NOR-1/NR4A3 is an “orphan member” of the nuclear hormone receptor superfamily. NOR-1 and its close relatives Nur1 and Nur77 are members of the NR4A subgroup of nuclear receptors. Members of the NR4A subgroup are induced through multiple signal transduction pathways. They have been implicated in cell proliferation, differentiation, T-cell apoptosis, chondrosarcomas, neurological disorders, inflammation, and atherogenesis. However, the mechanism of transcriptional activation, coactivator recruitment, and agonist-mediated activation remain obscure. Hence, we examined the molecular basis of NOR-1-mediated activation. We observed that NOR-1 trans-activates gene expression in a cell- and target-specific manner; moreover, it operates in an activation function (AF)-1-dependent manner. The N-terminal AF-1 domain delimited to between amino acids 1 and 112, preferentially recruits the steroid receptor coactivator (SRC). Furthermore, SRC-2 modulates the activity of the AF-1 domain but not the C-terminal ligand binding domain (LBD). Homology modeling indicated that the NOR-1 LBD was substantially different from that of hRORα, a closely related AF-2-dependent receptor. In particular, the hydrophobic cleft characteristic of nuclear receptors was replaced with a very hydrophilic surface with a distinct topology. This observation may account for the inability of this nuclear receptor LBD to efficiently mediate co-factor recruitment and transcriptional activation. In contrast, the N-terminal AF-1 is necessary for co-factor recruitment and can independently consist of coactivators. Finally, we demonstrate that the purine anti-metabolite 6-mercaptopurine, a widely used antineoplastic and anti-inflammatory drug, activates NOR-1 in an AF-1-dependent manner. Additional 6-mercaptopurine analogs all efficiently activated NOR-1, suggesting that the signaling pathways that modulate proliferation via inhibition of de novo purine and/or nucleic acid biosynthesis are involved in the regulation of NR4A activity. We hypothesize that the NR4A subgroup mediates the genotoxic stress response and suggest that this subgroup may function as sensors that respond to genotoxicity.

Nuclear hormone receptors (NRs) function as ligand-activated transcription factors that regulate gene expression involved in reproduction, development, and general metabolism (1). NRs function as the pipeline between physiology and gene expression. The importance of NRs in human physiology is underscored by the extensive range of therapeutics that has been created to combat disorders associated with dysfunctional hormone signaling. These diseases affect every discipline of medicine (2). All members of the NR superfamily display a highly conserved structural organization (1) with an amino-terminal region AB (that encodes activation function 1 (AF-1)), followed by the C-region that encodes the DNA binding domain (DBD), a linker region D, and the C-terminal E region. The DE region encodes the ligand binding domain (LBD) and a transcriptional domain, denoted as activation function 2 (AF-2) (1, 2). A decade ago, gene products were identified that appeared to belong to the nuclear receptor superfamily on the basis of their nucleic acid sequence identity. The endogenous signaling molecules that bound to these proteins were unknown; thus, the term “orphan receptor” was adopted. Based on members of the nuclear receptor superfamily that have been more fully characterized, the orphans forecast an enormous yet unexploited opportunity for the discovery of important new therapeutic agents.

The Nur77, NURR1, and NOR-1 (NR4A1–3 subgroup) family of orphan NRs are well conserved in the DNA binding domain (~91–95%) and the C-terminal LBD (~60%) but divergent in the N-terminal AB region (e.g. AF-1).

The NR4A family members can bind as monomers and homodimers to single/tandem copies of the nerve growth factor-inducible response element (NBRE), AAAGTCA, a variant NR half-site, and constitutively regulate transcription. In addition to binding DNA as monomers, Nur77 and Nur1 (but not NOR-1) heterodimerize with RXR and mediate efficient transcription in response to RXR-specific agonists from DR5 motifs. Moreover, a Nur response element has been found in the promoter region of the pro-opiomelanocortin gene (POMC) that is efficiently bound and transactivated by Nur and NOR-1 homodimers (3–8).
This subgroup of proteins functions as immediate early/stress response genes that are induced by a wide range of physiological signals (9–13). They have been implicated in proliferation, differentiation, apoptosis, hypertrophy/remodeling, Parkinson’s disease, schizophrenia, manic depression, atherosclerosis, cancer, and autoimmune disease (14–34). In itself, the NR4A1–3 subgroup presents an exciting scientific challenge; unlocking the molecular mechanisms that mediate NR4A-dependent transcription provides the platform for likely pharmaceutical and therapeutic exploitation.

NOR-1, Nur77, and NURR1 have been shown to play a key role in regulating expression of various genes in the hypothalamic-pituitary-adrenal axis (35). Corticotropin-releasing hormone treatment of adrenal and pituitary cells induces Nur77 and NOR-1. These events lead to the activation of the gene encoding steroid 12α-hydroxylase and the 20α-hydroxysteroid dehydrogenase promoters (36–38). Therefore, the NR4A subgroup plays a role in steroidogenesis. Among other genes that members of the NR4A subfamily regulate in the hypothalamic-pituitary-adrenal axis are the pro-opiomelanocortin (POMC) gene, which is the precursor to adrenocorticosteroid dehydrogenase promoters (36-38). Furthermore, the AF-1 domain was required for NR1-dependent transactivation of target genes. In accordance with the role of NOR-1 in inflammation and carcinogenesis, NOR-1 is activated by the anti-cancer and anti-inflammatory drug 6-mercaptopurine. Moreover, this thiouarine agent specifically modulates the activity of the AF-1 domain.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transient Transfection**—Proliferating C2C12 cells and COS-1 cells were kept in DMEM supplemented with 20 and 10% fetal calf serum, respectively, in 6% CO2. Cells grown in 12-well dishes to 60–70% confluence were transiently transfected with 0.33–1 μg of pGL2b-tk-LUC (50) or pNBR3-tk-LUC (6–8) or pOMC-TK-LUC (NurRE5-tk-LUC) (51) reporter plasmid together with 0.33–0.5 μg of pS655-NOR1-FL or pS655-NOR1-CDE or pS655-NOR1-ABC or pS65 alone using a DOTAP/DOSPER (Roche Applied Science and Scientific) liposome mixture in HEB (42 ng HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM NaH2PO4, 11 mM dextrose, pH 7.1) (total DNA 2–2.5 μg/well). The DNA/DOTAP/DOSPER mixture was added to the cells in 1 ml of fresh DMEM containing 10% fetal calf serum and incubated for 16 h. Medium was replaced with 0.53–1 μg of pG5E1b-LUC or pOMC-TK-LUC reporter plasmid together with 0.33–0.16 μg of pS655-NOR1-FL or pS655-NOR1-CDE, or pS655-NOR1-ABC or pCMX-Nur77 or pCMX-NURR1 (7) or pS65 or pCMX alone in the presence or absence of 6-mercaptopurine or 6-mercaptopurine riboside or 6-mercaptopurine-2’-deoxyriboside or 6-mercaptopurine monophosphate or 6-mercaptopurine-9-β-D-ribofuransoide (50–100 μM Sigma) were performed with phenol red-free DMEM containing 5% charcoal-stripped fetal calf serum. The DNA/DOTAP/DOSPER mixture was added to the cells in 1 ml of fresh DMEM containing 5% charcoal-stripped fetal calf serum and incubated for 14 h. Medium was replaced 16–24 h later, and/or 6-mercaptopurine, 6-mercaptopurine riboside, 6-mercaptopurine-2’-deoxyriboside, 6-mercaptopurine monophosphate, or 6-mercaptopurine-9-β-D-ribofuransoide was added, and cells were grown for a further 24–48 h.

Cells were harvested after 48 h and were assayed for luciferase activity. Each experiment represented at least two sets of independent triplicates to overcome the variability inherent in transfection experiments (52–56).

**Luciferase Assays**—Luciferase activity was assayed using a LucLite kit (Packard) according to the manufacturer’s instructions. Briefly, cells were washed once in PBS and resuspended in 150 μl of phenol red-free DMEM and 150 μl of LucLite substrate buffer. Cell lysates were transferred to a 96-well plate, and relative luciferase units were measured for 5 s in a Wallac Trilux 1450 microplate luminometer (52–56).

**GAL Hybrid Assays**—The COS-1 and C2C12 cells were passaged into 12-well plates and transfected at 50–80% confluence with 0.33–1 μg of reporter, G5E1b-LUC, and 0.33–0.16 μg of GALADBD or GAL-NOR-1 chimeric constructs (GAL-NOR-1-FL, AB, DE, aa 1–150, aa 40–160, aa 200–292, aa 1–60, aa 1–112, and the GAL-chimeric constructs containing mutations in the AB region) and/or were co-transfected in the presence and absence of pG5SRC-2 (0.53–0.1 μg) using a DOPAT/DOSPER liposome mixture in HEBs per well. Transfections were performed in DMEM containing 10% fetal calf serum. Phenol red-free DMEM containing 5% charcoal-stripped fetal calf serum was used for transfections performed with NOR-1 GAL-chimeric constructs and GAL4VPl6, GAL-MEF2C, GAL-MEF2D, GAL-MyoD, GAL-Myogenin, GAL-SRC2, and GAL-p38α constructs (50–52). These treatments were co-transfected in the presence and absence of 6-mercaptopurine, 6-mercaptopurine riboside, 6-mercaptopurine-2’-deoxyriboside, 6-mercaptopurine monophosphate, or 6-mercaptopurine-9-β-D-ribofuransoide (50–100 μM). After 16–24 h, the medium was replaced, and/or 6-MP derivatives were added to cells. Cells were harvested 48 h after transfection for the assay of luciferase activity. Each experiment represented at least two sets of independent triplicates to overcome the variability inherent in transfection experiments (52–56).

**Plasmids and Primer Sequences**—The expression plasmids GALO (57), pSG5 (Stratagene), pCMX (58), pCMX-Nur77, and pCMX-NURR1 (7) and the reporter plasmids pNBR3-tk-LUC (6–8), pOMC-tk-LUC (NurRE5-tk-LUC) (51), and G5E1b-LUC (50) have been described elsewhere. Generation of full-length mouse NOR-1 was performed by reverse transcriptase-PCR from muscle RNA with Primer 1 DNA polymerase (Promega), using the manufacturer’s buffer. All PCR products were cloned into the EcoRI site of pSG5 and then isolated after EcoRI digestion and subsequently cloned into pGALo and pGEX4T1. The primers

**AF-1 Domain of NOR-1/NR4A3**
used for the synthesis of full-length mouse NOR-1 were GMUQ-720 5' (GCC GAA TCT ACC ATG CCC TGG GTG CAA GCC CAG) and GMUQ-722 3' (GCC GAA TTT ACC ATG CCC TGG GTG CAA GCC CAG). We then synthesized additional primers to subclone the DBE, ABC, and CDE regions into pS5 and GALO. The primers used for the DBE were GMUQ-724 5' (GCC GAA TTC ACC ATG CTT ATG AAT GAA GAT GTG CGT) and GMUQ-722 3'. The primers used to synthesize the AB region were GMUQ-720 5' and GMUQ-726 3' (GCC GAA TCT ACA GCC TTC CCA AGA TGA TGA). The primers used to synthesize the ABC region were GMUQ-720 5' and GMUQ-733 3' (GCC GAA TTC TCA CCC GAC ACT GAG ACA CTT CTG). The primers used to synthesize the CDE region were GMUQ-732 5' (GCC GAA TTC ACC ATG TTC GCC GTG TGC GGC G) and GMUQ-722 3'.

We constructed various subdomains of the AB region by PCR and cloned these segments into the EcoRI/SalI site of GAL4. The NOR-1-AB-aa1–150 was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-720 5' and GMUQ-756 3' (GCC GTC GAC TCA GGG GAG GCC TGG AGT GG). The NOR-1-AB-aa1–160 was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-757 5' (GCC GTC GAC TCA GGG GAG GCC TGG AGT GG) and GMUQ-758 3' (GCC GTC GAC TCA CTC GTC GTC CCA CAG CG). The NOR-1-AB-aa150–292 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-757 5' and GMUQ-710 3' (GCC GTC GAC TCA CTC GTC GTC CCA CAG CG). The NOR-1-AB-aa150–292 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-720 5' and GMUQ-760 3' (GCC GTC GAC TCA GGG GAG GCC TGG AGT GG). The NOR-1-AB-aa1–112 region was subjected to PCR and cloned into SV40-GAL0 using primers GMUQ-720 5' and GMUQ-761 3' (GCC GTC GAC TCA ATG ATG GTG GTG GTG G) to transactivate the reporter backbone plasmid in either cell type (Fig. 1A and B). However, when C2C12 muscle cells and COS-1 cells were cotransfected with the NOR-1 expression vector and the reporter plasmid NBRE-3-tk-LUC, we observed muscle-specific NOR-1-dependent transactivation of the NBRE-3 reporter (Fig. 1, C and D). NOR-1 also efficiently trans-activated the NOR-1-5POMC reporter in muscle cells (Fig. 1E). In contrast to the situation with the NBRE-3 reporter, NOR-1 very efficiently and preferentially activated the NurRE-5POMC reporter in COS-1 cells (Fig. 1F). In summary, the data suggest NOR-1 trans-activated gene expression in a cell- and response element (target)-specific manner.

Moreover, we investigated and compared the ability of NOR-1 lacking the DBE domain, which encodes the LBD (e.g., NOR-1-ABC construct encodes aa 1–379), and NOR-1 lacking the AB region, which encodes the AF-1 domain (e.g., NOR-1-CDE construct contains aa 293–627), to transactivate the NBRE and NurRE-response elements in muscle and nonmuscle COS-1 cells (Fig. 1, C–F). Cell specificity has been found to play an important role in the activity of the AF-1 and AF-2 domains in the estrogen, glucocorticoid, progesterone receptors, and RARs (66, 67). The ability of different activation functions 1 and 2 to operate has been found to vary in relation to the cell line used and the spatiotemporal expression pattern of the specific receptor, suggesting that cell-specific activation mechanisms are involved in the functioning of the different AFs.

Surprisingly, deletion of the AB region that encodes the AF-1 domain (SG5-NOR-1-CDE) completely ablated the ability of NOR-1 to trans-activate the NBRE-3 and NurRE-5POMC reporters in myogenic and COS-1 cells (Fig. 1, C–F). Hence, we investigated whether the AB region could independently activate gene expression and/or operate in a cell-specific manner.
C2C12 myogenic cells and COS-1 cells were co-transfected with the SG5-NOR-1-ABC, expression vector, and pNBRE-tk-LUC and NurRE-5POMC reporters. The NOR-1-ABC expression vector efficiently trans-activated the NBRE reporter in muscle cells (Fig. 1C); however, its ability to trans-activate the NurRE-5POMC reporter in muscle and nonmuscle cells was compromised (Fig. 1, E and F). These experiments indicated that the AB region that encoded the AF-1 domain was necessary for optimal NOR-1-dependent transactivation of NBRE/NurRE-5POMC-dependent reporters. Second, the LBD was necessary for NOR-1-dependent transactivation of the NurRE-5POMC, but not the NBRE-3 reporter (Fig. 1, C, E, and F). Moreover, these experiments suggest that the C-terminal LBD (DE) region of NOR-1, unlike other orphan and classical nuclear receptors, is not sufficient to mediate trans-activation of gene expression, in contrast to the AF-1 domain, which can function in an independent manner. These experiments demonstrate that NOR-1 functions in a cell- and target-specific manner.

The N-terminal AB Region of NOR-1 Encodes an Efficient AF-1 Domain Located between Amino Acid Positions 1 and

C2C12: NBRE

A

pGL2b-TK-LUC
SG5
SG5-NOR-1-FL
SG5-NOR-1-ABC
SG5-NOR-1-CDE
C2C12: NBRE

B

pGL2b-TK-LUC
SG5
SG5-NOR-1-FL
SG5-NOR-1-ABC
SG5-NOR-1-CDE

C

NBRE-3-TK-LUC
SG5
SG5-NOR-1-FL
SG5-NOR-1-ABC
SG5-NOR-1-CDE

D

NBRE-3-TK-LUC
SG5
SG5-NOR-1-FL
SG5-NOR-1-ABC
SG5-NOR-1-CDE

E

POMC-TK-LUC
SG5
SG5-NOR-1-FL
SG5-NOR-1-ABC
SG5-NOR-1-CDE

F

POMC-TK-LUC
SG5
SG5-NOR-1-FL
SG5-NOR-1-ABC
SG5-NOR-1-ABC

Fig. 1. NOR-1 trans-activates gene expression in a cell- and response element-specific manner. A–F, SG5-NOR-1-FL, SG5-NOR-1-CDE, and SG5-NOR-1-ABC (0.33 μg) were co-transfected into C2C12 proliferating myoblasts and COS-1 cells together with the reporter gene 1 μg of pGL2b-tk-Luc (A and B), NBRE-3-tk-Luc (C and D), or NurRE-5-tk-Luc (E and F), respectively. The AB (AF-1) region of NOR-1 is necessary for agonist-independent and NOR-1-mediated transactivation of gene expression. AF-1 functions in a cell- and target-specific manner. C–F, SG5-NOR-1-FL, SG5-NOR-1-CDE, and SG5-NOR-1-ABC (0.33 μg) were co-transfected into C2C12 proliferating myoblasts and COS-1 cells together with the reporter gene 1 μg of NBRE-3-tk-Luc (C and D) or NurRE-5-tk-Luc (E and F). -Fold activation is expressed relative to luciferase activity obtained after cotransfection of the pSG5 alone, arbitrarily set at 1. The mean luciferase -fold activation values and S.D. (bars) were derived from a minimum of two or three independent triplicate experiments.
This indicated that the AB region of NOR-1 contained an agonist-independent AF-1 domain. The GAL-NOR-1-DE, which encodes the LBD of NOR-1 with the AB and C regions deleted, did not activate transcription (Fig. 2, B and C), which was consistent with the reporter analysis. This suggested that the LBD lacked an intrinsic and classical transcriptional domain.

The AB region is composed of 292 amino acids, which encode the N-terminal AF-1 domain. In order to further characterize the AF-1 region of NOR-1, we have constructed subregions of the AB domain by PCR and cloned these segments into the pGAL4-DBD (Fig. 3A). These constructs were transfected into C2C12 and COS-1 cells and assayed in the GAL4 hybrid system (Fig. 3, B and C). The constructs GAL-NOR-1-aa-1–150, GAL-NOR-1-aa40–160, and GAL-NOR-1-aa150–292 were created and assayed by transfection. These segments of the AB region of NOR-1 increased activation 20-, 9-, and 7-fold relative to the control, GAL-DBD alone, in myogenic cells (Fig. 3B). A similar trend was observed in COS-1 cells (Fig. 3C), although the level of activation was weaker. This suggested that the AF-1 domain was located between aa positions 1 and 150 in the AB region. The plasmid, GAL-NOR-1-aa150–292, did not trans-activate gene expression efficiently in this assay system (4–7-fold in nonmuscle and myogenic cells) (Fig. 3, B and C).

We subsequently constructed GAL-NOR-1-aa-1–60 and GAL-NOR-1-aa1–112 (Fig. 3A) and assayed these plasmids by transfection analysis. These segments of the AB region of NOR-1 increased activation 15- and 42-fold relative to the GAL4-DBD alone in myogenic cells (Fig. 3B). This delimited the functional AF-1 domain to between aa positions 1 and 112 in the AB region and showed that the region downstream of aa position 150 was not necessary for the efficient activity of the AF-1 domain.

**The Steroid Receptor Coactivators SRC-1, -2, and -3 Directly Interact with NOR-1: The AF-1 Domain Recruits SRC Directly**—The N-terminal AF-1 region and the C-terminal LBD (containing the imbedded AF-2 domain) of the NOR-1/NR4A3 subgroup are very unusual and have not been demonstrated to directly interact with coactivators. However, we have demonstrated that the steroid receptor coactivator, SRC, binds and modulates the activity of the Nur77/NR4A1 AF-1 domain (68).

Consequently, we examined the ability of the SRCs (SRC-1, -2, and -3) to directly interact with NOR-1. We tested this hypothesis using a biochemical approach, the in vitro GST pull-down assay. Glutathione-agarose-immobilized GST-NOR-1, GST-NOR-1-AB, and GST-NOR-1-DE were tested for direct interaction with in vitro 35S-radiolabeled full-length SRC-1, -2, and -3 (Fig. 4A). We observed that SRC-1, -2, and -3 efficiently interacted with NOR-1. Interestingly, the SRCs directly interacted with the AB region of NOR-1 that encoded the AF-1 domain, concordant with the observations with the other NR4A member, Nur77 (68). In contrast to other nuclear hormone receptors, the DE region that encodes the LBD did not efficiently interact with the steroid receptor coactivators (Fig. 4A). This is consistent with the NBRE reporter assays and the GAL4 hybrid analysis (Figs. 1B and 2, B and C).

**The NOR-1 LBD Coactivator Binding Cleft Is Unusually Hydrophobic: Coactivator-derived Peptides Bind Poorly in Docking Simulations**—We used molecular modeling as a tool to further investigate the inability of the atypical nuclear receptor LBD to interact with coactivators. The starting point for homology modeling was the crystal structure of the human RARγ receptor (containing the imbedded AF-2 domain) of the NOR-1/NR4A3 subgroup. We observed that SRC-1, -2, and -3 directly interacted with NOR-1 that encoded the AF-1 domain, concordant with the observations with the other NR4A member, Nur77 (68). In contrast to other nuclear hormone receptors, the DE region that encodes the LBD did not efficiently interact with the steroid receptor coactivators (Fig. 4A). This is consistent with the NBRE reporter assays and the GAL4 hybrid analysis (Figs. 1B and 2, B and C).

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Fig. 3. The activation domain within the AB region is located between amino acid positions 1 and 112. A, diagrammatic representation of Gal-NOR-1 chimeric constructs. B and C, GAL-NOR-1-AB, GAL-NOR-1-aa1–150, GAL-NOR-1-aa40–160, GALNOR-1-aa150–292, GAL-NOR-1-aa1–60, and GAL-NOR-1-aa1–112 chimeras (0.33 μg) were cotransfected with the GAL4-dependent reporter G5E1b-Luc (1 μg) into C2C12 proliferating myoblasts (B) and COS-1 cells (C). Fold activation is expressed relative to luciferase activity obtained after cotransfection of the Gal4 DNA-binding domain alone, arbitrarily set at 1. The mean luciferase -fold activation values and S.D. values (bars) were derived from a minimum of two or three independent triplicate experiments.

and further quality checking with the WhatIf suite of programs. Hydrophobicity analysis was performed using the molecular modeling program SCULPT (61). An LXXLL-containing peptide from a previously published thyroid hormone receptor/steroid receptor coactivator peptide complex was superimposed onto the NOR-1 LBD, enabling us to delineate a hypothetical coactivator binding interface.

Fig. 4 shows comparative surface views of rRORγ (a related nuclear receptor but one that is distinct from the template structure) (B) and the modeled NOR-1 (C). The rRORγ surface is derived from a crystallographic structure of the receptor in complex with a coactivator peptide (69). Examination of the molecular surface in the region of the hypothetical coactivator interfaces shows startling differences in hydrophobicity; rRORγ (B) shows the characteristic hydrophobic groove (blue) found in AF-2-activating receptors, whereas NOR-1 (C) possesses an unusually hydrophilic surface (red shade). Additionally, there are marked differences in local surface topography as shown in the close up views (B and C). Docking using molecular mechanic simulation of a superimposed SRC coactivator peptide from an existing structure suggests that NOR-1 can bind a coactivator peptide. However, this procedure results in noticeable distortion of the peptide. Peptide distortion together with differences in the coactivator-binding surface of NOR-1 prompted us to perform comparative simulations of the ability of NOR-1 and RARγ to bind coactivator peptide *de novo* (i.e. without reference to existing coactivator peptide complexes). Simulations were based on iterative docking and fractional rotation, so that $6.3 \times 10^8$ rotational combinations between coactivator peptide and receptor were sampled with ranking of the resulting solutions according to docking energy. When we performed this simulation using the LBD of RARγ, we found that 6 of the 10 highest scoring solutions docked to within 5 Å root mean square of a coactivator/receptor model based on superposition of the existing thyroid hormone receptor/GRIP-1 (SRC-2) peptide crystal structure. In contrast, the best 50 solutions for NOR-1 did not approach to within 5 Å of the equivalent position (data not shown). However, higher energy docking solutions did cluster in the area delineated by the earlier superimposed coactivator peptide. This is most likely due to the differences in hydrophobicity and topology between the two interfaces. Given the dominance of the SRC-2/GRIP-1 type LXXLL coactivator peptide in receptor interaction, this observation may account for the inability of this nuclear receptor LBD to efficiently mediate SRC recruitment in the GST pull-down assay.

The Steroid Receptor Coactivator, SRC-2, Stimulates the Activity of the NOR-1 AF-1 Domain—The process of cofactor recruitment and the interaction of coactivators with NOR-1/NR4A3 has remained obscure. Since NOR-1 can activate transcription directly, and we had demonstrated that the SRCs interact directly with the NOR-1 AF-1, we examined the effect of SRC-2/GRIP-1 expression on NOR-1-mediated transactivation in the GAL4 hybrid system. In these assays, the activity of NOR-1 is independent of its binding to its cognate binding motifs, the NBRE-3 and NurRE-5POMC response elements. If SRC-2 regulates the transcriptional activity of NOR-1, then the potential of the GAL4-NOR-1 fusions to trans-activate gene expression should be increased in this assay (Fig. 5A). Cells were cotransfected with GAL-NOR-1, GAL-NOR-1-AB, GAL-NOR-1-DE, and the G5E1b-LUC reporter in the presence and absence of SRC-2. We observed that GAL-NOR-1-AB, GAL-NOR-1-AB, and GAL-NOR-1-AB induced transcription relative to the GAL4 DBD; this level of activity was significantly stimulated by the addition of SRC-2 by ∼4-fold. SRC-2 did not modulate or increase the activity of the C-terminal LBD (Fig. 5B).

To delimit the region within the AF-1 domain that is modulated by SRC-2, we transfected cells with GAL-NOR-1-aa1–150, GAL-NOR-1-aa40–160, GAL-NOR-1-aa150–292, and further quality checking with the WhatIf suite of programs. Hydrophobicity analysis was performed using the molecular modeling program SCULPT (61). An LXXLL-containing peptide from a previously published thyroid hormone receptor/steroid receptor coactivator peptide complex was superimposed onto the NOR-1 LBD, enabling us to delineate a hypothetical coactivator binding interface.
1-aa1–150, and GAL-NOR-1-aa40–160 were efficiently stimulated ~7-fold by co-expression of SRC-2. However, GAL-NOR-1-aa150–292 was not stimulated by SRC-2. This suggested that SRC-2 modulates the activity of the N-terminal AF-1 domain in NOR-1, and this coactivation is dependent on the region between aa positions 40 and 160 in the AB region (Fig. 5C). Additionally, we examined the ability of SRC-2 to stimulate GAL-NOR-1-aa1–60 and GAL-NOR-1-aa1–112 in an effort to further delimit the SRC-2-responsive domain (Fig. 5D). Although we observed that these chimeras were modulated by SRC-2, the most efficient modulation by SRC-2 was mediated by the domain between aa positions 40 and 160.

The Nuclear Hormone Receptor Cofactors SRC, p300, PCAF, and DRIP205/TRAP220 Directly Interact with NOR-1: The AF-1 Domain Can Recruit a Co-activator Complex—Our studies had demonstrated the direct binding of SRCs to NOR-1 and the modulation of AF-1 activity by SRC-2. Activation of gene expression by the classical nuclear hormone receptors is dependent on the recruitment of SRC-1, -2, and -3. These SRCs recruit p300/CREB-binding protein and PCAF to synergistically activate transcription. SRCs, CREB-binding protein/p300, and PCAF possess intrinsic histone acetyltransferase activity and act in concert to remodel the chromatin. This complex results in the assembly of a higher order structure that includes the “DRIP-TRAP-ARC” protein complex that regulates localized nucleosome structure (as reviewed in Ref. 70). The key member of this complex is DRIP205-TRAP220.

To further investigate cofactor recruitment, we examined the
ability of p300, DRIP205, and PCAF to interact with NOR-1. Glutathione-agarose-immobilized GST-NOR-1, GST-NOR-1-AB, and GST-NOR-1-DE were tested for direct interaction with in vitro $^{35}$S-radiolabeled full-length p300, DRIP205, and PCAF. We observed that p300, DRIP205, and PCAF very efficiently interacted with native NOR-1 (Fig. 6A, lanes 7–9). In contrast, the discrete N- and C-terminal regions of NOR-1 that encoded the NOR-1-AB and NOR-1-DE domains, respectively, supported weak but significant recruitment of p300. The specificity of the weak p300 interaction was demonstrated by the lack of DRIP205 binding to the N-terminal AB region (Fig. 6A, lane 11). However, the C-terminal DE region (i.e. the LBD) of NOR-1 could support the efficient recruitment of DRIP205 (Fig. 6A, lane 14). PCAF interacted efficiently with native NOR-1 and the individual NOR-1-AB and NOR-1-DE regions (Fig. 6A, lanes 9, 12, and 15). These data and the previous binding data with SRC-2 demonstrate that the N-terminal AB region is necessary for cofactor recruitment.

As described, SRC-2 independently and efficiently interacted with the AB region of NOR-1; furthermore, it stimulated the activity of the AF-1 domain (see Figs. 4 and 5). Hence, we investigated the relative recruitment of p300 and SRC-2 to the AF-1 domain. Furthermore, we investigated whether the AF-1 domain encoded by the AB region mediated DRIP205 recruitment in the presence of p300 and SRC-2. We observed that SRC-2 bound more efficiently than p300 to GST-NOR-1-AB (Fig. 6B, lane 5 versus lane 7). Moreover, in the presence of SRC-2 and p300, the N-terminal region could recruit DRIP205 (Fig. 6B, lane 8 versus lane 6). The specificity of the AF-1-mediated DRIP205 recruitment was verified by the lack of binding in the absence of the primary coactivators, SRC-2 and p300 (Fig. 6B, lane 6). Neither SRC-2 nor p300 independently mediated the recruitment of DRIP205 to the N-terminal AB region (Fig. 6C, lanes 9 and 10, relative to the GST control in lane 4). These studies suggested that the AF-1 domain of NOR-1 supports the recruitment of DRIP205 in the presence of the primary cofactors, SRC-2 and p300.

We extended these studies to compare the relative ability of native NOR-1 and the discrete AB and DE regions to recruit and interact with the coactivator complex. We observed that GST-NOR-1 and GST-NOR-1-AB could simultaneously and efficiently pull down SRC, DRIP205, p300, and PCAF (Fig. 6C, D).
The AF-1 Domain of NOR-1/NR4A3

AF-1 Domain of NOR-1 Mediated Activation by 6-Mercaptopurine—To identify the domain of NOR-1 that mediated 6-MP activation, we utilized our series of GAL-NOR-1 deletions. We investigated the ability of 6-MP to modulate the activity of GAL-NOR-1, GAL-NOR-1-AB, and GAL-NOR-1-DE. These experiments clearly demonstrated that 6-MP potently modulated the activity of NOR-1. Moreover, the data clearly demonstrated that the N-terminal AB region, which encodes the potent AF-1 domain and the C-terminal LBD region of NOR-1.

The Antileukemic Hypoxanthine Analog 6-Mercaptopurine Regulates NR4A1-3-mediated Trans-activation—In 2002, Heyman (87) presented data demonstrating that the purine anti-metabolite 6-mercaptopurine (and some related compounds) activated the trans-activation of the NurRE_{POMC} reporter by NR4A2/Nurr1 in CV-1 cells. We examined the ability of 6-MP to activate Nur1-, NOR-1-, and Nur77-mediated trans-activation of the NurRE_{POMC} reporter in myogenic cells. We cotransfected the NurRE_{POMC} reporter with expression vectors encoding the three members of the NR4A family into mouse muscle cells cultured in phenol red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum. We observed that 6-MP induced the Nur1-, NOR-1-, and Nur77-mediated trans-activation. Furthermore, the 6-MP-activated NOR-1-mediated trans-activation was compromised by the deletion of either the C-terminal LBD or N-terminal AB region (Fig. 8B). This suggested that the purine anti-metabolite activated all members of the NR4A subgroup.

FIG. 6. The nuclear hormone receptor cofactors SRC, p300, PCAF, and DRIP205-TRAP220 directly interact with NOR-1: The AF-1 domain can recruit a co-activator complex. A. Glutathione-agarose-immobilized GST (lanes 4–6), GST-NOR-1 (lanes 7–9), GST-NOR-1-AB (lanes 10–12), and GST-NOR-1-AB (lanes 13–15) were incubated independently with in vitro 35S-radiolabeled full-length p300, DRIP205, and PCAF, respectively. The input lanes (lanes 1–3) represent ~10% of the total protein. B. GST (lane 4) and GST-NOR-1-AB were incubated independently (lanes 5–7) or simultaneously (lane 8) with in vitro 35S-radiolabeled full-length p300, DRIP205, and SRC-2. The input lanes (lanes 1–3) represent ~10% of the total protein. C. GST (lane 4) and GST-NOR-1-AB were incubated independently (lanes 5–7) or simultaneously (lanes 8–10) with in vitro 35S-radiolabeled full-length p300, DRIP205, and SRC-2. The input lanes (lanes 1–3) represent ~10% of the total protein. D. GST (lane 5) and GST-NOR-1 (lane 6), GST-NOR-1-AB (lane 7), and GST-NOR-1-DE (lane 8) were incubated simultaneously with in vitro 35S-radiolabeled full-length p300, DRIP205, SRC-2, and PCAF. The input lanes (lanes 1–4) represent ~10% of the total protein.
the AF-1 domain of NOR-1, mediated 6-MP activation. This experiment ruled out the possibility that 6-MP activation involved the modulation of DNA binding or LBD activation (Fig. 9A).

The specificity and selectivity of the 6-mercaptopurine compound have been examined by the Heyman group at X-ceptor Therapeutics. This compound does not activate other nuclear hormone and orphan receptors. Hence, we investigated whether 6-MP activated general trans-activators (GAL4VP16), myogenic trans-activators (MyoD, myogenin, MEF2C, and MEF2D), and the primary coactivators, SRC-2 and p300. We observed that 6-MP did not selectively activate other transcription factors and coactivators in muscle cells (Fig. 9, B and C). There were minor inductions of 1.5–3-fold; however, relative to the nonspecific effects on GAL4 (1–1.7-fold) and >20-fold effects on NOR-1, these changes were insignificant.

Potential phosphorylation sites in the N-terminal region of the NR4A family surrounding amino acid positions 50 and 142 have been implicated in the control of subcellular localization, AF-1 activity, and modulation by several kinases (e.g. Erk2, Trk, Ras, and mitogen-activated protein kinase) (13, 74, 75).

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*P. Ordentlich and R. Heyman, personal communication.
We constructed two double mutants in both regions to investigate the effect of mutation on potential phosphorylation sites and 6-MP-mediated activation. We observed that both double mutants GAL-NOR-1 S59P/T60P and GAL-NOR-1 S142P/T145P did not compromise the ability of 6-MP to modulate the activity of NOR-1/NR4A3 (Fig. 10).

The Minimal Region between Amino Acid Residues 1 and 150 Mediated 6-MP Activation: Other Thiopurine Compounds Activated NOR-1

We utilized our comprehensive set of GAL-NOR-1-AB deletions to identify the minimal region that mediates 6-MP activation. We observed that the domain between amino acids 1 and 150 retained the potential to be activated significantly by 6-MP. The C-terminal region of the AF-1 domain between amino acids 150 and 292 was completely dispensable for 6-MP activation. Although the region between amino acid residues 1 and 60 retained 6-MP inducibility, it was considerably compromised (Fig. 11A).

We examined the ability of several other thiopurine compounds including 6-mercaptopurine riboside (6-MP-R), 6-mercaptopurine deoxyriboside (6-MP-deoxy-R), 6-mercaptopurine monohydrate (6-MP-mh), and 6-mercaptopurine ribofuranoside (6-MP-RF) to activate NOR-1. Interestingly, all of these thiopurine compounds efficiently activated the N-terminal region of NOR-1 (Fig. 11B).

**DISCUSSION**

In this investigation, we have provided evidence that NOR-1 trans-activates gene expression in a cell- and target-specific manner. Moreover, the AB region was necessary for optimal NOR-1-dependent trans-activation and encodes an N-terminal
AF-1 domain between amino acids 1 and 112, which is necessary for the activation of gene expression. Interestingly, the N-terminal AB region (not the LBD) is essential for the recruitment of the coactivator complex. Additionally, we demonstrate that SRC-2 (i) modulates the activity of the N-terminal AF-1 domain, (ii) interacts efficiently with the N-terminal AB region, (iii) as evidenced by the activation of NOR-1 chimeras cotransfected with GAL4-dependent reporter G5E1B-Luc in the presence and absence of 6-MP.

**FIG. 11.** The region between amino acid residues 1 and 150 mediated 6-MP activation: Other thiopurine compounds activated NOR-1. A, GAL-NOR-1-AB, GAL-NOR-1-aa1–60, GAL-NOR-1-aa1–112, GAL-NOR-1-aa40–160, GAL-NOR-1-aa1–150, and GAL-NOR-1-aa150–292 chimeras (0.33 µg) were cotransfected with the GAL4-dependent reporter G5E1B-Luc (1 µg) into C2C12 proliferating myoblasts in the presence and absence of 50 µM 6-MP (B). GAL-NOR-1-AB (0.33 µg) was cotransfected with the GAL4-dependent reporter G5E1B-Luc (1 µg) into C2C12 proliferating myoblasts in the presence and absence of 50 µM 6-MP, 6-mercaptopurine riboside (6-MP-R), 6-mercaptopurine deoxyriboside (6-MP-deoxyR), 6-mercaptopurine monohydrate (6-MP-mh), and 6-mercaptopurine ribofuranoside (6-MP-RF), respectively. Fold induction is expressed as the fold activation obtained from cotransfection of the GAL-NOR-1 chimeras in the presence of 6-MPs relative to the fold activation obtained from cotransfection of GAL-NOR-1 chimeras in the absence of 6-MPs. The mean luciferase fold induction values and S.D. values (bars) were derived from a minimum of two or three independent triplicate experiments.
and (iii) potentiates the physical association of the N-terminal AF-1 domain with the C-terminal LBD of NOR-1.

Interestingly, we demonstrated that the purine anti-metabolite 6-mercaptopurine (and other 6-MP thiopurine variants), which has antiproliferative, anti-cancer, and anti-inflammatory properties, activated Nur77, Nurrl, and NOR-1-mediated trans-activation of gene expression in muscle cells. This implicates the NR4A subgroup as potential mediators of 6-MP-mediated antiproliferative effects. Surprisingly, the data demonstrated that the N-terminal AF-1 domain of NOR-1 mediated the 6-MP activation and not the C-terminal LBD. This was consistent with the in vitro binding data and the identification of the N-terminal trans-activation domain. The results imply that the AF-1 domain plays a hierarchical role in NOR-1-mediated transcriptional activation and is the target of compounds that induce genotoxic stress by the inhibition of de novo purine biosynthesis and nucleic acid misincorporation of thiopurines into nucleic acids.

Surprisingly, the N-terminal AF-1 domain was necessary for cofactor recruitment and 6-MP-mediated activation. Although the LBD independence was atypical of NRs, NOR-1 retained the classical domain-dependent coupling of ligand dependent modulation and coactivator binding. This suggests that both AF-1 and AF-2 domains are the targets of agonist-dependent regulation and interfaces for cofactor binding. The NOR-1 N-terminal AB region encodes an AF-1 domain, which is directly bound and modulated by SRC-2/GRIP-1. In contrast to other nuclear hormone receptors, the DE region that encodes the LBD did not efficiently interact with the SRC factors and other members of the coactivator complex. However, the NOR-1-AB region supported the efficient recruitment of the coactivator complex including SRC-2, p300, PCAF, and DRIP205. Furthermore, the AF-1 domain is necessary for the formation of the coactivator complex and provides one explanation for the atypical nature of NR4A-mediated trans-activation. Interestingly, the AF-1-mediated recruitment of SRC-2 promotes intramolecular interactions with the LBD that may function to stabilize the receptor-coactivator complex during transcriptional activation and/or facilitate agonist dependent regulation. This observation provided justification for (i) the loss of activity after deletion of the C-terminal helix 12 in the LBD and (ii) the requirement of the LBD for optimal 6-MP/NOR-1-mediated transactivation of the NurRE_{POMC}, although the NOR-1 LBD does not encode an activation domain per se.

Our molecular modeling analysis of the NR4A3/NOR-1 C-terminal LBD region suggests that the topology of the orphan LBD prevents efficient coactivator recruitment. Examination of the molecular surface in this region shows an unusually hydrophilic surface, which is in contrast to the archetypal and characteristic hydrophobic groove found in AF-2-activating receptors (e.g. RAR and thyroid hormone receptor). Computer modeling demonstrates that the NOR-1 LBD is able to bind a coactivator-derived peptide. However, binding results in significant distortion of the motif due to the differences in hydrophobicity and topology between the NOR-1 and classical NRs. Additionally, the ability of our NOR-1 model to dock with coactivator peptides in de novo simulations is appreciably compromised when compared with receptors that are known to bind SRC type coactivator peptides. These observations are also consistent with the observation that the AF-2 core regions in the ligand-dependent receptors (e.g. RAR and RXR) contain a very highly conserved glutamic acid. This glutamic acid is important for coactivator recruitment and transcriptional activation in classical NRs (76); in contrast, the NR4A subgroup (Nur77, Nurrl, and NOR-1) has a conserved lysine.

6-MP regulates the activity of NOR-1 in an AF-1-dependent manner. This thiopurine compound is a small molecule widely used for the treatment of acute lymphoblastic leukemia, chronic myelocytic leukemia, and autoimmune/inflammatory disorders (e.g. Crohn’s disease) (77–79). 6-MP belongs to a class of nucleic acid analogs that selectively block de novo nucleotide synthesis in rapidly proliferating cells. This purine anti-metabolite has antiproliferative and cytotoxic effects resulting primarily from the inhibition of purine biosynthesis at multiple steps and incorporation into nucleic acids as thionucleosides (80).

6-MP is metabolized into the active derivative, 6-thiinosine monophosphate, by hypoxanthine-guanine phosphoribosyltransferase, a key enzyme involved in the purine salvage pathway. The 6-thio-IMP can be metabolized into 6-thioguanosine 5’-monophosphate, which is then incorporated into DNA and RNA as 6-thioguanosine triphosphate (6-thio-GTP) resulting in eventual cytotoxicity and cell death (80). Incorporation of 6-thi-GTPs into nucleic acids is thought to be the main mechanism of action.

At this point, the 6-thio-IMP can be utilized in two different pathways. One option is that the 6-thinoisine monophosphate can be methylated by thiopurine methyltransferase to methyl mercaptopurine riboside phosphate, which is a potent inhibitor of de novo purine biosynthesis at the level of phosphoribosylpyrophosphate aminotransferase, which depletes the intracellular ATP pools. Thiopurine methyltransferase also converts 6-MP into methylmercaptopurine, which has no cytotoxic activity. The majority of the known functions of 6-MP involve conversion into the active metabolite 6-thio-IMP through the enzyme hypoxanthine-guanine phosphoribosyltransferase (80). However, 6-MP also leads to DNA hypomethylation, which can effect gene expression.

The antiproliferative activity of 6-MP has been also shown to result partly from a decrease in the production of adenosine and guanosine synthesis (80). It should be noted that control of adenosine pools can regulate levels of AMP/ADP/ATP available to carry out cellular functions. This would imply that there exists the possibility of an ATP-dependent pathway that can regulate NOR-1 (NR4A3) activity. Indeed, recent reports suggest that a cAMP-dependent protein kinase A signaling cascades can regulate both NR4A subgroup expression and transcription activity (81, 82). Serine/threonine-rich domains in the N terminus have been implicated in the regulation of NR4A1/Nur77-dependent transcription (13, 74, 75). Moreover, phosphorylation of amino acid residues in the AB region have been implicated in growth factor-dependent nucleocytoplasmic shuttling and modulation of activity by Erk2, Trk, Ras, and mitogen-activated protein kinase (13, 74, 75). Furthermore, regulatory phosphorylation sites have been identified in the DNA binding domain of NR4A1/Nur77. For example, Akt kinase, a key player in the transduction of antiapoptotic and proliferative signals in T cells, phosphorylates Ser-350 and decreases the transcriptional activity of Nur77/NR4A1 (83, 84). Therefore, a potential mechanism for NR4A activation by 6-MP may include regulation of kinase or phosphatase pathways. However, mutation of the serine and threonine residues at amino acid positions 54, 55, 142, and 145 in the phosphorylation motifs that had been implicated in kinase-dependent regulation targets did not compromise the ability of 6-MP to activate NOR-1.

The ability of 6-MP to regulate the activity of a transcription factor class, such as the NR4A subgroup, considerably broadens the possible mechanism of action of this drug. NR4A subgroup expression and tissue distribution in leukemic cells and inflammatory disease indicate that members of this family are likely to be present in the tissues targeted by 6-MP.
many of the steps involved in 6P-mediated cytotoxicity have been elucidated, it is possible that these effects can be enhanced or suppressed through secondary targets. NOR-1 has been shown to play a role in T-cell antigen receptor and calcium-mediated apoptosis, cell death, and thyrocyte selection in T lymphocytes (14–16, 44). Additionally, NOR-1 translocation to the Ewing’s sarcoma gene has been implicated in extraskel-etal myxoid chondrosarcoma (45).

It has not been directly demonstrated that the regulation of apoptosis by the NRA4 subgroup is through transcriptional mechanisms. However, we speculate that the effectiveness of 6P treatment and the antiproliferative effects may be influenced by the activity of these receptors. Apoptosis represents an effective way to eliminate cancerous cells, and a variety of evidence suggests that the NRA4 subgroup is involved in the regulation of apoptosis in prostate, lung, gastric, breast, and colon cancer cells (28–34).

Supporting evidence for this hypothesis includes the following. (i) NOR-1 expression is regulated by Ca2+/calmodulin-de-pendent protein kinases and inflammatory cytokines. (ii) Gene targeting experiments have established a role for NOR-1 and Nur77 in mediating T cell development. Furthermore, Nur77 targeting experiments have established a role for NOR-1 and dependent protein kinases and inflammatory cytokines. (iii) Gene side all efficiently activated the N-terminal region of NOR-1.

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AF-1 Domain of NOR-1/NR4A3

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The AF-1 Domain of the Orphan Nuclear Receptor NOR-1 Mediates Trans-activation, Coactivator Recruitment, and Activation by the Purine Anti-metabolite 6-Mercaptopurine

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J. Biol. Chem. 2003, 278:24776-24790.
doi: 10.1074/jbc.M300088200 originally published online April 22, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300088200

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