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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NOR-1/NR4A3 is an “orphan member” of the nuclear hormone receptor superfamily. NOR-1 and its close relatives Nur77 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 confer coactivator activity. 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.

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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 (36, 37). The Nur subfamily also regulates steroid 17α-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 adrenocorticotropin hormone, the chief regulator of adrenal steroidogenesis, and the corticotropin-releasing hormone gene (39, 40).

In response to inflammatory cytokines (e.g. interleukin-1β and tumor necrosis factor-α), there is local up-regulation of corticotropin-releasing hormone in rheumatoid arthritis (RA) synovial tissue, indicating corticotropin-releasing hormone as a component of the inflammatory cascade in arthritis. It has also been shown that the NR4A subgroup up-regulates corticotropin-releasing hormone expression by interacting with specific cis-acting sequences in its proximal promoter region. As such, the NR4A family of proteins represents a potential target for therapeutic intervention to control inflammatory responses (25, 41, 42).

Gene targeting experiments have established a role for NOR-1 and Nur77 in mediating T cell development. Nur77 and NOR-1 are rapidly induced by the T-cell antigen receptor signaling in immature thymocytes and T-cell hybrids leading to apoptosis (14–17, 44). Apoptosis represents an effective way to eliminate cancerous cells, and a variety of evidence suggests that the NR4A subgroup is involved in the regulation of apoptosis in prostate, lung, gastric, breast, and colon cancer cells (28–34). The NR4A subfamily is further implicated in carcinogenesis by the finding that NOR-1 is involved in a chromosomal translocation with the Ewing’s sarcoma gene identified in the extraskeletal myxoid chondrosarcoma (45).

Activated smooth muscle cells are a hallmark of pathological vascular processes, including atherosclerosis and restenosis (46). It has been established that atheromatous lesions are active sites of inflammation and immune responses, and cytokines mediate the chronic development of atherosclerosis (47–49). Recently, it has been shown that the NR4A subgroup is expressed in human atherosclerotic lesions, and inhibition of the NR4A subgroup subfamily transcription factors in smooth muscle cells results in enhanced lesion formation, whereas overexpression of Nur77/NOR-1/Nurr1 decreases neointimal formation. Thus, the NR4A subgroup has a protective function in the initiation and the progression atherosclerosis (26, 27). The importance of NOR-1 as a potential target for anti-cancer, inflammation, and atherogenic drugs underscores the importance of understanding the NOR-1 molecular mechanism of action. Hence, we used a variety of techniques to investigate the critical subdomains and coactivators that mediate its function.

In summary, we demonstrated that NOR-1-mediated transcription and co-factor recruitment operated in an AF-1-dependent manner; furthermore, the AF-1 domain was required for NOR-1-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 thiorpurine 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 pN BRE3-tk-LUC (6–8) or pPOMC-TK-LUC (NurRE-SOM-tk-LUC) (51) reporter plasmid together with 0.33–0.5 μg of pSG5-NOR-1-FL or pSG5-NOR-1-CDE or pSG5-NOR-1-ABC or pSG5 alone using a DOTA/P DOSP HER (Roche Applied Science and Scientific) liposome mixture in HEBS (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 0.4 mM Na HPO₄, 11 mM dextrose, pH 7.1) (total DNA 2–2.5 μg/well). The DNA/DOTA/DOSP HER mixture was added to the cells in 1 ml of fresh DMEM containing 10% fetal calf serum and incubated for 1 h. Transfections were performed with C2C12 or COS-1 with 0.33–1 μg of pGL2b-tk-LUC or pPOMC-TK-LUC reporter plasmid together with 0.33–0.16 μg of pSG5-NOR-1-FL or pSG5-NOR-1-CDE, or pSG5-NOR-1-ABC or pCMX-Nur-77 or pCMX-NURR1 (7) or pSG5 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-9β-β-ribofuranoside (50–100 μM Sigma) were performed with phenol red-free DMEM containing 5% charcoal-stripped fetal calf serum. The DNA/DOTA/DOSP HER 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-9β-β-ribofuranoside 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 Luciferase substrate buffer. Cells lysates were transferred to a 96-well plate, and relative luciferase units were measured for 5 s in a Wallac Trilux 1450 microbeta luminometer (52–56). For transfections performed with NOR-1 GAL-chimeric constructs and reporter plasmids, cells were transfected 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 GAL4DBD 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 pSG5-SRC-2 (0.33–0.1 μg) using a DOTA/P DOSP HER 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 GAL4VP16, GAL-MEF2C, GAL-MEF2D, GAL-MyoD, GAL-Myogenin, GAL-siE1, and GAL-p38 constructs (50–52). These constructs were co-transfected in the presence and absence of 6-mercaptopurine, 6-mercaptopurine riboside, 6-mercaptopurine-2’-deoxyriboside, 6-mercaptopurine monophosphate, or 6-mercaptopurine-9β-β-ribofuranoside (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 pN BRE3-tk-LUC (6–8), pPOMC-tk-LUC (NurRE-SOM-tk-LUC) (51), and G5E1b-LUC (59) have been described elsewhere. Generation of full-length mouse NOR-1 was performed by reverse transcriptase-PCR from muscle RNA with Pri/a 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...
used for the synthesis of full-length mouse NOR-1 were GMUQ-720 5′ (GCC GAA TTC ACC ATG CCC TGC GTG GAA GCC GAG) and GMUQ-722 3′ (GCC GAA TTC TCA GAA AGG CAG GGT GTC AAG GAA). We then synthesized additional primers to subclone the DE, AB, and ABC regions into pSG5 and GALO. The primers used for the DE region were GMUQ-724 5′ (GCC GAA TTC ACC ATG GGT AAT GTG GTC GTG GTG) and GMUQ-722 3′. The primers used to synthesize the AB region were GMUQ-720 5′ and GMUQ-726 3′ (GCC GAA TTC TCA GTG 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 TCT GTG). The primers used to synthesize the DE region were GMUQ-732 5′ (GCC GAA TTC ACC ATG GTC GTG GTC GGC G) and GMUQ-722 3′.

We then synthesized 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-GALO using primers GMUQ-750 5′ and GMUQ-756 3′ (GCC GTC GAC TCA GGG GAC GCC GGG GC) and GMUQ-759 3′ (GCC GTC GAC TCA CTC GTC GTC CCA CAG CG). The NOR-1-AB-aa1–292 region was subjected to PCR and cloned into SV40-GALO using primers GMUQ-757 5′ and GMUQ-760 3′ (GCC GTC GAC TCA CTC GTC GTC CCA CAG CG) and GMUQ-761 3′ (GCC GTC GAC TCA ATG GTG ATG CAC ATG CCC TGC GTG CAA GCC CAG).

We subsequently constructed various chimeras that simultaneously carried double, triple, and quadruple amino acid mutations. NOR-1-AB mutant primers were synthesized and 5′-phosphorylated by GENEWORX. The primers made were 5′-TGGGATCCGGGATCCGGGATCCGGG (GCG GCC GTC GAC) and 5′-TGGGATCCGGGATCCGGGATCCGGG (GCG GCC GTC GAC).

**RESULTS**

**NOR-1 Trans-activates Gene Expression in a Cell- and Response Element-Specific Manner**—NOR-1 is expressed in skeletal muscle tissue and cells (63–65). We performed reverse transcriptase-PCR experiments using total RNA extracted from C2C12 skeletal muscle cells to isolate full-length mouse NOR-1 cDNA. To verify the integrity of the cloned NR after full-length sequencing, we examined the ability of NOR-1 to trans-activate two NR4A-dependent reporters, NBRE-3-5k-LUC (6–8) and the NurRE-5-tk-LUC (51). The NBRE-3-5k-LUC reporter construct contains the NBRE derived from the monomeric consensus binding site, AAAGTTCA, that was identified by genetic selection in *Saccharomyces cerevisiae*. This plasmid contains three copies of a consensus binding site (AAAGTTCA) cloned upstream of the heterologous herpes simplex virus thymidine kinase (tk) promoter (6–8) linked to the luciferase (LUC) gene. The NurRE-5-tk-LUC reporter contains five copies of the naturally occurring NR4A response element (TGTATTTACATCTCAAATGCGCA) from the POMC gene. This native response element is responsive to physiological stimuli, binds NR4A dimers, and is more responsive to NR4A-mediated transactivation (5, 51).

We investigated the ability of NOR-1/NR4A3 to transactivate the NBRE-3 and NurRE-5-tk-LUC reporters in muscle C2C12 and nonmuscle COS-1 cells in DMEM supplemented with 10% fetal bovine serum. In control studies, C2C12 myogenic cells and COS-1 cells were transfected with the control reporter plasmid pGL2-tk-LUC (6), linked to the firefly LUC (6) and the NurRE-5-tk-LUC (51). The NBRE-3-tk-LUC reporter constructs that simultaneously carried double, triple, and quadruple amino acid mutations. NOR-1-AB mutant primers were synthesized and used the Stratagene QuikChange multisite-directed mutagenesis kit as per the manufacturer’s instructions to produce GAL4-NOR-1-AB chimera that simultaneously carried double, triple, and quadruple amino acid mutations. NOR-1-AB mutant primers were synthesized and 5′-phosphorylated by GENEWORX. The primers made were 5′-TGGGATCCGGGATCCGGGATCCGGG (GCG GCC GTC GAC) and 5′-TGGGATCCGGGATCCGGGATCCGGG (GCG GCC GTC GAC).

**Molecular Modeling**—We used molecular modeling as a tool to further investigate the inability of the atypical nuclear receptor LBD to play an important role in the activity of the AF-1 and AF-2 response element-specific manner. NOR-1 also efficiently trans-activated the NurRE-5-tk-LUC 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-5-tk-LUC 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 DE region, 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 spatio-temporal expression pattern of the specific receptor, suggesting that cell-specific activation mechanisms are involved in the functioning of the different AFs.
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. 1E 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, 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 24779
To identify and further characterize the domains of NOR-1, we constructed subregions of 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 constructed subregions of the AB domain by PCR and cloned these segments into the GAL4-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-aa1–60 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 150 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 NOR-1. However, we have demonstrated that the steroid receptor coactivator, SRC, binds and modulates the activity of the Nur77/NR4A1 AF-1 domain (68).

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The NOR-1 LBD Coactivator Binding Cleft Is Unusually Hydrophilic: 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. Alignments were performed with ClustalV and adjusted manually thereafter. Homology modeling was performed by satisfaction of spatial restraints using the program Modeler 6 (60). The resulting model was subjected to Ramachandran analysis.

Fig. 2. The AB region of NOR-1 encodes a potent AF-1 activation domain. A, diagrammatic representation of Gal-NOR-1 chimeric constructs. B and C, GALNOR-1-FL, GAL-NOR-1-AB, and GAL-NOR-1-DE (0.33 μg) were co-transfected with the GAL reporter G5E1b-Luc (1 μg) into C2C12 proliferating myoblasts (B) and into 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. (bars) were derived from a minimum of two or three independent triplicate experiments.

112—To identify and further characterize the domains of NOR-1 involved in transcriptional activation, we utilized the GAL4 hybrid system, whereby putative activation domains are fused to the DBD of the yeast transcription factor GAL4 (57). If these regions encode modular activation domains, they complement the GAL4 DBD (to produce a functional trans-activator) and induce the transcription of the GAL-responsive reporter construct G5E1b-LUC, containing an E1b TATA box with five 17-mer GAL4 binding sites linked to the LUC reporter (59). The system utilized an SV40 promoter expression vector pGAL4-DBD (57) that contains a multiple cloning site downstream of the GAL 4 DBD. We fused NOR-1 and various domains (e.g. AB or DE regions, etc.) of NOR-1 to the GAL 4 DNA binding domain (Fig. 2A) and examined the ability of these chimeras to regulate the expression of the G5E1b-LUC reporter in C2C12 and COS-1 cells. The GAL4-NOR-1 chimera containing the full open reading frame of NOR-1 activated transcription ~10-fold above the control, pGALO (GAL4 DBD), in muscle and nonmuscle cells (Fig. 2, B and C). The GAL-NOR-1-AB plasmid, which contains only the AB regions of NOR-1, with the DBD and LBD deleted, increased transcription of the reporter construct ~15–20-fold over the GAL-DBD alone in myogenic cells and nonmuscle cells (Fig. 2, B and C). 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.
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/stereoid 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 rRORβ (a related nuclear receptor but one that is distinct from the template structure) (B) and the modeled NOR-1 (C). The rRORβ 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; rRORβ (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 NOR-1 is independent of its binding to its cognate binding motifs, the NBRE-3 and NurRE-5POMC response elements. If

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, GAL-NOR-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-3 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.

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Fig. 4 shows comparative surface views of rRORβ (a related nuclear receptor but one that is distinct from the template structure) (B) and the modeled NOR-1 (C). The rRORβ 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; rRORβ (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 NOR-1 is independent of its binding to its cognate binding motifs, the NBRE-3 and NurRE-5POMC response elements. If
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 35S-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,
The N-terminal AB Region Directly Interacts with the C-terminal LBD: The Interaction Is Potentiated by AF-1-mediated Recruitment of SRC—It has been previously demonstrated that human SRC-2 concomitantly interacts with the estrogen receptor AF-1 and AF-2 to synergistically regulate transcription (71–73). Our studies suggested that SRC-2 preferentially interacts with the NOR-1 AF-1 but not the C-terminal LBD. We investigated whether the atypical role of the NOR-1 LBD in transcriptional activation involved intramolecular interactions with the N-terminal AF-1 and whether this process was modulated by SRC recruitment (Fig. 7A).

We tested this hypothesis using a biochemical approach, the in vitro pull-down assay. Glutathione-agarose-immobilized GST-NOR-1-AB was tested for direct interaction with in vitro 35S-radiolabeled full-length SRC-2 and the NOR-1 LBD (CDE) (Fig. 7B). As expected from our studies above, we observed that SRC-2 efficiently interacted with the NOR-1-AB (Fig. 7B, lane 5). Very interestingly, we observed that the N-terminal AB region that encodes the potent AF-1 domain interacts more efficiently with the C-terminal LBD region than with the C-terminal LBD region in the presence of SRC-2 (Fig. 7B, lane 6 versus lane 8). The coactivator p300 did not potenti ate this interaction in an efficient manner (Fig. 7B, lane 7). This evidence clearly demonstrates intramolecular interactions between the N- and C-terminal regions of NOR-1 and provides evidence for the involvement of the NOR-1 LBD in the absence of an intrinsic activation function. In summary, this suggests that SRC-2 potentiates the direct interaction between the N-terminal 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 NurREPOMC reporter by NR4A2/Nurr1 in CV-1 cells. We exploited the ability of 6-MP to activate Nurr1, NOR-1, and Nur77-mediated trans-activation of the NurREPOMC reporter in myogenic cells. We cotransfected the NurREPOMC 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 Nurr1-, NOR-1-, and Nur77-mediated trans-activation of the NurREPOMC reporter (Fig. 8, A–C). 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.

The 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 LBD, demonstrated a more potent activation by 6-MP than the C-terminal LBD (Fig. 9A and B). We observed that the GAL-NOR-1-AB-mediated trans-activation was significantly less potent than the GAL-NOR-1-mediated trans-activation (Fig. 9A). This suggested that the N-terminal AB region encodes the LBD of NOR-1.

These studies suggested that the AF-1 domain of NOR-1 can independently and efficiently mediate the recruitment of the coactivator complex, which is consistent with the intrinsic transcriptional activity of the AF-1 domain.
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 (GAL4-VP16), 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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**Fig. 7.** The N-terminal AB region directly interacts with the C-terminal LBD: The interaction is potentiated by AF-1-mediated recruitment of SRC. A, a diagrammatic representation of the intramolecular interaction between AF-1 and AF-2 of NOR-1 that is potentiated by AF-1-mediated recruitment of SRC-C2. SRC-GST (lane 2) was incubated simultaneously with in vitro 35S-radiolabeled p300, SRC-2, and NOR-1-CDE and GST-NOR-1-AB (lanes 4–6) were incubated independently with in vitro 35S-radiolabeled p300, SRC-2, and NOR-1-CDE, respectively, and GST-NOR-1-AB (lane 7) was incubated simultaneously with in vitro 35S-radiolabeled p300, and NOR-1-CDE and GST-NOR-1-AB (lane 8) were incubated simultaneously with in vitro 35S-radiolabeled SRC-2 and NOR-1-CDE. The input lane represents ∼10% of the total protein.

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**Fig. 8.** The antileukemic hypoxanthine analog 6-mercaptopurine regulates NR4A1-3-mediated transcription. A, pCMX-NURR1-FL (0.33 μg) was co-transfected into C2C12 proliferating myoblasts together with the reporter gene NurRE-5-tk-Luc in the presence and absence of 50–100 μM 6-MP. B, SG5-NOR-1-FL, SG5-NOR-1-CDE, and SG5-NOR-1-ABC (0.33 μg) were co-transfected into C2C12 proliferating myoblasts together with the reporter gene NurRE-5-tk-Luc in the presence and absence of 50 μM 6-MP. C, pCMX-Nur77-FL (0.33 μg) was co-transfected into C2C12 proliferating myoblasts together with the reporter gene NurRE-5-tk-Luc in the presence and absence of 50–100 μM 6-MP. Fold activation is expressed relative to luciferase activity obtained after cotransfection of the pCMX or pSG5 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.
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,
and (iii) potentiates the physical association of the N-terminal AF-1 domain with the C-terminal LBD of NOR-1.

Intriguingly, 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, Nurr1, 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 thio- purines 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 cofactor recruitment and transcriptional activation in classical NRs (76); in contrast, the NR4A subgroup (Nur77, Nurr1, 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 thio- guanine nucleotides (80).

6-MP is metabolized into the active derivative, 6-thioguanosine 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-thio-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-thioguanosine monophosphate can be methylated by thio- purine 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. Whereas
many of the steps involved in 6MP-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 tumour necrosis factor (TNF) (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 NRB subgroup is through transcriptional mechanisms. However, we speculate that the 6MP treatment and the antiproliferative effects may be induced by the activity of these receptors. Apoptosis represents an effective way to eliminate cancerous cells, and a variety of evidence suggests that the NRB 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 Ca2+/calmodulin-de- pendent protein kinases and inflammatory cytokines. (iii) NOR-1 expression is regulated by Ca2+/calmodulin-de- pendent protein kinases and inflammatory cytokines. (iii) NOR-1 expression is regulated by Ca2+/calmodulin-de- pendent protein kinases and inflammatory cytokines.
AF-1 Domain of NOR-1/NR4A3

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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