Activation of the Promoter of the Orphan Receptor SHP by Orphan Receptors That Bind DNA as Monomers*

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Small heterodimer partner (SHP) is an orphan nuclear receptor that lacks a conventional DNA binding domain. It interacts with several other members of the nuclear receptor superfamily and inhibits receptor transactivation. In order to characterize the regulation of SHP expression, a number of receptors and other transcription factors were tested for effects on the SHP promoter. Among these, the orphan receptor steroidogenic factor-1 (SF-1) was found to potently transactivate the SHP promoter. Detailed footprinting studies show that the SHP promoter contains at least five SF-1 binding sites, and mutagenesis studies demonstrate each of the three strongest binding sites is required for SF-1 transactivation. SHP is coexpressed with SF-1 in adrenal glands, but is also expressed in tissues that lack SF-1, including liver. However, liver expresses a close relative of SF-1, the orphan fetoprotein transcription factor (FTF), and FTF can also transactivate the SHP promoter. These results suggest that alterations in the levels or activities of SF-1 or FTF could modulate SHP expression in appropriate tissues and thereby affect a variety of receptor dependent signaling pathways.

Mammalian genomes encode approximately 50 members of the nuclear hormone receptor superfamily (1). The receptors for steroids, retinoids, thyroid hormone, and a number of other ligands currently comprise about half of the superfamily. These proteins are ligand-dependent transcription factors that generally bind DNA as either homodimers or heterodimers and activate transcription in the presence of their ligands. The rest of the family is made up by the orphan receptors, which do not have known ligands but share sequence similarity with the conventional receptors (2). Throughout the superfamily the highest sequence similarities are observed in the DNA binding domains, which are zinc-binding modules. Lesser conservation is observed in the ligand binding domains, with as little as 20% sequence identity between the most divergent family members. However, x-ray crystallographic studies have confirmed that even divergent sequences adopt very similar three-dimensional structures (3).

In mammals there are at least two unusual orphans that lack conventional DNA binding domains. DAX-1, the first described, includes a ligand binding domain fused to a novel domain reported to bind DNA, but unrelated to the DNA binding domains of other nuclear receptors (4). DAX-1 interacts functionally (5, 6) with the orphan SF-1, which was first identified as an activator of expression of a number of steroidogenic enzymes (7). Within the receptor superfamily, SF-1 is also somewhat unusual in that it binds DNA efficiently as a monomer. SF-1 is required for development of both gonads and adrenals (8), and mutations of DAX-1 result in congenital adrenal hypoplasia in humans (4).

SHP1 (9) is the second orphan receptor that lacks a conventional DNA binding domain. It was initially isolated on the basis of its interaction with the orphan receptor CAR and also interacts with a number of other receptor superfamily members, including the conventional receptors thyroid hormone receptor, retinoid X receptor, and estrogen receptor (9–12). It was also independently isolated twice as a peroxisome proliferator-activated receptor interactor (12, 13). In general, SHP acts to decrease transcriptional activation by its receptor targets. The very broad range of receptors sensitive to this inhibition suggests a central role for SHP in modulation of nuclear receptor signaling pathways.

Although the mechanisms underlying the inhibitory effects of SHP remain to be defined, they depend on a variety of factors, particularly the relative amounts of SHP and its targets. Thus, factors that increase or decrease SHP expression could have significant effects on receptor signaling. To identify such factors, we have studied the SHP promoter. Among a number of nuclear receptors and other transcription factors examined, SF-1 and the closely related orphan FTF were found to stimulate SHP promoter function. A combination of mutagenesis and footprinting studies revealed a series of functional SF-1 binding sites in close proximity to the transcription start site and demonstrated that each of three high affinity sites is necessary for transactivation. These results suggest that SF-1 and FTF are important regulators of SHP expression and that increasing or decreasing the amounts or specific activity of SF-1 or FTF could have indirect effects on a variety of receptor dependent signaling pathways.

EXPERIMENTAL PROCEDURES

Plasmids—Various reporter constructs for the mouse and human SHP promoters were prepared by insertion of polymerase chain reaction-amplified 5′-flanking regions of the two genomic DNAs into a previously described luciferase reporter (14). Preparation of 5′ deletion constructs of mouse SHP promoter has been described elsewhere (15). Point mutations were introduced into the SHP(−453)luc reporter using

The abbreviations used are: SHP, small heterodimer partner; SF-1, steroidogenic factor 1; FTF, fetoprotein transcription factor; DAX-1, dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1; TK, thymidine kinase; GST, glutathione S-transferase.

*This work was supported by Grant DK46546 from the National Institutes of Health (to D. D. M.), by a grant from the Howard Hughes Medical Institute (to K. L. P.), by Grant GE 96-78 from the Korean Institutes of Health (to D. D. M.), by a grant from the Howard Hughes Medical Institute (to H. S. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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polymerase chain reaction with primers in which the GG in the SF-1 consensus site was altered to TT. The SF-1Luc reporter has five copies of an SF-1 consensus sequence in front of the prolactin promoter, as described previously (16). A mammalian expression vector for SF-1 was constructed by inserting a full-length SF-1 fragment from pCMV5-SF1 into pCEP4 (Invitrogen). A pCI human FTF expression vector is a kind gift from Dr. Luc Belanger. For in vitro translation of SF-1, SF-1 insert from pCEP4-SF-1 was placed downstream of the T7 promoter.

Cell Culture and Transient Transfection—HepG2 and Y1 cells were propagated in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. One day before transfection, cells were split into 12-well plates. In general, 4 h after replacement with fresh medium the cells were transfected using calcium phosphate (17) with 0.5 mg/well of both the luciferase reporter and the TKGH (18) internal control plasmids and indicated amounts of receptor expression vectors. However, in comparisons of SHP promoter deletion constructs, equimolar amounts were used. Luciferase expression was assayed and normalized using growth hormone expression as described previously (19). All luciferase values were averaged from triplicate samples. All transfection data were repeated at least three times.

DNase I Footprinting Assay—Bacterially expressed GST-SF1 fusion protein was incubated with approximately 30 fmol of end-labeled DNA probe at room temperature for 30 min, and the reaction mixture was digested with DNase I for 3 min using standard conditions provided by the manufacturer (Promega). The digested products were analyzed using 5% nondenaturing polyacrylamide gel electrophoresis.

Gel Mobility Shift Assay—Oligonucleotides corresponding to the five SF-1 protected sites from the SHP promoter region were used as DNA probes. SF-1 protein was expressed using the TNT coupled reticulocyte lysate system (Promega). The assays were performed essentially as described (9). Briefly, 0.5 μl of the lysate was incubated with end-labeled probe for 30 min at room temperature, and the reaction mixture was analyzed by 5% nondenaturing gel electrophoresis. Competition assays used either 1-, 5-, and 20-fold molar excesses of a unlabeled oligonucleotide containing sft1, the furthest upstream SF-1 binding site, or 100-fold excess of an unlabeled oligonucleotide containing a mutant version of the sft1 sequence in which the GG in the SF-1 consensus was changed to TT.

RESULTS

In order to study the regulation of SHP gene expression, approximately 2 kilobase pairs of the 5′-flanking region of the mouse SHP gene was introduced into a luciferase reporter construct. A variety of nuclear hormone receptors and other transcription factors were tested for ability to stimulate the SHP promoter in transient transfection. Among these, the orphan nuclear receptor SF-1 was found to enhance SHP promoter driven luciferase activity 2–10-fold, depending on the cell type transfected.

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In order to map sequences required for SF-1 activation, a
DNase I footprinting. The SHP promoter fragment from −453 to +21 was end-labeled and used for DNase I footprint analysis with the indicated amounts of bacterially expressed GST-SF1 fusion protein. The arrow on the left indicates the position of the transcription start site. Smaller arrows on the right indicate the positions and orientations of SF-1 protected regions. All footprints contain matches to the SF-1 consensus sequence matching the consensus SF-1 binding site (20). Footprints sft1, sft3, and sft4 contain elements that differ from the SF-1 consensus at only one position; and the sft2 and sft5 motifs contain two mismatches (Fig. 2B). Similar elements were also observed in the human SHP promoter.

Gel mobility shifts were used to confirm these footprinting results and test the relative binding affinity of SF-1 for each site. As shown in Fig. 3, SF-1 bound oligonucleotides corresponding to each of the five sites, as expected. An sft1 oligonucleotide with a GG to TT mutation in the core of the SF-1 binding site, which abolishes specific DNA binding (21), was unable to compete for binding. Consistent with their sequences, sft1, sft3, and sft4 were relatively resistant to competitions with increasing amounts of an unlabeled sft1 oligonucleotide, indicating that they bind SF-1 with high affinity. Sites sft2 and sft5 have lower apparent affinity for SF-1, as they were more sensitive to competition with the sft1 oligonucleotide.

The deletion results indicate that the presence of sites sft3, sft4, and sft5 is not sufficient to confer response to SF-1. To explore the requirements of the individual sites for the SF-1 responsiveness in more detail, a GG to TT double substitution corresponding to each of the five sites, as expected. An sft1 oligonucleotide with a GG to TT mutation in the core of the SF-1 binding site, which abolishes specific DNA binding (21), was unable to compete for binding. Consistent with their sequences, sft1, sft3, and sft4 were relatively resistant to competitions with increasing amounts of an unlabeled sft1 oligonucleotide, indicating that they bind SF-1 with high affinity. Sites sft2 and sft5 have lower apparent affinity for SF-1, as they were more sensitive to competition with the sft1 oligonucleotide.

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FIG. 2. Five SF-1 binding sites in the mouse SHP promoter. A, DNase I footprinting. The SHP promoter fragment from −453 to +21 was end-labeled and used for DNase I footprint analysis with the indicated amounts of bacterially expressed GST-SF1 fusion protein. The arrow on the left indicates the position of the transcription start site. Smaller arrows on the right indicate the positions and orientations of SF-1 protected regions. All footprints contain matches to the SF-1 consensus sequence. B, SF-1 consensus binding motifs in the protected regions. The footprints sft1, sft3, and sft4 contain elements that differ from the SF-1 consensus (20) at a single position, whereas elements in sft2 and sft5 have two mismatches. Protein contacting G residues are underlined.

FIG. 3. Relative affinity of SF-1 binding sites. DNA fragments corresponding to each protected region were used electrophoretic mobility shift assays with in vitro translated SF-1. As indicated, a 1-, 5-, or 20-fold excess of an unlabeled sft1 oligonucleotide, or 100-fold excess of unlabelled mutant sft1 oligonucleotide (m, GG to TT mutation), was added prior to the addition of the probes. Specific complexes were resolved on a 5% polyacrylamide gel.
FIG. 4. Mutagenesis of individual in SHP(−453)luc SF-1 binding sites. The central G residues of each SF-1 binding site were altered to T residues to generate a series of five independent mutated promoters. The wild type and mutated constructs were cotransfected into Y1 cells with 50 ng of CDMS or SF-1 expression vectors. The normalized luciferase activities were averaged from three independent experiments.

cells, since the SHP(−453)luc construct retaining the five footprints showed little or no transactivation by FTF. The basis for this requirement for additional 5’ sequences remains to be identified.

DISCUSSION

Previous results demonstrate that SHP acts to inhibit transactivation by a number of the members of nuclear receptor superfamily (9–12). These inhibitory effects are not absolute, but are dependent on the relative amounts of both SHP and its various targets. Thus, information on factors that increase or decrease SHP expression is clearly central to understanding its regulatory effects. Based on this, we examined a number of nuclear hormone receptors as well as other transcription factors for effects on the SHP promoter. As described here, the SHP promoter can be strongly transactivated by SF-1 and FTF. The magnitude of this response is consistent with the fact that the promoter contains at least five SF-1/FTF binding sites. Recently, we have found that the promoter can also be transactivated by the estrogen receptor.2

The activation of the SHP promoter by SF-1 is consistent with the presence of SHP transcripts in several SF-1 expressing tissues or cell lines, including rat testis and adrenal (12, 13), human fetal adrenal gland (15), and the adrenal-derived Y-1 cell line.4 Similarly, the response to FTF is consistent with the expression of SHP in liver (9). Thus, the current results suggest that alterations in the levels of SF-1 and FTF could have important effects on a variety of nuclear receptor signaling pathways as a consequence of their effects on SHP.

Each of the three highest affinity binding sites is essential for SF-1 response in Y1 adrenocortical cell lines, since mutation of any one of them abolishes SF-1 transactivation. This finding contrasts with results with some other promoters, in which individual elements are sufficient to confer response to various receptors. However, it is also consistent with results with other promoters found to have multiple SF-1 sites, such as those directing expression of steroid 21-hydroxylase (24) and steroidogenic acute regulatory protein (25). This need for multiple sites suggests that SF-1 may be particularly dependent on cooperative or synergistic interactions for effective function.

Thus, in promoters that apparently contain single functional SF-1 binding sites, SF-1 may be synergizing with other transcription factors. Recent reports of cooperative function of SF-1 and NGFI-A in the luteinizing hormone β subunit promoter (26) and SF-1 and Sp1 in the bovine CYP11A promoter (27) provide apparent examples of such synergy.

The observation that SHP is a general inhibitor of nuclear receptor transactivation raises the possibility that it may act in negative feedback loops to inhibit its own expression. This would contrast with findings with several other nuclear receptor superfamily members, including SF-1 (28) and all three retinoic acid receptor genes (29–32), which function as positive regulators of their own expression. Surprisingly, preliminary results indicate that SHP stimulates SF-1 transactivation, but inhibits FTF transactivation, suggesting that it can affect its own promoter both negatively and positively. Further studies will be necessary to define the molecular basis of these apparently disparate effects of SHP.

Acknowledgments—We thank Dr. Jaemog Soh and Hye-Kyung Lee for SHP genomic clones and Dr. Luc Belanger for FTF.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) Cell 83, 835–839
2. Mangelsdorf, D. J., and Evans, R. M. (1995) Cell 83, 841–850
3. Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., and Gromemeyer, H. (1996) Nat. Struct. Biol. 3, 87–94
4. Zanaria, E., Muscatelli, F., Bardoni, B., Strem, T. M., Guioli, S., Guo, W., Lalli, E., Moser, C., Walker, A. P., McCabe, E. R. B., Meitinger, T., Monaco, A. P., Sassone-Corsi, P., and Camerino, G. (1994) Nature 372, 635–641
5. Ita, M., Yu, R., and Jameson, J. L. (1997) Mol. Cell. Biol. 17, 1476–1485
6. Nachitigal, M. W., Hirokawa, Y., Eoyeart-VanHouten, D. L., Panagana, J. N., Hammer, G. D., and Ingraham, H. A. (1998) Cell 93, 445–454
7. Lala, D. S., Rice, D. A., and Parker, K. L. (1992) Mol. Endocrinol. 6, 1249–1258
8. Luo, X., Ikeda, Y., and Parker, K. L. (1994) Cell 77, 481–490
9. Seol, W., Choi, H. S., and Moore, D. D. (1996) Science 272, 1336–1339
10. Seol, W., Chung, M., and Moore, D. D. (1997) Mol. Cell. Biol. 17, 7126–7131
11. Seol, W., Hanstein, B., Brown, M., and Moore, D. D. (1998) Mol. Endocrinol. 12, 1551–1557
12. Johansson, L., Thomsen, J. S., Damdimopoulos, A. E., Spyrou, G., Gustafsson, J., and Treuter, E. (1999) J. Biol. Chem. 274, 345–353
13. Noda, N., Yasumo, H., Tanura, T., Hashiguchi, N., Farasawa, T., Tsukamoto, T., Sadano, H., and Osumi, T. (1997) Biochem. Biophys. Acta 1350, 27–32
14. Carter, M. E., Galick, T., Moore, D. D., and Kelly, D. P. (1994) Mol. Cell. Biol. 14, 4860–4873
15. Lee, H.-K., Lee, Y.-K., Park, S.-H., Kim, Y.-S., Park, S. H., Lee, J. W., Kwon, H.-B., Soh, J., Moore, D. D., and Choi, H.-S. (1996) J. Biol. Chem. 273, 14398–14402

2 W. Seol, Y.-K. Lee, D. D. Moore, and M. Brown, manuscript in preparation.
3 Y.-K. Lee, unpublished data.
16. Ikeda, Y., Lala, D. S., Luo, X., Kim, E., Moisan, M. P., and Parker, K. L. (1993) Mol. Endocrinol. 7, 852–860
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1998) Current Protocols in Molecular Biology, pp. 9.1.4–9.1.11, J. Wiley & Sons, New York
18. Selden, R. F., Howie, K. B., Rowe, M. E., Goodman, H. M., and Moore, D. D. (1986) Mol. Cell. Biol. 6, 3173–3179
19. Baes, M., Gullick, T., Choi, H.-S., Martinoli, M. G., Simha, D., and Moore, D. D. (1994) Mol. Cell. Biol. 14, 1544–1552
20. Keri, R. A., and Nilson, J. H. (1996) J. Biol. Chem. 271, 10782–10785
21. Shen, W. H., Moore, C. C., Ikeda, Y., Parker, K. L., and Ingraham, H. A. (1994) Cell 77, 651–661
22. Ikeda, Y., Swain, A., Weber, T. J., Hentges, K. E., Zanaria, E., Lalli, E., Tamai, K. T., Sassone-Corsi, P., Lovell-Badge, R., Camerino, G., and Parker, K. L. (1996) Mol. Endocrinol. 10, 1261–1272
23. Galanin, L., Pare, J. F., Allard, D., Hamel, D., Levesque, L., Tugwood, J. D., Green, S., and Belanger, L. (1996) Mol. Cell. Biol. 16, 3853–3865
24. Rice, D. A., Kronenberg, M. S., Mouw, A. R., Aitken, L. D., Franklin, A., Schimmer, B. P., and Parker, K. L. (1990) J. Biol. Chem. 265, 8052–8058
25. Sugawara, T., Kiriakidou, M., McAllister, J. M., Kallen, C. B., and Strauss, J. F., III (1997) Biochemistry 36, 7249–7255
26. Lee, S. L., Sadovsky, Y., Swirnoff, A. H., Polish, J. A., Goda, P., Gavriliia, G., and Milbrandt, J. (1996) Science 273, 1219–1221
27. Liu, Z., and Simpson, E. R. (1997) Mol. Endocrinol. 11, 127–137
28. Nomura, M., Nawata, H., and Murahashi, K. (1996) J. Biol. Chem. 271, 8243–8249
29. de The, H., Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H., and Dejean, A. (1990) Nature 343, 177–180
30. Sucov, H. M., Murakami, K. K., and Evans, R. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5392–5396
31. Leroy, P., Nakashatri, H., and Chambon, P. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10138–10142
32. Lehmann, J., Zhang, X.-K., and Pfahl, M. (1992) Mol. Cell. Biol. 12, 2976–2985