these elements in the liver have significance in PEX11α expression. The differential actions of transcription factors recognizing elements A, B, and C on the PEX11α and perilipin gene expression are presumably due to different properties of transactivating functions of PPARα and PPARγ. At present, the mechanism of synergism between PPARγ and such factors is not clear. It is possible that PPARγ and other transcription factors interact directly, or alternatively the interaction may be indirect, through a higher order coactivator complex. For the progesterone receptor, a two-step synergy with NF-1 has been proposed, the mechanism involving a change in nucleosomal conformation (*38*). Whatever the mechanism,

differential synergy between the two PPAR subtypes and other transcription factors appears to play important roles in the selective induction of PEX11α and perilipin genes through a common PPRE.

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