The Hepatocyte Nuclear Factor 4 (HNF-4) Represses the Mitochondrial HMG-CoA Synthase Gene

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We have recently shown that the gene for the mitochondrial HMG-CoA synthase is a target for PPAR and that this receptor mediates the induction of this gene by fatty acids. With the aim of gaining further insight into the function and regulation of this gene we examined the effect of other members of the nuclear hormone receptor superfamily on its expression. We previously identified a regulatory element in the mitochondrial HMG-CoA synthase gene promoter that confers transcriptional regulation by PPAR, RXR and the orphan nuclear receptor COUP-TF. In this study we demonstrate a trans-repressing regulatory function for HNF-4 at this same nuclear receptor response element (NRRE). HNF-4 binds to the mitochondrial HMG-CoA synthase NRRE, and, in cotransfection assays in HepG2 cells, it represses PPAR-dependent activation of a reporter gene linked to the mitochondrial HMG-CoA synthase gene promoter.These results suggest that the mitochondrial HMG-CoA synthase gene is subject to differential regulation by the interplay of multiple members of the nuclear hormone receptor superfamily.

The nuclear hormone receptor superfamily is formed by a growing number of receptors that are ligand-activated transcription factors. This superfamily includes receptors for different kinds of hormones, retinoids and vitamin D_3. In addition to receptors with a known ligand, many other proteins have been included in this family on the basis of homology in characteristic domains. No ligand has yet been identified for several of them, which have thus been grouped as orphan members of the superfamily (1).

We have recently shown that the gene for the mitochondrial HMG-CoA synthase, the enzyme that catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA and CoA in mitochondria, is a target for PPAR and that this receptor mediates the induction of the gene by fatty acids (2). With the aim of gaining further insight into the function and regulation of this gene, which codes for an enzyme which contributes to the regulation of hepatic production of ketone bodies (3), we have examined the effect of other members of the superfamily on its expression. The promiscuous binding of different nuclear receptors to a particular DNA-response element is not uncommon and the competition among receptors for the binding to the same element, the different posibilities for heterodimerization and the interaction with different ligands, coactivators or corepressors increases the possibilities for the regulation of target gene transcription.

We previously identified a regulatory element in the mitochondrial HMG-CoA synthase gene promoter that confers transcriptional regulation by PPAR, RXR and the orphan nuclear receptor COUP-TF (2, 4). In this study we demonstrate that this gene is also a target for HNF-4.

The HNF-4 orphan receptor is a liver-enriched member of the zinc finger nuclear receptor superfamily of transcription factors (5). Several observations suggest that in combination with other liver-specific or ubiquitous transcription factors HNF-4 plays an important role in the liver-specific gene expression (6-9) and in the regulation of several genes involved in carbohydrate metabolism (10-12), lipid metabolism (13-17), urea biosynthesis (18), blood coagulation (19-21), development

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The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; CAT, chloramphenicol acetyltransferase; COUP-TF, chicken ovalbumin upstream promoter transcription factor; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; NRRE, nuclear receptor-responsive element; RAR, retinoic acid receptor; TR thyroid hormone receptor; HNF-4, hepatic nuclear factor 4; hRXRα, human 9-*cis*-retinoic acid receptor α; mPPARα, mouse peroxisome proliferator-activated receptor α; EMSA, electrophoretic mobility shift analysis.

(22, 23), and also some members of the cytochrome P450 superfamily (24).

In this paper we show an HNF-4-mediated suppression of the PPAR-enhanced expression of the mitochondrial HMG-CoA synthase gene.

MATERIAL AND METHODS

Plasmid constructions. The reporter chloramphenicol acetyltransferase (CAT) construct pSMPCAT1 contains 1148 bp of the 5′-flanking region and the first 28 bp of exon 1 of the rat HMG-CoA synthase gene in pCAT-Basic vector (Promega) and has been described previously (25). Receptor expression plasmids pSG5-mPPARα and pJCXR8 have also been described (2); pSG5-rHNF-4 containing a cDNA for rat HNF-4 was constructed by cloning the *Bam*HI fragment of pLEN4s-rHNF-4, kindly provided by Dr.J.E. Darnell (The Rockefeller University, New York) (5), into the expression vector pSG5. pFLCOUP-TFI, generously provided by Dr. Ming-Jer Tsai (Baylor College of Medicine), contains the full-length COUP-TFI cDNA in the pGEM7Zf(+) (Promega) vector (26). pSG5-CEA+, kindly provided by Dr. Martin Zenke (Institut for Molecular Pathology (IMP) Wien, Austria), contains the chicken TRα (27).

Transfections and measurement of CAT activity. Human hepatoma HepG2 cells were cultured in minimal essential media supplemented with nonessential amino acids and 10% fetal calf serum. HepG2 cells were transfected by the calcium phosphate co-precipitation procedure, essentially as already described (28). The cells received 5μg of a reporter gene construct and, when indicated, 0-5 μg of pSG5-mPPARα and 2.5 μg of pSG5-rHNF-4 expression plasmids. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the empty expression vector pSG5 (Stratagene). Extracts of harvested cells were prepared as described (2). β-galactosidase activity was determined (29) in a 10-20 μl volume of extract to normalize for transfection efficiency. All samples assayed for CAT activity (28) were first incubated at 65°C for 10 min.

In vitro transcription/translation. In vitro transcription of cDNAs encoding receptors and subsequent translation in rabbit reticulocyte lysate were performed using a commercially available kit according to the instructions of the manufacturer (Promega); we used 1 μl of TNT™ T7 RNA polymerase for mPPARα (pSG5-mPPARα), hRXRα (pJCXR8), rHNF-4 (pSG5-rHNF-4), and cTRα (pSG5-CEA+), 1 μl of TNT™ SP6 RNA polymerase for hCOUP-TFI (pFLCOUP-TF I). 1 μg of plamid was used for separate translations and 0.75 μg of each plasmid was used for cotranslations.

DNA binding assays. Proteins synthesized *in vitro* (2.5 μl) were preincubated on ice for 10 min in 10 mM Tris-HCl, pH 8.0, 40 mM KCl, 0.05% (vol/vol) Nonidet P-40, 6% glycerol, 1 mM dithiothreitol and 1 μg of poly(dI-dC). Then 0.8 ng of ^{32}P-labeled MSPPRE oligonucleotide was added and the incubation was continued for 15 min at room temperature. The final volume for all reactions was 20 μl. Binding reactions were electrophoresed at 4°C on a 4.5% polyacrylamide gel in 0.5× TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.0). MSPPRE is the fragment corresponding to coordinates −116 to −69 of the mitochondrial HMG-CoA synthase gene and contains the PPAR response element of this gene (−104 to −92) (5′-TGACTTGTTCTGAGACCTTTGGCCCAGTTTTTCTGAGGCAGGCAGAGG) (2). Dissociation kinetics were studied by gel retardation "off" curves. Complexes were performed for 15 min at room temperature and a 1000-fold excess of cold competitor oligonucleotide was added at 0 min. These experiments were performed with a ^{32}P-labeled MS113-82 oligonucleotide, corresponding to coordinates −113 to −82 of the mitochondrial HMG-CoA synthase gene (5′-agCTTGTTCTGAGACCTTTGGCCCAGTTTTTCTG).

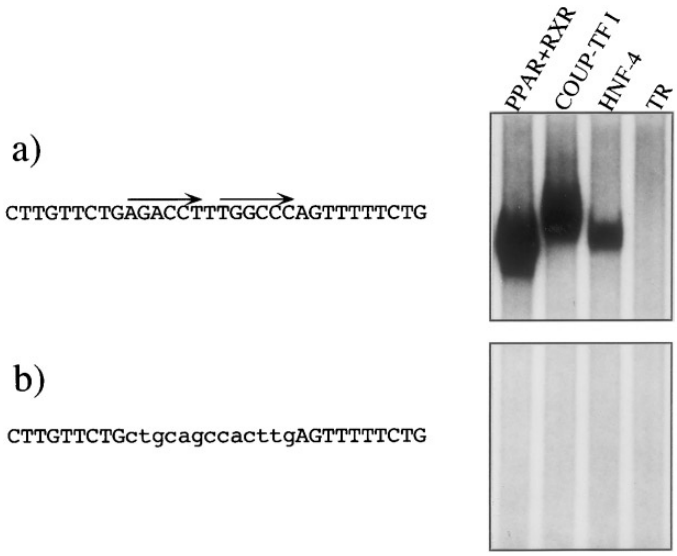

FIG. 1. Interaction of different nuclear receptors with the mitochondrial HMG-COA synthase NRRE. **(A)** Several members of the nuclear receptors superfamilly are able to bind to the mitochondrial HMG-CoA synthase NRRE. Electrophoretic mobility shift assay was performed on labeled MSPPRE oligonucleotide incubated with *in vitro* translated PPAR-RXR, COUP-TF I, HNF-4 or TR as indicated. **(B)** Identical experiment performed with a probe containing a NRRE mutated by scrambling as indicated (lower case letters).

RESULTS

HNF-4 Binds to a Nuclear Receptor Responsive Element (NRRE) in the Mitochondrial HMG-CoA Synthase Gene

In order to determine whether HNF-4 is able to bind to the NRRE of the mitochondrial HMG-CoA synthase we performed gel mobility shift assays with a DNA probe containing this element. *In vitro* transcribed and translated HNF-4 binds to this element as occurs with COUP-TF and the heterodimer PPAR-RXR, but not with TR (Fig. 1a). An oligonucleotide containing a scrambled sequence between −104 and −92 was unable to form a complex with any of those receptors (Fig.1b).

There are several putative half nuclear receptor recognition sites in the DNA fragment used as a probe, marked as A, B and C in the figure (Fig. 2a). In order to achieve more precise mapping of the DNA sequence responsible for the HNF-4 binding we performed gel shift experiments with several probes containing different wild type or mutated sequences as indicated. HNF-4 binds to the DNA region comprised between −104 and −92 containing the TGACCT-like repeats B and C (Fig. 2b). Either a point mutation in repeat B or a scrambling of repeat C impairs the binding of HNF-4. Neither the presence of repeat A nor the 3′ T region necessary for the binding of the PPAR-RXR heterodimer to the p450 4A6 gene (30) and also to the mitochondrial HMG-CoA syn-

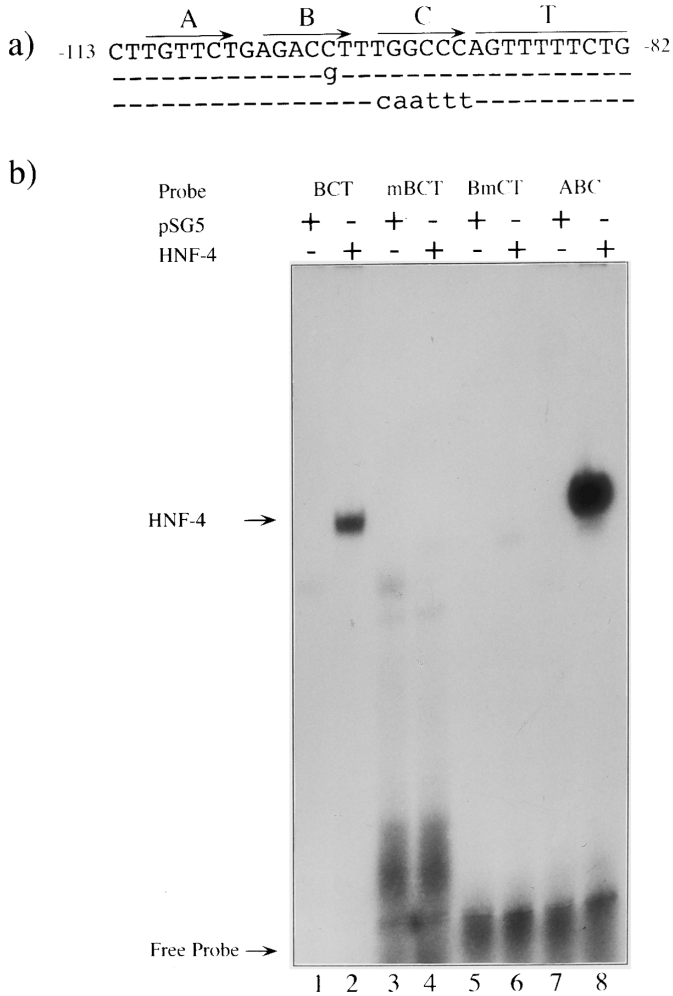

FIG. 2. Analysis of the interaction of HNF-4 with the mitochondrial HMG-CoA synthase NRRE. Electrophoretic mobility shift assays were carried out in the absence or presence of *in vitro*-synthesized HNF-4 with the indicated labeled probes containing the different TGACCT-like repeats and the additional 3′ region (T) as indicated in **a,** the mutations introduced in the probe are indicated by lower case letters and the conserved positions are indicated by dashes. The arrows in **b,** indicate the HNF-4-DNA complex (lanes 2, and 8) and the free probe. pSG5 indicates the control carried out with the product of a mock transcription and translation reaction performed with the empty vector pSG5 (lanes 1, 3, 5 and 7).

thase gene (J. C. Rodríguez and D. Haro, unpublished) is necessary for the binding of HNF-4 to the mitochondrial HMG-CoA synthase gene.

HNF-4 Represses the Mitochondrial HMG-CoA Synthase Gene

As can be seen in Fig. 3, chloramphenicol acetyltransferase expression from pSMPCAT1, a construct containing the CAT gene under the control of the promoter and 5′-flanking sequences of the rat mitochondrial HMG-CoA synthase gene (coordinates −1148 to +28), was repressed by cotransfection with HNF-4 in human hepatoma HepG2 cells (the average values of CAT activity, from at least three independent experiments with two plates each, presented as % of normalized CAT activity relative to pSMPCAT1 in the absence of HNF-4 is 48±20.2). This effect is dependent on the binding of HNF-4 to DNA as it is not seen when a reporter construct containing a point mutation C to G at −100, that prevents the binding of HNF-4, is used instead of the wild type promoter. We used the promoter of the Medium Chain Acyl-CoA dehydrogenase as a positive control to check the capacity of HNF-4 to activate gene transcription in our hands.

HNF-4 Represses the Induction of the Mitochondrial HMG-CoA Synthase Gene by PPAR

As both HNF-4 and **t**he heterodimer PPAR-RXR bind to the same DNA region, to asses whether the observed repression is due to competition of HNF-4 with PPAR for the binding to DNA, we performed cotransfection experiments with PPAR and HNF-4. Chloramphenicol acetyltransferase expression from pSMPCAT1 was activated by cotransfection with PPAR in human hepatoma HepG2 cells (2). This activation was repressed by cotransfection with HNF-4 but, surprisingly, this effect cannot be overcome by cotransfection with increasing amounts of PPAR (Table I) even in the presence of added RXR (data not shown).

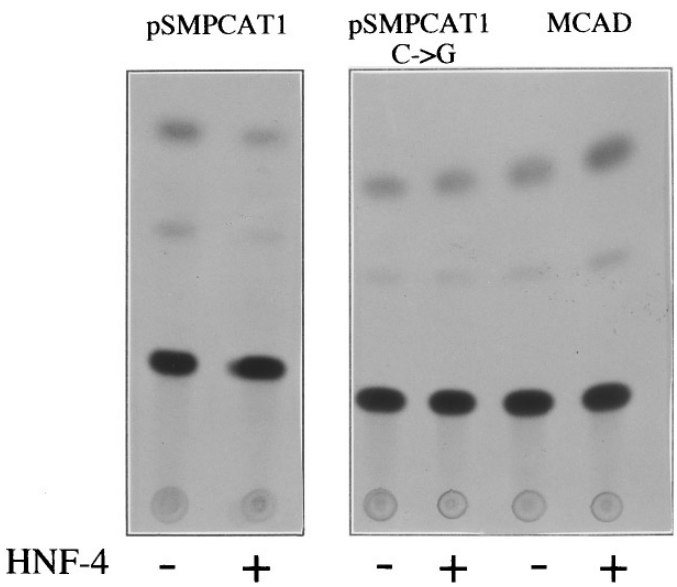

FIG. 3. HNF-4 dependent inactivation of the mitochondrial HMG-coA synthase gene promoter in HepG2 cells. CAT reporter constructs containing the wild-type (pSMPCAT1) 5′-flanking region of the mitochondrial HMG-CoA synthase gene or a mutated version containing a point mutation C to G at -100, were cotransfected with or without 2.5 μg of pSG5HNF-4 into HepG2 cells. An autoradiograph showing a representative experiment is shown. The activation of the Medium Chain Acyl-CoA dehydrogenase gene promoter is shown as a positive control.

TABLE I

Antagonistic Effects of PPAR and HNF-4 on the Mitochondrial HMG-CoA Synthase Gene

HNF-4 (2.5 μg)	−	+	+	+	+
PPAR α μg	0.1	0.1	1.0	2.5	5
Fold Induction	20.6 ± 8.1	1.65 ± 0.21	2.65 ± 0.63	3.43 ± 0.45	2.61 ± 0.22

Note. HepG2 cells were cotransfected with reporter construct pSMPCAT1 and indicated amounts of expression plasmids for mPPARα and HNF-4. PPAR induction of reporter activity (column 2) was inhibited by cotransfection of 2.5 μg of pSG5-HNF-4 (column 3). To relieve this repression increasing amounts of pSG5-mPPARα (1-5 μg) were cotransfected (columns 4-6). Average values of CAT activity (means ± S.D.), from two independent experiments with two plates each, are presented as "fold induction" relative to pSMPCAT1 in the absence of expression plasmids.

The HNF-4 Containing Complex is Much Less Stable than the Complex Formed by the PPAR-RXR Heterodimer

In order to compare the affinity of HNF-4 binding with that of the PPAR-RXR heterodimer we performed rate-off experiments. Surprisingly, in contrast with the results shown above, the binding of HNF-4 to DNA *in vitro* was weaker than the binding of the PPAR-RXR heterodimer. The HNF-4-DNA complex was totally competed after 5 minutes of incubation with an excess of cold probe (Fig. 4), whereas the PPAR-RXR-DNA complex remained after 60 min of incubation with the competitor.

DISCUSSION

HNF-4 is a member of the nuclear receptor superfamilly that transcriptionally activates a wide variety of genes. In this report we show that in transiently transfected HepG2 cells HNF-4 represses the mitochondrial HMG-CoA synthase gene promoter.

It has recently been shown that the hypolipidemic effect of the peroxisome proliferators is accounted for by enhanced catabolism of plasma triacylglyceride-rich lipoproteins due to a decrease in plasma apolipoprotein C-III produced as a consequence of the displacement of HNF-4 from the apolipoprotein C-III promoter mediated by PPAR (31). The same effect has been described for the transferrin gene where PPAR also suppresses HNF-4-enhanced expression (32).

Interestingly, a site mapped in the promoter of L-pyruvate kinase responsible for transcriptional repression by polyunsaturated fatty acids (11) corresponds to the HNF-4 site of the L-pyruvate kinase gene. These observations, along with the demonstration that fatty acids promote the binding of PPAR-RXR heterodimers (33-34), suggest that PPARα not only promotes fatty acid catabolism but it also mediates fatty acid-dependent suppression of lipogenic and glycolytic enzymes.

In this paper we show the opposite effect, an HNF-4 suppression of the PPAR-enhanced expression of the mitochondrial HMG-CoA synthase gene. This suppression is probably due to competition for the binding to

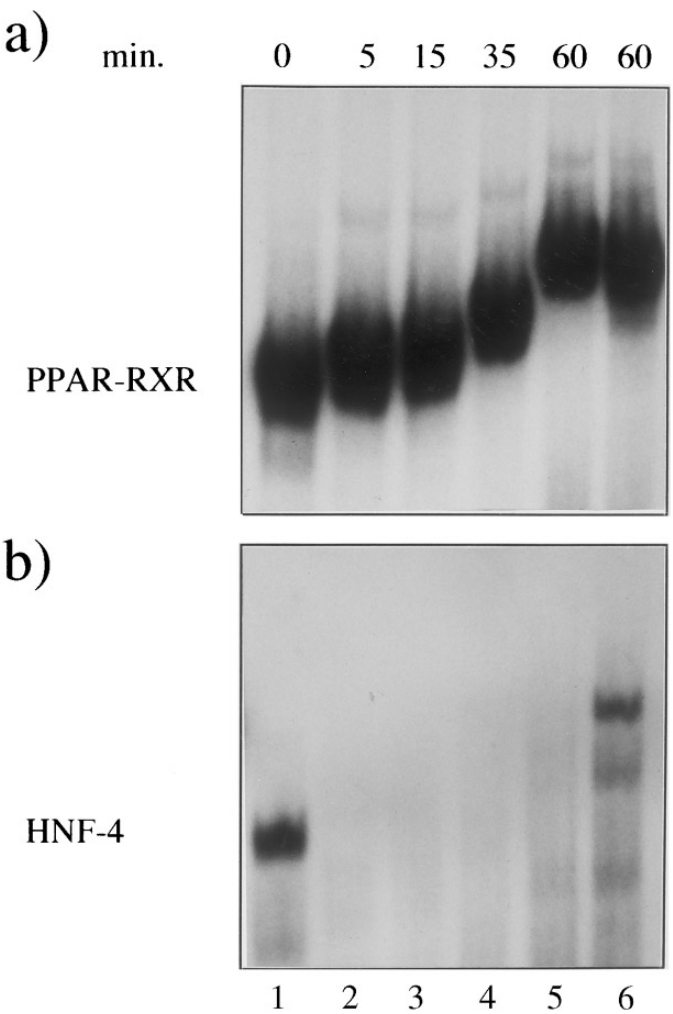

FIG. 4. PPAR/RXR heterodimers form more stable complex with DNA than HNF-4. Electrophoretic mobility shift assays were carried out with *in vitro*-synthesized PPAR/RXR (a) or HNF-4 (b) and a probe containing the proposed NRRE of the mitochondrial HMG-CoA synthase gene (MS 113-82). To determine the dissociation kinetics of the complexes formed, a 1000-fold excess of unlabeled probe was added at time point 0 (lane 1). After 5, 15, 35, and 60 min (lanes 2-5) the reaction was loaded onto a running polyacrylamide gel. The last lane (6) show gel-shift experiments performed after 60 min of incubation under identical conditions, but in the absence of competitor DNA.

DNA, as firstly HNF-4 binds to a sequence of the mitochondrial synthase gene that is overlapped by the PPAR-binding sequence, and secondly, in cotransfection experiments in HepG2 cells HNF-4 represses a reporter gene linked to the mitochondrial HMG-CoA synthase gene promoter.These results constitute evidence of a mechanism of suppression of fatty acid signaling which consists in the replacement of an active PPAR-RXR complex by an abortive HNF-4 homodimer. Interestingly, in this model we may consider the participation of another factor since: a) transfection experiments show that once HNF-4 is overexpressed, increasing amounts of PPAR cannot overcome the inactivation (Table I), perhaps due to a recruitment of a co-repressor; and b) observations *in vitro* demonstrate that this effect is not simply due to a higher affinity of HNF-4 homodimers for the element (Fig. 4).

Therefore, the mitochondrial HMG-CoA synthase gene is regulated by an antagonism between PPAR and HNF-4, in which PPAR is an activator and HNF-4 is a repressor. A similar mechanism has been reported for the genes encoding acyl-CoA oxidase and hydratase-dehydrogenase, the first two enzymes in the peroxisomal β-oxidation pathway (35). Our data provide further evidence that HNF-4 contributes to the regulation of genes involved in the metabolism of fatty acids.

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REFERENCES

1. Tsai, M.-J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486.
2. Rodríguez, J. C., Gil-Gómez, G., Hegardt, F. G., and Haro, D. (1994) *J. Biol. Chem.* **269,** 18767–18772.
3. Casals, N., Roca, N., Guerrero, M., Gil-Gómez, G., Ayté, J., Ciudad, C. J., and Hegardt, F. G. (1992) *Biochem. J.* **283,** 261–264.
4. Rodríguez, Joan C., Ortiz, José A., Hegardt, Fausto G., and Haro, Diego (1997) *Biochem. J.* **326,** 587–592.
5. Sladek, F. M., Zhong, W., Lai, E., and Darnell, J. E., Jr. (1990) *Genes & Dev.* **4,** 2353–2365.
6. Costa, R. H., Grayson, D. R., and Darnell, J. E., Jr. (1989) *Mol. Cell. Biol.* **9,** 1415–1425.
7. Xanthopoulos, K. G., Prezioso, V. R., Chen, W. S., Sladez, F. M., Cortese, R., and Darnell, J. E., Jr. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3807–3811.
8. Tian, J. M., and Schibler, U. (1991) *Genes & Dev.* **5,** 2225–2234.
9. Späth, G. F., and Weiss, M. C. (1997) *Mol. Cell. Biol.* **17,** 1913–1922.
10. Liu, Z., and Towle, H. C. (1995) *Biochem. J.* **308,** 105–111.
11. Hall, R. K., Sladek, F. M., amd Granner, D. K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 412–416.
12. Liimatta, M., Towle, H. C., Clarke, S., and Jump, D. (1994) *Mol. Endocrinol.* **8,** 1147–1153.
13. Mietus-Snyder, M., Sladek, F., Ginsburg, G. S., Kuo, C. K., Ladias, J. A. A., Darnell, J. E., Jr., and Karathanasis, S. K. (1992) *Mol. Cell. Biol.* **12,** 1708–1718.
14. Ladias, J. A. A., Hadzopoulou-Cladaras, M., Kardassis, D., Cardot, P., Cheng, J., Zannis, V., and Cladaras, C. (1992) *J. Biol. Chem.* **267,** 15849–15860.
15. Chan. J., Nakabayashi, H., and Wong, N. C. W. (1993) *Nucleic. Acids Res.* **21,** 1205–1211.
16. Carter, M. E., Gulick, T., Raisher, B. D., Caira, T., Ladias, J. A. A., Moore, D. D., and Kelly, D. P. (1993) *J. Biol. Chem.* **268,** 13805–13810.
17. Carter, M. E., Gulick, T., Moore, D. D., and Kelly, D. P. (1994) *Mol. Cell. Biol.* **14,** 4360–4372.
18. Kimura, A., Nishiyori, A., Murakami, T., Tsukamoto, T., Hata, S., Osumi, T., Okamura, R., Mori, M., and Takiguchi, M. (1993) *J. Biol. Chem.* **268,** 11125–11133.
19. Erdmann, E., and Heim, J. (1995) *J. Biol. Chem.* **270,** 22988–22996.
20. Hung, H-L., and High, K. A. (1996) *J. Biol. Chem.* **271,** 2323–2331.
21. Pollak, E. S., Hung, H-L., Godin, W., Overton, G. C., and High, K. A. (1996) *J. Biol. Chem.* **271,** 1738–1747.
22. Duncan, S. A., Manova, K., Chen, W. S., Hoodless, P., Weinstein, D. C., Bacharova, R. M., and Darnell, J. E., Jr. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7598–7602.
23. Chen, W. S., Manova, K., Weinstein, D. C., Duncan, S. A., Plump, A. S., Prezioso, V. R., Bacharova, R. M., and Darnell, J. E., Jr. (1994) *Genes & Dev.* **8,** 2466–2477.
24. Chen, D., Lepar, G., and Kemper, B. (1994) *J. Biol. Chem.* **269,** 5420–5427.
25. Gil-Gómez, G., Ayté, J., and Hegardt, F. G. (1993) *Eur. J. Biochem.* **213,** 773–779.
26. Cooney, A., Tsai, S. Y., O'Malley, B. W., and Tsai, M.-J. (1992) *Mol. Cell. Biol.* **12,** 4153–4163.
27. Sap, J., Muñoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom-B (1986) *Nature.* **324,** 635–640.
28. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology. Green Publishing Associates/Wiley Interscience, New York.
29. Sambrock, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. Palmer, C. N. A., Hsu, M-H., Griffin, K. J., and Johnson, E. F. (1995) *J. Biol. Chem.* **270,** 16114–16121.
31. Hertz, R., Bishara-Shieban, J., and Bar-Tana, J. (1995) *J. Biol. Chem.* **270,** 13470–13475.
32. Hertz, R., Seckbach, M., Zakin, M. M., and Bar-Tana, J. (1996) *J. Biol. Chem.* **271,** 218–224.
33. Forman, B. M., Chen, J., and Evans, R. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4312–4317.
34. Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M., and Lehmann, J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4318–4323.
35. Winrow, C. J., Marcus, S. L., Miyata, K. S., Zhang, B., Capone, J. P., and Rachubinski, R. (1994) *Gene Expression.* **4,** 53–62.