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Two distinct nuclear receptor interaction domains in NSD1, a novel SET protein that exhibits characteristics of both corepressors and coactivators

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NSD1, a novel 2588 amino acid mouse nuclear protein that interacts directly with the ligand-binding domain (LBD) of several nuclear receptors (NRs), has been identified and characterized. NSD1 contains a SET domain and multiple PHD fingers. In addition to these conserved domains found in both positive and negative *Drosophila* chromosomal regulators, NSD1 contains two distinct NR interaction domains, NID^{-L} and NID^{+L}, that exhibit binding properties of NIDs found in NR corepressors and coactivators, respectively. NID^{-L}, but not NID^{+L}, interacts with the unliganded LBDs of retinoic acid receptors (RAR) and thyroid hormone receptors (TR), and this interaction is severely impaired by mutations in the LBD α-helix 1 that prevent binding of corepressors and transcriptional silencing by apo-NRs. NID^{+L}, but not NID^{-L}, interacts with the liganded LBDs of RAR, TR, retinoid X receptor (RXR), and estrogen receptor (ER), and this interaction is abrogated by mutations in the LBD α-helix 12 that prevent binding of coactivators of the ligand-induced transcriptional activation function AF-2. A novel variant (FxxLL) of the NR box motif (LxxLL) is present in NID^{+L} and is required for the binding of NSD1 to holo-LBDs. Interestingly, NSD1 contains separate repression and activation domains. Thus, NSD1 may define a novel class of bifunctional transcriptional intermediary factors playing distinct roles in both the presence and absence of ligand.

Keywords: activation function AF-2/chromatin/PHD finger/SET domain/transcriptional intermediary factor

Introduction

Nuclear receptors (NRs) are transcriptional regulators that control many aspects of development, differentiation and homeostasis upon binding of cognate hydrophobic ligands, such as steroid and thyroid hormones, vitamin D3 and retinoids. They act as homodimers or heterodimers by binding to DNA response elements present in the regulatory regions of target genes (for reviews see Mangelsdorf *et al*., 1995; Chambon, 1996; Perlman and Evans, 1997). NRs have a modular organization with three main regions: an N-terminal A/B region that contains the activation function 1 (AF-1), a highly conserved DNA-binding domain (DBD) (region C) and a C-terminal ligand-binding domain (LBD) (region E). The LBD includes the interaction surfaces for homo- and/or heterodimerization, the ligand-induced activation function AF-2, and in certain cases (see below) a repression function that is abrogated upon ligand binding (for reviews and references see above). The structures of all NR LBDs correspond to a novel protein fold that commonly includes 12 α-helices (Wurtz *et al*., 1996 and references therein). The binding of the ligand to the unliganded apo-form of the LBD triggers a transconformation that generates the LBD holoform structure (Wurtz *et al*., 1996). A number of putative intermediary factors for the transcriptional functions associated with NR LBDs have been characterized recently, and have been shown to act either as coactivators that bind to holo-LBDs or corepressors that bind to apo-LBDs. The integrity of the N-terminal α-helix 1 (H1) is indispensable for transcription repression by certain NR apo-LBDs, whereas the C-terminal α-helix 12 [H12, that includes the highly conserved core of the AF-2 activating domain (AF-2 AD core)] is required for transactivation by NR holo-LBDs. It is therefore believed that the ligand-induced transconformation of the apo-LBD generates the holo-LBD coactivator interaction surface whose formation requires the integrity of H12, whereas the apo-LBD corepressor interaction surface that involves H1 is concomitantly destroyed (Chambon, 1996; Wurtz *et al*., 1996; Perlman and Evans, 1997).

The putative corepressors N-CoR and SMRT (Chen and Evans, 1995; Hörlein *et al*., 1995; Kurokawa *et al*., 1995) efficiently bind the apo-receptors for retinoic acid (RAR) and thyroid hormone (TR). Their binding to RAR and TR LBDs requires the integrity of H1, whereas that of H12 is necessary for their dissociation in the presence of ligand. Interestingly, corepressors form complexes with SIN3 and histone deacetylases (Alland *et al*., 1997; Heinzel *et al*., 1997; Nagy *et al*., 1997), which suggests that chromatin remodelling by histone deacetylation is involved in NR-mediated repression (reviewed by Pazin and Kadonaga, 1997; Wolffe, 1997). Putative TIFs/coactivators for the AF-2 activation function include TIF1α, RIP140, SRC-1/NcoA1, TIF2/GRIP1, pCIP/RAC3/AIB1/ACTR/TRAM-1 and CBP/p300 (for review and references see Glass *et al*., 1997; Kalkoven *et al*., 1998; Voegel *et al*., 1998). All of them bind NR LBDs in the presence of cognate agonists and the integrity of H12 (AF-2 AD core) is required. Some of them (SRC-1, ACTR, CBP, p300) appear to possess intrinsic histone acetyltransferase (HAT) activity

and, in addition, can interact in a multisubunit complex with the histone acetyltransferase p/CAF (Bannister and Kourazides, 1996; Ogryzko *et al*., 1996; Chen *et al*., 1997; Spencer *et al*., 1997). Moreover, CBP and p300 also interact with RNA helicase A, which in turn binds RNA polymerase II (Nakajima *et al*., 1997). Thus, chromatin remodelling by histone acetylation and recruitment of the basal transcriptional machinery to target promoters appear to be involved in transcriptional activation by holo-NRs.

In a search for additional transcriptional intermediary factors for NRs, we have used the RARα LBD as a bait in a yeast two-hybrid screen, and here we report the identification of a novel NR-binding SET-domain-containing protein (NSD1), that also contains multiple PHD fingers. In addition to these conserved domains, which are present in members of the *Trithorax* gene family and other chromatin-related factors acting either positively and/or negatively on transcription (Laible *et al*., 1997 and references therein), NSD1 contains two adjacent but distinct NR interaction sites. One of these sites (NID^{-L}) binds RAR and TR in the absence, but not in the presence, of ligand and this binding requires the integrity of the LBD α-helix 1, whereas the other interaction site (NID^{+L}) binds all NRs tested [RAR, TR, retinoid X (RXR) and estrogen (ER) receptors] in the presence of ligand and this binding requires the integrity of α-helix 12. Thus, NSD1 differs from all previously identified putative NR cofactors. Moreover, NSD1 appears to contain distinct repression and activation domains, which suggest that it may function as a bifunctional transcriptional intermediary factor.

Results

Cloning of the cDNA of NSD1, a novel protein that contains several conserved domains

NSD1 cDNA was isolated in a two-hybrid screen for proteins that interact with the LBD of RARα. A chimeric receptor consisting of the core of ERα DBD (ER.CAS) fused to RARα DEF regions [RARα(DEF)–ER.CAS; Figure 1A; Heery *et al*., 1993] was expressed in yeast PL3 which contains a URA3 reporter gene controlled by three copies of an estrogen response element (*ERE-URA3*; Figure 1A; Pierrat *et al*., 1992). Activation of this reporter by RARα(DEF)–ER.CAS in the presence of all-*trans* retinoic acid (T-RA) was sufficient to allow yeast growth on medium lacking uracil, but not in the presence of 6-azauracil (AU, an inhibitor of the URA3 gene product, OMPdecase) which restores uracil auxotrophy (Figure 1B; vom Baur *et al*., 1996). A mouse embryo cDNA library in which the inserted sequences were fused to those encoding the acidic activation domain (AAD) of the VP16 protein (vom Baur *et al*., 1996) was introduced into yeast PL3 expressing RARα(DEF)–ER.CAS. Yeast transformants (~3×10^6) containing both plasmids were recovered and plated at a multiplicity of 10 onto uracil-negative plates containing 500 nM T-RA and 30 μg/ml AU. Among the Ura^+ clones, one 'fused' cDNA (designated AAD-NSD1.6; Figure 1B) showed an absolute requirement for coexpressed RARα(DEF)–ER.CAS to confer uracil prototrophy.

NSD1.6, which contains a novel 852 bp open reading frame (ORF; sequence within brackets in Figure 2A) fused

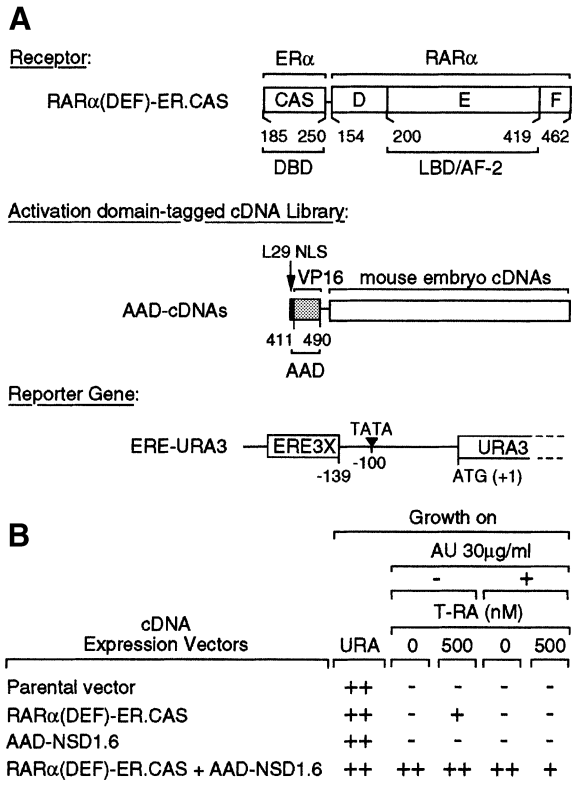


Fig. 1. Isolation of NSD1.6 cDNA. (**A**) Schematic representation of the components used in the yeast two-hybrid screen. RARα(DEF)–ER.CAS corresponds to the bait (numbers refer to amino acid positions in wild-type receptors), AAD-cDNAs represent the VP16 acidic activation domain (AAD)-tagged mouse embryo cDNA expression library [the AAD tag also includes codons specifying the nuclear localization signal (NLS) of the yeast ribosomal protein L29] and *ERE-URA3* is the URA3-based reporter gene whose expression is controlled by three estrogen response elements (ERE3X) in the yeast reporter strain PL3. (**B**) Expression of AAD–NSD1.6 cDNA complements the growth defect of yeast PL3 cells expressing RARα(DEF)–ER.CAS in the presence of 500 nM T-RA and 30 μg/ml azauracil (AU). PL3 was transformed with high-copy number plasmids containing RARα(DEF)–ER.CAS, AAD–NSD1.6 cDNA or no insert. Transformants were grown on Ura^+ medium, and spot tested on selective medium lacking uracil ± T-RA and ± AU as indicated. Plates were incubated at 30°C for 3 days. '++', wild-type growth; '+', weak growth; '–', no growth.

to the VP16 AAD, was used to probe a mouse embryo cDNA library. Several overlapping clones allowed us to reconstitute a cDNA sequence of 10 019 bp (hereafter called NSD1 cDNA) encompassing an ORF of 7764 bp flanked by a 1099 bp 5′- and a 1156 bp 3′-UTR (Figure 2A; DDBJ/EMBL/GenBank accession No. AF064553). A NSD1 cDNA variant containing a 312 bp insertion (Figure 2B) between nucleotides 930 and 931 (Figure 2A, filled triangle) is most probably generated through alternative splicing, as sequencing the genomic DNA led to the identification of intronic sequences downstream and upstream of nucleotides 930 and 931, respectively (data not shown). All subsequent studies were performed with the cDNA isoform that lacks the insertion and encodes a putative 2588 amino acid NSD1 protein (284 kDa) (Figure 2A).

A database search revealed several conserved domains present in proteins that regulate transcription and/or are bound to chromosomes (Figure 3A). The ~150-amino acid

SET domain, located between residues 1834 and 1980 (boxed in Figure 2A), was first identified in three *Drosophila* chromosomal regulators, Su(var)3-9, Enhancer of zeste [E(z)] and Trithorax (Trx) (Tschiersch *et al*., 1994), and was later found in a number of transcriptional regulators from different species (Figure 3A; Hobert *et al*., 1996; see Discussion). The SET domains most similar to that of NSD1 are encoded by the *Drosophila* trithorax-group gene *Ash1* (Tripoulas *et al*., 1996) and the yeast ORF YJQ8 (>40% identity; Figure 3B). As in Ash1 and YJQ8, the NSD1 SET domain is not C-terminal, unlike those of Su(var)3-9, E(z) and Trx (Figure 3A).

Immediately preceding the SET domain, NSD1 contains a Cys-rich domain (residues 1791–1833; herein referred to as the SAC domain; see below; Figure 2A), that is conserved at the same position in some, but not all, SET-domain-containing proteins (Figure 3A). This SAC domain, originally noticed in E(z) and its murine homolog Enx-1 (Hobert *et al*., 1996), was also found adjacent to the SET domain of Ash1, YJQ8 and Su(var)3-9 but not Trx (Figure 3A). An alignment of the corresponding SAC domains revealed the conservation of three motifs, designated A, B and C, and a variation in their configuration among the SET proteins (Figure 3C). Motif C is present in all SAC domains, where it can be associated with either motif A (as in NSD1, Ash1 and YJQ8), motifs A and B [as in E(z)], or motif B [as in Su(var)3-9] (Figure 3C). As searches in protein databases revealed this Cys-rich domain only in proteins containing the SET domain, we called it SAC for SET domain-associated cysteine-rich domain.

In addition to the SAC and SET domains, NSD1 contains five zinc finger-like motifs which all match the consensus sequence of the PHD finger, also designated as the C4HC3 motif (Aasland *et al*., 1995; $PHD_{I\ to\ V}$ in Figures 2A and 3A). However, three of the five NSD1 PHD fingers have a His residue in place of the seventh Cys residue (PHD_{II}, PHD_{III} and PHD_{V}; Figure 3C) and may therefore belong to a new subclass of PHD-H2 fingers. $PHD_{I\ to\ IV}$ lie adjacent to each other from residues 1443 to 1646, N-terminal to the SAC domain (Figures 2A and 3A), whereas PHD_{V} is located C-terminal to the SET domain (residues 2018–2060). There are also four PHD fingers located N-terminal to the SET domain of the *Drosophila* Trx protein and its human homolog (HRX/All-1/MLL; Stassen *et al*., 1995 and references therein; Figure 3A and C). In contrast, Ash1 contains a single C-terminally located PHD finger (Figure 3A and C), whereas Pcl, the product of the *Drosophila* polycomb-group gene *Polycomb-like*, has two PHD fingers, but no SET domain (Lonie *et al*., 1994; Figure 3C). Adjacent to the C-terminus of the NSD1 PHD_{V}, there is a sixth region rich in cysteine and histidine residues, which could correspond to a zinc finger-like motif, however different from PHD fingers and other Cys-rich motifs so far described (residues 2062–2103; Figure 2A).

Overall, the predicted sequence of NSD1 is moderately rich in serine residues (11%) that are spread throughout the 1000 N-terminal residues and 500 C-terminal residues. A negative charge cluster was found from position 161 to 187, as well as a proline-rich region (20% in 143 residues) in the C-terminal part of the protein (residues 2105–2247; Figure 2A). NSD1 also contains several putative single and bipartite nuclear localization signals (residues 948–951; 1054–1071; 1169–1174; 1192–1199; 1301–1309; 1364–1386; and 1993–2006; underlined in Figure 2A). In interphase cells, NSD1 was found within the nucleus, but excluded from nucleoli and condensed heterochromatin (Figure 3E).

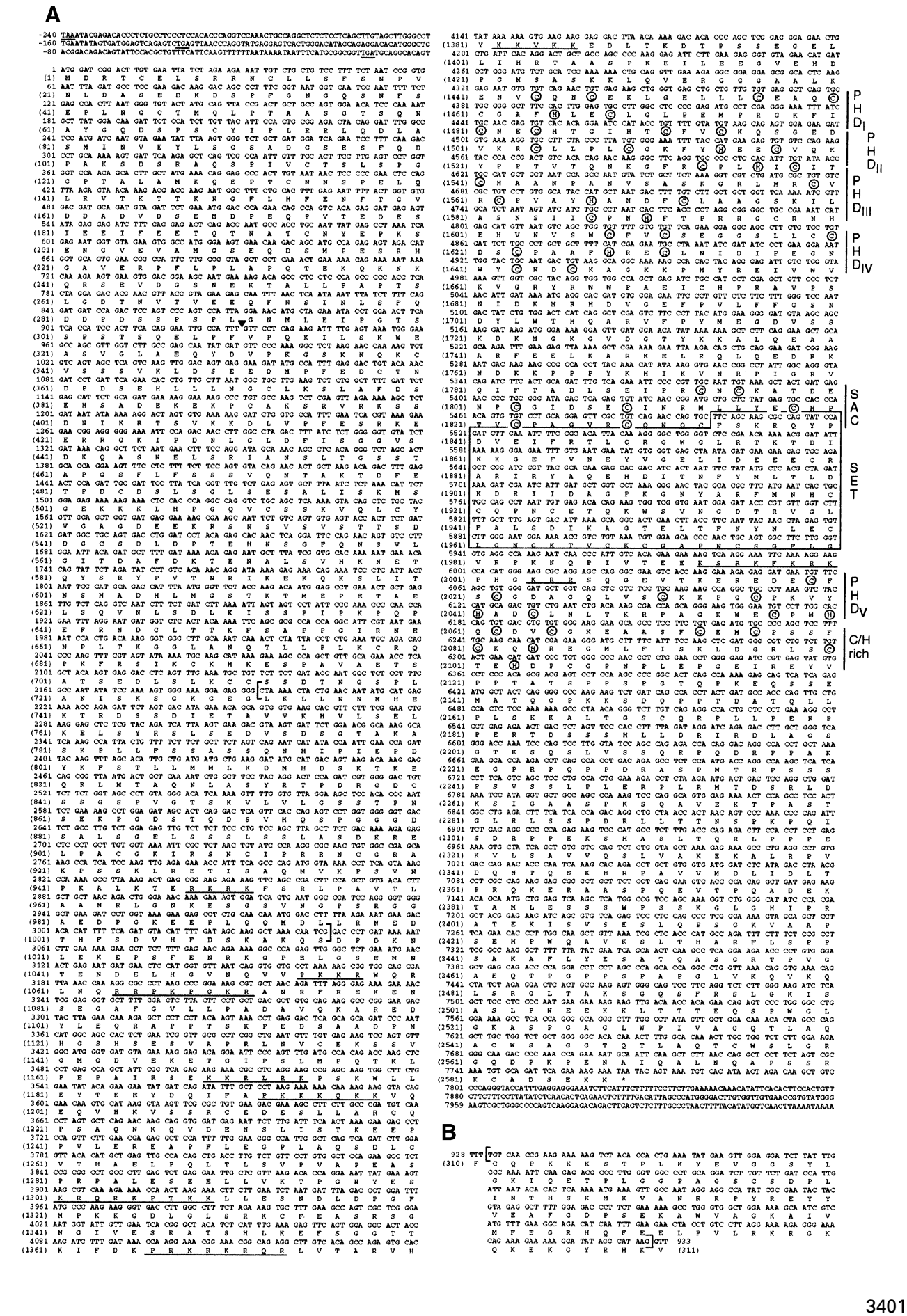
Interaction between RARα and NSD1 is reduced in the presence of retinoic acid

The NSD1 coding sequence was fused to that of the VP16 AAD (AAD–NSD1; Figure 4A) and expressed in yeast PL3 with either the 'unfused' DBD of ERα (amino acids 176–282; as a control) or the DBD–RARα(DEF) fusion protein (Figure 4A). In the absence of T-RA, coexpression of the two chimeric proteins resulted in a 36-fold increase in URA3 reporter activity as compared with the control [DBD–RARα(DEF) + AAD; Figure 4A], indicating a functional interaction between NSD1 and unliganded RARα LBD. The RARα-interacting domain of NSD1 was mapped to a 153 amino acid region (residues 738–891; Figure 4A), which does not include any of the NSD1 conserved domains (PHD, SAC or SET).

The addition of T-RA to yeast cells coexpressing DBD–RARα(DEF) and full-length NSD1 or truncated NSD1(738–891) resulted in ~4- and 10-fold decreases in reporter activity, respectively (Figure 4A). Similar ligand-dependent decreases were observed for the reciprocal interactions [DBD–NSD1(738–891) and AAD–RARα1, Figure 4B], and by substituting the ERα DBD for the LexA DBD in another version of the two-hybrid system using lexA binding sites within the context of the GAL1 promoter (Vojtek *et al*., 1993; Figure 4C). This ligand negative effect was also evident from the growth of yeast PL3 coexpressing RARα(DEF)–ER.CAS and AAD–NSD1.6 [AAD–NSD1(732–1015); Figure 2A] on AU-containing medium (Figure 1B).

The direct binding of NSD1 to RARα was studied *in vitro* using purified recombinant proteins. A purified *Escherichia coli*-expressed histidine-tagged NSD1 fusion protein [His-NSD1(738–891)] was incubated in the presence or absence of 1 μM T-RA with the glutathione *S*-transferase fusion protein GST–RARα(DEF) attached to glutathione–Sepharose beads (Figure 4D). Western blotting revealed that His-NSD1(738–891) was retained on GST–RARα(DEF), but not on 'control' GST beads (Figure 4D, lanes 2 and 3). As observed in the two-hybrid assay, addition of T-RA severely decreased, but did not eliminate, the binding of His-NSD1(738–891) to GST–RARα(DEF) (Figure 4D, lanes 3 and 4). Thus, residues 738–891 of

Fig. 2. Nucleotide and amino acid-deduced sequence of mouse cDNA encoding NSD1. (**A**) The 8277 nucleotides of NSD1 cDNA, the 2588 amino acid ORF and 5′-flanking termination codons (underlined) are shown. The filled triangle indicates the location of a 312 bp insertion encoding 104 additional amino acids [see (B)]. The boundaries of the original two-hybrid clone NSD1.6 (residues 732–1015) are in parentheses. Basic residues that might serve as nuclear localization signals are underlined. Cysteine and histidine residues belonging to the PHD fingers, the SAC motif and the C-terminal Cys–His cluster (amino acids 2062–2103) are highlighted with circles. The SET domain is boxed. (**B**) Sequence of the 312 bp internal insertion found in several NSD1 cDNA clones.


A
B
PHD I
PHD II
PHD III
PHD IV
PHD V
SAC
SET
C/H rich

NSD1 can interact with the LBD of RARα both in yeast and *in vitro*, and this direct interaction is severely reduced in the presence of ligand.

The AF-2 AD core is required for the ligand-dependent decrease of NSD1–RARα interaction

Deletion mutants of RARα were fused to the VP16 AAD and assayed for interaction with DBD–NSD1(738–891) in yeast PL3. No increase in reporter activity was observed with the AF-1-containing N-terminal A/B region [Figure 5A; AAD–RARα(1–87)] or the DBD (region C) and D region [AAD–RARα(80–211)] of the receptor. In contrast, and as expected (Figures 1A and 4A), a 12-fold activation that decreased 5-fold upon addition of T-RA was observed upon coexpression of DBD–NSD1(738–791) and AAD–RARα(170–462) that includes the entire LBD/AF-2 (helices 1–12, amino acids 181–416; Wurtz *et al*., 1996; Figure 5A). A C-terminal truncation that deleted H12 which contains the AF-2 AD core had little effect on the constitutive interaction with NSD1, but prevented its ligand-dependent decrease [compare AAD–RARα(170–403) with AAD–RARα(170–462) in Figure 5A], while still allowing T-RA binding (Durand *et al*., 1994). Similarly, a RARα mutant bearing an internal deletion of the AF-2 AD core interacted strongly with NSD1, irrespective of the presence of T-RA [AAD–RARαΔ408–416; Figure 5A]. Furthermore, a C-terminal fragment of RARα encompassing H12 failed to interact with NSD1 [AAD–RARα(390–462); Figure 5A]. Thus, the RARα AF-2 AD core is not necessary to mediate the interaction with NSD1(738–891), but it is essential for the ligand-dependent decrease of NSD1-RARα interaction.

Several RARα mutants carrying point mutations in the AF-2 AD core, all of which are known to abrogate AF-2 activity in transfected animal cells (Durand *et al*., 1994), were tested for their ability to interact with NSD1 in yeast. Assays were also performed with the nuclear receptor corepressor N-CoR that, like NSD1, interacts with RARα in the absence, but not in the presence, of the ligand (Figure 5B; Hörlein *et al*., 1995; see Introduction). RARα(DEF)L409A/I410A, in which the conserved hydrophobic residues L409 and I410 were replaced by Ala residues, similarly interacted with NSD1(738–891) and N-CoR(2239–2300) both in the absence and presence of T-RA (Figure 5B). In contrast, mutations replacing the conserved hydrophobic residues M413 and L414 with Ala residues [RARα(DEF)M413A/L414A] or the conserved acidic residues E412 and E415 with Gln residues [RARα(DEF)E412Q/E415Q] did not affect these interactions (Figure 5B). Thus, not all of the conserved residues of the AF-2 AD core of the RARα LBD that are important for transactivation by AF-2 are required for the drastic decrease in NSD1 interaction upon ligand binding.

Integrity of the CoR box of RARα is required for interaction with NSD1

The interaction of N-CoR with RARα and TRβ has been shown to require a conserved sequence lying within the LBD α-helix H1, termed the CoR box (Hörlein *et al*., 1995; Kurokawa *et al*., 1995; Wurtz *et al*., 1996; Figure 5C). Point mutations in the CoR box of RARα and TRβ that abolish N-CoR interaction, but do not influence DNA binding, ligand binding or transactivation have been identified (Kurokawa *et al*., 1995; Vivat *et al*., 1997). Three RARα CoR box mutants were tested to investigate the importance of the RARα CoR box for interaction with NSD1 (Figure 5C). All three mutants were severely impaired in their ability to interact with NSD1 in the absence of ligand, while still binding, in a ligand-dependent fashion, the putative transcriptional mediator mTIF1α as efficiently as its WT counterpart (Le Douarin *et al*., 1995a) (Figure 5C, and data not shown). Thus, as in the case of N-CoR, an intact CoR box is required for the ligand-independent NSD1 interaction.

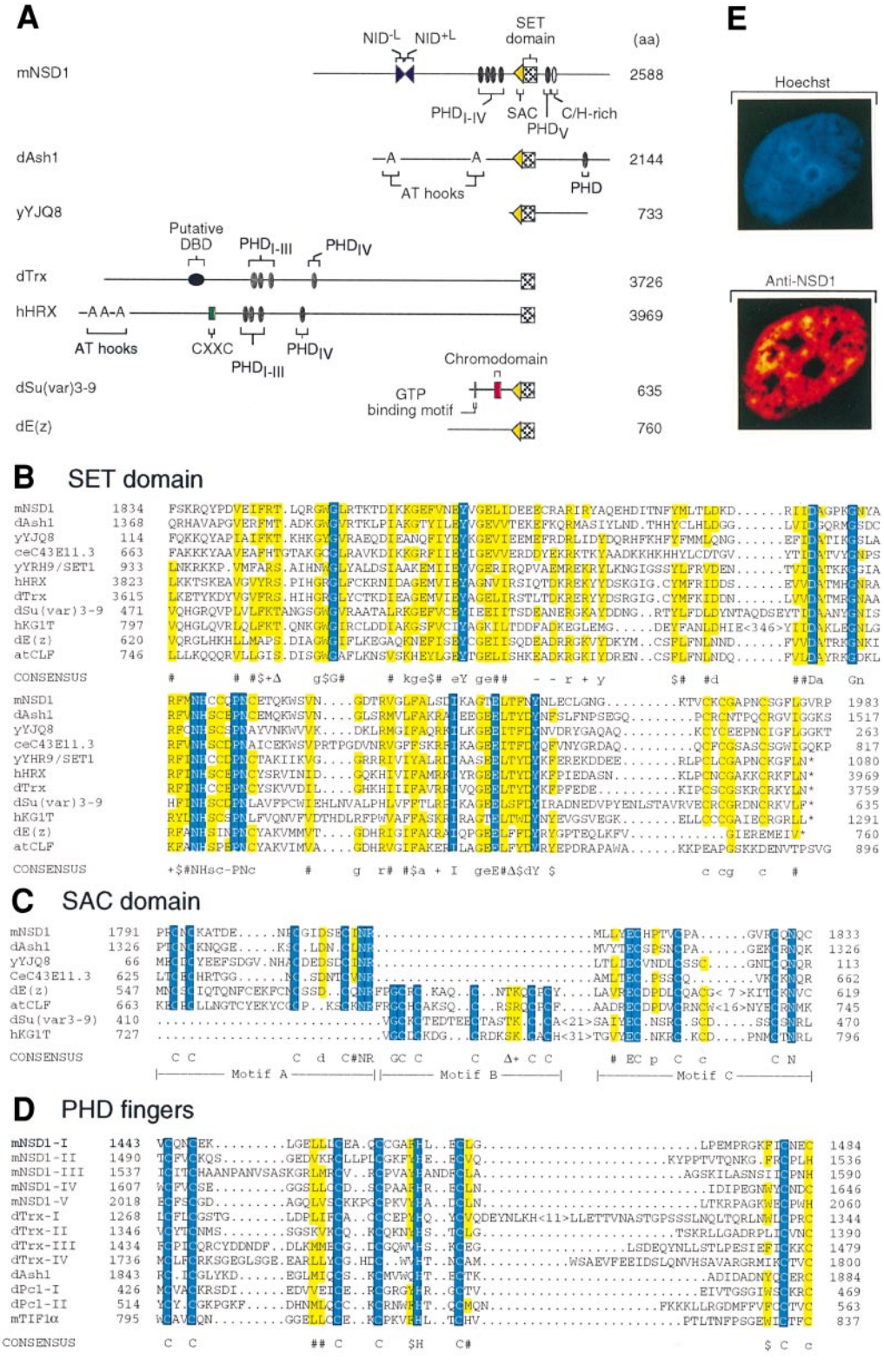
NSD1 interacts differentially with the LBDs of RARα, TRα, RXRα and ERα

DBD–TRα and –RXRα fusion proteins were coexpressed in yeast PL3 with AAD–NSD1 (Figure 6A). DBD–TRα(DE) stimulated expression of the URA3 reporter when coexpressed with either AAD–NSD1 or AAD–NSD1(738–891) in the absence of ligand, indicating that NSD1 can interact with the unliganded LBD of TRα in yeast cells. The extent of this stimulation (compared with the AAD control) was decreased upon thyroid hormone (T3) addition (Figure 6A). Thus, as observed with RARα(-DEF), the presence of the ligand decreases NSD1–TRα(DE) interaction. In marked contrast, a strictly 9C-RA-dependent (albeit weak) interaction was observed with the C-terminal moiety of RXRα [Figure 6A, DBD–RXRα(DE) and AAD–NSD1 or AAD–NSD1(738–891)]. Similar interactions were observed using LexA–LBD

Fig. 3. NSD1 is a nuclear protein containing a SET domain and multiple PHD fingers. (**A**) Schematic representation of known proteins containing SET domains. NID^{-L} and NID^{+L} refer to the nuclear receptor interaction domains of NSD1 that specifically bind the apo-LBDs of RAR and TR and the holo-LBDs of RXR, ER, TR and RAR, respectively. 'SAC' refers to the SET domain associated Cys-rich domain. C/H-rich refers to an additional Cys–His-rich region where no conserved motif has been identified. AT hook, DNA-binding domain originally characterized in the HMG-I(Y) proteins (Reeves and Nissen, 1990); DBD, putative DNA binding domain in dTrx (Stassen *et al*., 1995) ; CXXC, Cys-rich motif found in DNA methyltransferases and a methyl-CpG binding protein PCM1 (Cross *et al*., 1997). DDBJ/EMBL/GenBank accession Nos: *Drosophila* Ash1 (dAsh1, U49439); *S.cerevisiae* YJQ8 [yYJQ8 (also named YJL168C or JO520), P46995]; *Drosophila* Trithorax (dTrx, Z31725); human Trithorax [hHRX (also named All-1 or MLL), L04284]; *Drosophila* Su(var)3–9 [dSu(var)3–9, P45975]; *Drosophila* Enhancer of zeste [dE(z), U00180]. Proteins are shown roughly to scale and (aa) numbers refer to protein lengths. (**B**) Alignment of the SET domains. The sequences were aligned using both the program of CLUSTAL W (Thompson *et al*., 1994) and manual adjustment. Numbers refer to amino acid positions in the corresponding proteins. Invariant amino acids are highlighted in blue. Amino acids conserved in >60% of the proteins are highlighted in yellow. Consensus symbols are: #, hydrophobic residues (L, I, V, M); $, aromatic residues (F, W, Y). Δ, S or T; +, basic (K, R); –, acidic (E, D). DDBJ/EMBL/GenBank accession Nos: *Caenorhabditis elegans* C43E11.3 (ceC43E11.3, U80437); *S.cerevisiae* YHR9/SET1 [yYHR9/SET1 (also named YHR119W), P38827]; human KG1T (hKG1T, D31891); *Arabidopsis* CLF (atCLF, Y10580). (**C**) Alignment of the SAC domains. The three conserved sequence motifs A, B and C are indicated. Symbols are as in (B). (**D**) Alignment of the PHD fingers. Symbols are as in (B). Proteins with more than one PHD finger are indicated by the protein name followed by a dash and the ordinal position of the domain in the sequence. DDBJ/EMBL/GenBank accession Nos: *Drosophila* Polycomblike (dPcl, L35153); mouse TIF1α (mTIF1α; S78219). (**E**) Subcellular localization of NSD1. Endogenous NSD1 was detected in Hela cell nuclei using immunofluorescence and confocal microscopy. The upper panel shows the Hoechst DNA staining and the lower panel corresponds to the immunodetection with anti-NSD1 mAbs.

fusion proteins coexpressed with AAD–NSD1 in the yeast reporter L40 and a strictly ligand (E2)-dependent interaction was also found between the DEF region of ERα and NSD1 (Figure 6B). Interestingly, these ligand-dependent ERα and RXRα LBD interactions with NSD1 were severely decreased or abrogated by mutations in the conserved residues of the AF-2 AD core known to impair AF-2 activity of these receptors (Danielian *et al*., 1992, 1995a) (Figure 6C and D), and no interaction was observed between ERα and NSD1 in the presence of the AF-2 antagonist hydroxytamoxifen (data not shown). Thus, in contrast to RARα and TRα, the interaction of the LBDs of RXRα and ERα with NSD1 in yeast is dependent on both the presence of an agonistic ligand and the integrity of the AF-2 AD core (H12).

To investigate whether these functional interactions in



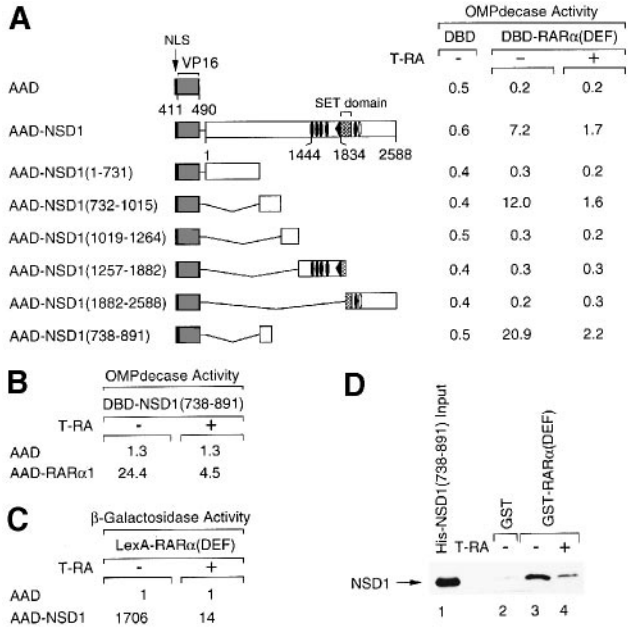


Fig. 4. Interaction between NSD1 and RARα is severely reduced in the presence of retinoic acid. (**A**) Mapping of the RARα-interacting domain in NSD1. Plasmids expressing full-length NSD1 or various regions of NSD1 fused to the VP16 AAD were introduced into yeast PL3 together with either the 'unfused' DBD of ERα or DBD–RARα(DEF). Transformants were grown in liquid medium ±500 nM T-RA. Extracts were prepared and assayed for OMPdecase activity, which is expressed in nmol substrate/min/mg protein. Values (±10%) are the averages from three independent transformants. Note that the expression of all AAD fusion proteins was confirmed by Western blotting using the antibody 2GV-4 against VP16 (data not shown). (**B**) Reciprocal two-hybrid interaction between RARα and NSD1. PL3 transformants expressing the indicated fusion proteins were treated as described in (A). (**C**) Co-expression of LexA–RARα(DEF) with AAD–NSD1 stimulates a *lacZ* reporter gene in a severely ligand-reduced manner. The indicated LexA and AAD fusions were assayed for interaction in the yeast reporter strain L40 grown in the presence (+) or absence (–) of 500 nM T-RA. β-galactosidase activities are expressed in nmol substrate/min/mg. (**D**) NSD1 interacts with RARα *in vitro*. Purified His-NSD1(738–891) was incubated in a batch assay with 'control' GST (lane 2) or GST-RARα(DEF) bound to glutathione-*S*–Sepharose beads in the absence (lane 3) or presence (lane 4) of 1 μM T-RA. Bound NSD1 was detected by Western blotting. Lane 1 shows 1/10 the amount of input His-NSD1(738–891), the position of which is indicated by an arrow.

yeast correspond to direct interactions, GST-receptor LBD fusions were assayed for their ability to bind His-tagged NSD1(738–891) *in vitro* (Figure 6E). In the absence of ligand, only GST–TRα(DE) bound His-NSD1(738–891) and, as observed with GST–RARα(DEF), addition of the ligand decreased this binding (Figure 6E). In contrast, a strictly ligand-dependent interaction was detected between NSD1(738–891) and the LBDs of RXRα and ERα (Figure 6E), and this interaction was also dependent on the presence of the AF-2 AD core (Figure 6E, lanes 6–13). Taken together, all of these results demonstrate that the functional interactions detected in yeast correspond to direct interactions between NSD1 and the various LBDs, and moreover that, depending on the receptor, the ligand has opposite effects on these interactions, whereas the AF-2 core motif appears either dispensible (RARα) or required (RXRα and ERα).

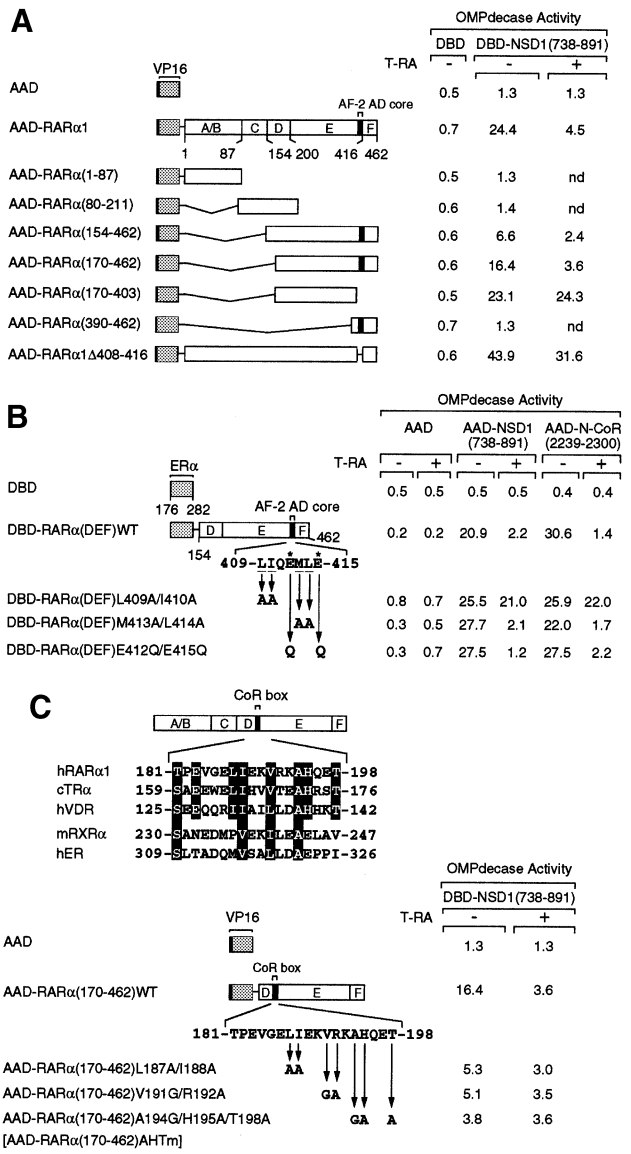


Fig. 5. Two-hybrid interaction between various RARα mutants and NSD1. (**A**) Residues 170–403 of RARα are sufficient for interaction with NSD1. The indicated regions of RARα were fused to the VP16 AAD and assayed for interaction with either DBD or DBD–NSD1(738–891) in yeast PL3 grown in the presence (+) or absence (–) of 500 nM T-RA. (**B**) Specific residues within the AF-2 AD core of RARα are critically involved in the ligand-dependent release of NSD1 and N-CoR from RARα. The sequence of the AF-2 AD core within H12 of RARα LBD is shown. The conserved hydrophobic and acidic residues are underlined and indicated by a star, respectively. The indicated DBD–RARα fusions were assayed for interaction with AAD–NSD1(738–891) in yeast PL3 grown in the absence or presence of 500 nM T-RA. (**C**) Integrity of the CoR box of RARα is required for interaction with NSD1. The sequences of the CoR box/helix 1 of human RARα1, chicken TRα, human VDR, mouse RXRα and human ERα were aligned according to the common fold identified by Wurtz *et al.* (1996). Residues of RARα that are conserved are highlighted. The indicated mutants of RARα were fused to the AAD of VP16 and assayed for interaction with DBD–NSD1(738–891) in yeast PL3 grown in the presence (+) or absence (–) of 500 nM T-RA. In all panels, OMPdecase activities are expressed in nmol substrate/min/mg protein. Values (±10%) are the averages from three independent transformants. Expression of all the DBD and AAD fusion proteins indicated was confirmed by Western blotting using the antibodies F3 against the F region of ERα and 2GV-4 against VP16, respectively (data not shown).

NSD1 contains two distinct interaction domains to which nuclear receptors bind differentially in the presence and absence of ligand

The above results suggest that NSD1 may contain distinct interacting surfaces to which NRs bind differentially in the presence and absence of ligands. This possibility was investigated using shorter NSD1 truncated mutants in the two-hybrid assay (Figure 7A). Functional interactions were observed between DBD–NSD1(738–788) and either AAD–RARα(170–462) or AAD–TRα(DE), and these interactions were abolished upon addition of the cognate ligand (Figure 7A and B). In contrast, no interaction was detected between NSD1(738–788) and AAD–RXRα(DE) or AAD–ERα(DEF), irrespective of the presence of the ligand (Figure 7A). These LBDs, however, exhibited a strong ligand-dependent interaction with DBD–NSD1(788–841) (Figure 7A). Interestingly, this 788–841 region of NSD1 also interacted with the LBDs of RARα (although weakly) and TRα in a strictly ligand-dependent manner (Figure 7A), thus accounting for the residual interactions observed between RARα or TRα and full-length NSD1 or NSD1(738–891) upon ligand addition (Figures 6A and 7A; also see below).

Thus, NSD1 appears to possess two neighbouring NR interacting surfaces, referred to here as NID^{-L} and NID^{+L} for nuclear receptor interaction domain minus ligand and plus ligand, respectively. NID^{-L} specifically interacts with the unliganded LBDs of RARα and TRα, whereas NID^{+L} can interact with the liganded LBD of either RXRα, ERα or TRα, and (less efficiently) RARα. This conclusion was supported by the demonstration that the same mutations in the RARα CoR box which were deleterious for the interaction with NSD1 (Figure 5C) also prevented the interaction with NID^{-L}, whereas they did not affect the ligand-dependent interaction of RARα with NID^{+L} (Figure 7B). Mutations in the AF-2 AD core (Figure 5B) were also tested for their effect on RARα interaction with either NID^{-L} or NID^{+L} in yeast (Figure 7C). None of these mutations affected the interaction with NID^{-L}, whereas two of them, L439A/I410A and E412Q/E415Q, abolished the NID^{+L} interaction (Figure 7C). Importantly, as in the case of RARα interaction with NSD1(738–891) (Figure 5B), the L409A/I410A mutation in the RARα AF-2 AD core prevented the ligand-induced decrease of NID^{-L}–RARα interaction (Figure 7C). Thus, the RARα sequence (and ligand) requirements for interaction with the NID^{-L} and NID^{+L} of NSD1 in yeast are clearly different. Accordingly, we could not find any significant sequence similarity between NID^{-L} and NID^{+L}.

A segment extending from residues 802 to 814, which is predicted (Rost and Sander, 1993) to form an α-helix, contains a motif (FxxLL) related to the NR box motif LxxLL that was originally identified in the nuclear receptor-interacting domain of TIF1α (Le Douarin *et al*., 1996), and subsequently found in other putative NR coactivators (Heery *et al*., 1997; Torchia *et al*., 1997; Voegel *et al*.,

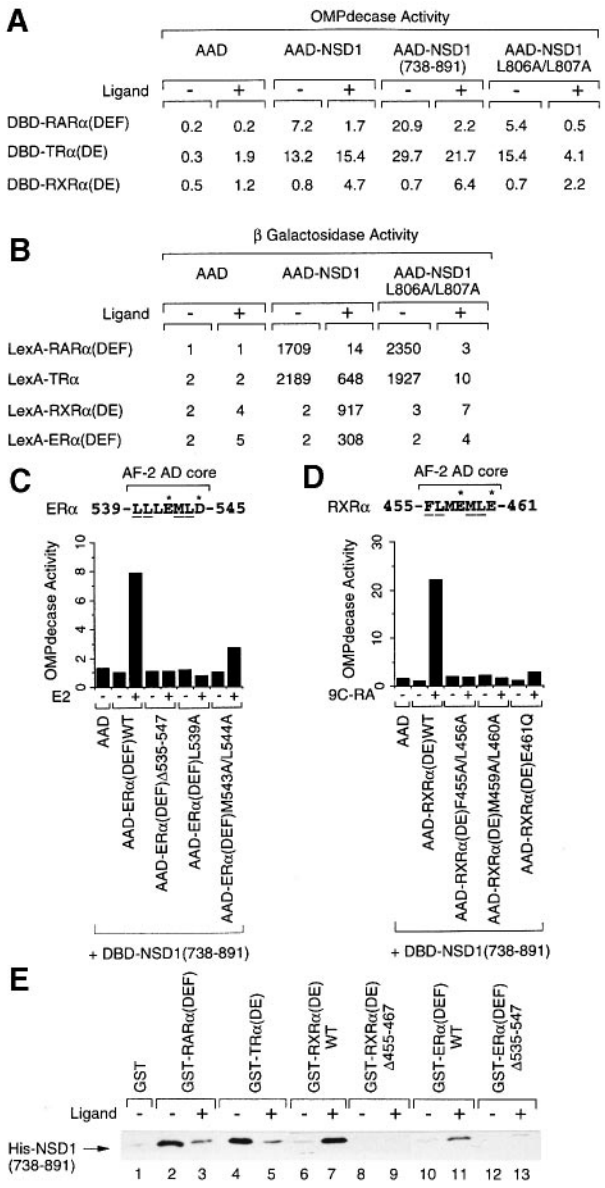


Fig. 6. Differential interactions between NSD1 and the LBDs of various NRs. (**A**) Differential effects of ligand binding on interaction between NSD1 and the LBD of RARα, TRα and RXRα. The indicated LBDs were fused to the ERα DBD and assayed for activation with VP16 AAD either alone or fused to wild-type or mutated NSD1 (see below) in yeast PL3 grown in the presence (+) or absence (–) of the cognate ligand (500 nM T-RA for RARα, 5 μM T3 for TRα, 500 nM 9C-RA for RXRα). OMPdecase activities are expressed in nmol substrate/min/mg protein. Values (±10%) are the averages from three independent transformants. (**B**) NSD1 interacts with the LBD of ERα in a ligand-dependent manner, and the double L806A/L807A mutation (Figure 7D and E) in NSD1 reduces the interaction with liganded RARα, TRα, RXRα and ERα, but not with unliganded RARα and TRα. Yeast L40 transformants expressing the indicated LexA and AAD fusions were ligand-treated as in (A); 1 μM E2 was used for the transformants expressing LexA-ERα(DEF). β-galactosidase activities are expressed in nmol substrate/min/mg. (**C** and **D**) Integrity of the AF-2 AD cores of ERα and RXRα are required for NSD1 interaction. DBD–NSD1(738–891) was coexpressed with the indicated mutants of ERα (in C) and RXRα (in D) fused to the AAD of VP16 in yeast PL3 grown in the presence (+) or absence (–) of the cognate ligand (1 μM E2 for ERα, 500 nM 9C-RA for RXRα). OMPdecase activites are expressed as in (A). In panels A–D, expression of the AAD fusion proteins was confirmed by Western blotting using the antibody 2GV-4 against VP16 (data not shown). (**E**) Effect of ligand on NSD1/NR interaction *in vitro*. Purified His-NSD1(738–891) was incubated with control 'GST' (lane 1) or GST fusions containing the indicated LBDs (lane 2–13) bound to glutathione *S*–Sepharose beads in the presence or absence of the cognate ligand (1 μM T-RA for RARα, 5 μM T3 for TRα, 1 μM 9C-RA for RXRα, 1 μM E2 for ERα). Bound NSD1 was detected by Western blotting.

1998 and references therein; Figure 7D). To determine whether this NSD1 802–814 segment has some NR binding activity on its own, we fused it to the ERα DBD and tested for its interaction with NR LBDs in yeast. A ligand-dependent interaction was observed with all LBDs tested (Figure 7E). As point mutations replacing Leu residues at positions 4 and 5 of the TIF1α LxxLL motif with Ala residues abrogated TIF1α–RXRα interaction (Le Douarin *et al.*, 1996), similar mutations were introduced into the corresponding residues of the NSD1 NR box motif. The mutated sequence NSD1(802–814)L806A/L807A failed to interact with all receptor LBDs (Figure 7E). The S804 and T805 residues were also mutated, even though they are not conserved among the various NR box motifs (Figure 7D). Their substitution by Ala residues abrogated the interaction with all NRs tested (Figure 7E), which may indicate a preference for hydrophilic residues at these positions. In contrast, mutation of the Phe residue F803 to a Tyr or an Ala residue had no effect on these interactions (Figure 7E), suggesting that any hydrophobic (aromatic or aliphatic) residues may be tolerated at this conserved position.

To investigate whether the NID^{+L} NR box motif FxxLL is actually functional in NSD1, we introduced the double L806A/L807A mutation in the context of full-length NSD1 (Figure 6A and B). The interaction with the unliganded LBDs of RARα or TRα was not affected. In contrast, the interactions occurring with RARα and TRα in presence of the ligand were strongly decreased (Figure 6A and B). Importantly, there was very little, if any, ligand-dependent interaction with RXRα and ERα (Figure 6A and B). Taken together these data indicate that the FxxLL motif is indeed required for ligand-dependent binding of nuclear receptors to NSD1.

RARα and ERα interact with NSD1 in mammalian cells

To investigate whether RARα and NSD1 could also interact in mammalian cells, Cos-1 cells were transiently transfected with RARα and NSD1(1–891), which contains the receptor NIDs, but none of the putative NLS (see above). Unlike the nuclear-localized endogenous NSD1 (Figure 3E), NSD1(1–891) was detected by immunofluorescence in the cytoplasm of the transfected cells (Figure 8A and B). As RARα was nuclear irrespective of the presence of T-RA (Figure 8C and D; data not shown), NSD1(1–891) and RARα were then cotransfected, and the localization of both proteins was analyzed by double labeling immunocytofluorescence. In the absence of T-RA, and in >90% of the cells expressing both proteins, RARα was partly shifted into the cytoplasm where it adopted the localization pattern of NSD1(1–891) (Figure 8F and G, compare panel anti-NSD1 with panel anti-RARα). In contrast, RARα was mostly nuclear in T-RA-treated cells (Figure 8J).

An interaction between NSD1 and the liganded ERα was also demonstrated in transfected Cos-1 cells. NSD1 and ERΔNLS, which contains an internal deletion preventing its nuclear localization (Ylikomi *et al.*, 1992; Le

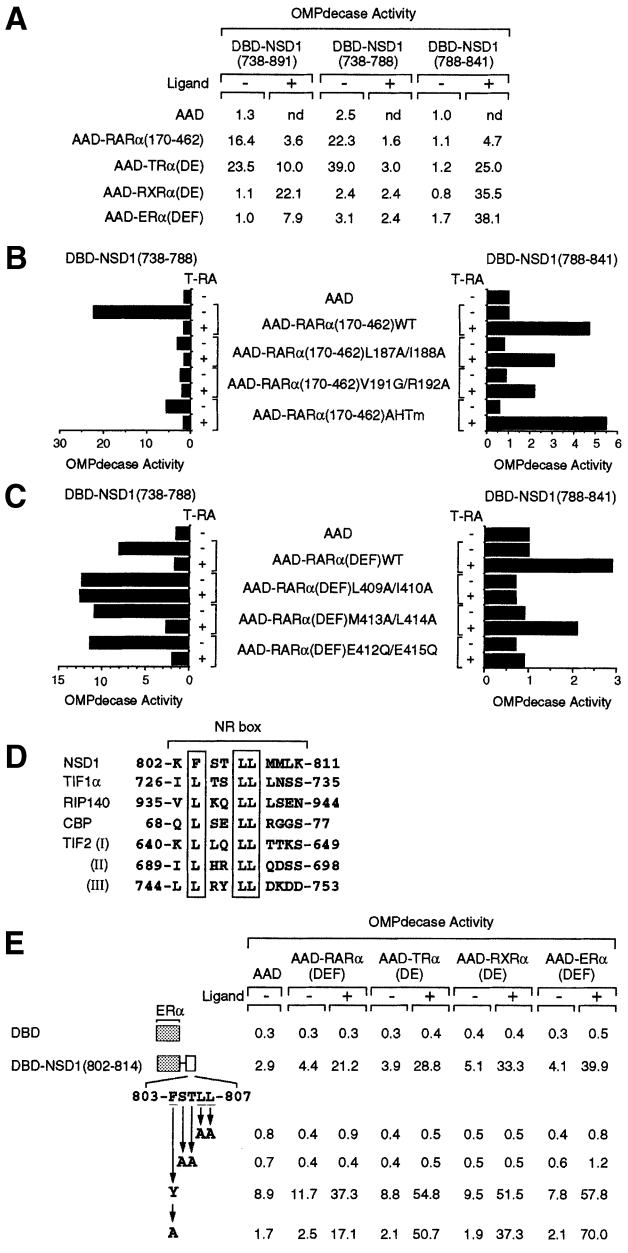


Fig. 7. Differential interactions between two NSD1 domains and nuclear receptors. (**A**) Identification of a domain (aa 738–788) that interacts with the apo-LBD of RARα and TRα, and a domain (aa 788–841) that interacts with all holo-LBDs tested. The indicated DBD and AAD fusion proteins were co-expressed in yeast PL3. Transformants were treated as in Figure 6. OMPdecase activities are expressed in nmol substrate/min/mg protein. Values (±10%) are the averages of three independent transformants. (**B** and **C**) The RARα sequence requirements for interaction with NSD1(738–788) and NSD1(788–841) are different. Several AAD fusion mutants carrying specific mutations in the CoR box/helix 1 of RARα (in B) or in the AF-2 AD core/helix 12 of RARα (in C) were assayed for interaction with either DBD–NSD1(738–788) or DBD–NSD1(788–841) in yeast PL3 grown in the presence or absence of 500 nM T-RA. OMPdecase activities are expressed as in (A). (**D**) NSD1 contains a FxxLL variant of the LxxLL NR box motif. Sequence alignment of the NR boxes identified in several putative coactivators. The conserved leucine residues are boxed. (**E**) Effects of mutations in the NSD1 FxxLL motif located at amino acids 802–814 on interaction with various NR LBDs. The indicated DBD–NSD1(802–814) fusion mutants were assayed for interaction with AAD–LBDs in yeast PL3. Transformants were treated as in Figure 6. OMPdecase activities are expressed as in (A). In panels A–C and E, expression of all DBD and AAD fusion proteins was confirmed by Western blotting using the antibodies F3 against the F region of ERα and 2GV-4 against VP16, respectively (data not shown).

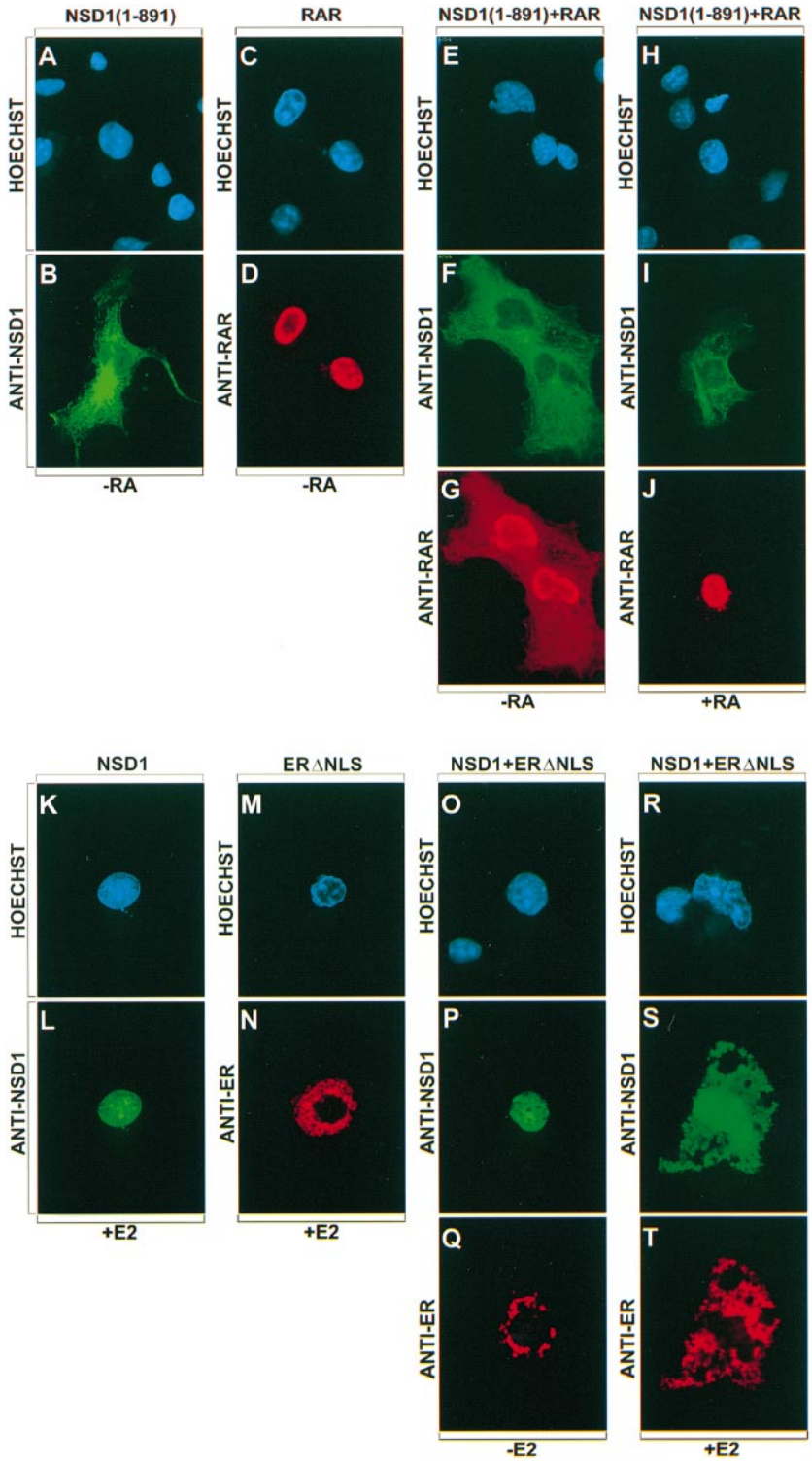


Fig. 8. RARα and ERα interact with NSD1 in mammalian cells. (**A**–**J**) NSD1 interacts with unliganded RARα in Cos-1 cells. pSG5-based expression vectors (10 μg) encoding either NSD1(1–891) or RARα were transfected alone (A, B and C, D) or in combination (E–J) into Cos-1 cells in the absence (A–G) or presence (H–J) of 1 μM T-RA, as indicated. Immunocytofluorescence studies using confocal microscopy were performed with anti-NSD1 and anti-RARα antibodies. (**K**–**T**) Ligand-dependent interaction between NSD1 and ERα in Cos-1 cells. In panels K, L and M, N, Cos-1 cells were transfected in the presence of 1 μM E2 with 10 μg NSD1 or ERαΔNLS expression vectors, respectively. In panels O–Q and R–T, cells were co-transfected with both expression vectors (10 μg) in the presence or absence of 1 μM E2, as indicated. The corresponding proteins were revealed as above using anti-NSD1 and anti-ERα monoclonal antibodies.

Douarin *et al*., 1995a), were cotransfected in the absence or presence of oestradiol (E2). In the absence of E2, NSD1 and ERΔNLS exhibited their characteristic nuclear and cytoplasmic localizations, respectively (Figure 8O–Q, compare with Figure 8K–N). In the presence of E2, the bulk of ERΔNLS remained cytoplasmic, whereas a fraction of NSD1 became cytoplasmic, co-localizing with ERΔNLS (Figure 8S and T).

Thus, as observed in yeast cells and *in vitro*, NSD1 appears to efficiently interact in mammalian cells with RARα in the absence, but not in the presence, of T-RA, whereas the presence of oestradiol is required for interaction with ERα.

Autonomous repression and activation functions in NSD1

Segments of NSD1, spanning the entire protein (Figure 9A), were fused to the GAL4 DBD and tested for transcriptional activity using GAL4 reporter genes in transiently transfected Cos-1 cells. GAL4–NSD1 fusion proteins were tested for repression of the chimeric trans-activator ER(C)–VP16 using a GAL4 reporter containing both two GAL4 binding sites (17M2) and an estrogen response element (ERE) in front of a β-globin (G) promoter-CAT fusion (17M2-ERE-G-CAT; Le Douarin *et al*., 1996). A ~50-fold repression was reproducibly observed with GAL4–NSD1(644–1881) (Figure 9B), whereas a much weaker repression (~3- to 4-fold) was associated with the NSD1 1708–2255 and 2201–2588 segments, and the 1–731 segment had no effect (Figure 9B). GAL4–NSD1(1089–1400) resulted in ~10-fold repression, whereas under similar conditions GAL4–NSD1(738–1264) and GAL4–NSD1(842–1089) repressed by 2–3-fold only (Figure 9B and C). In contrast to GAL4–NSD1(1089–1400), NSD1(1089–1400) on its own had no repression activity (Figure 9C). Thus, the 1089–1400 region of NSD1, which does not contain any of the conserved domains of the protein, appears to contain a transcriptional silencing domain.

To investigate whether NSD1 may contain autonomous activation domain(s), the various GAL4–NSD1 expression vectors were also co-transfected with the CAT reporter gene 17M5-TATA-CAT (Voegel *et al*., 1998) whose minimal promoter has a low basal level of activity. None of the GAL4–NSD1 stimulated the activity of the reporter upon transient transfection in a variety of mammalian cell lines (Cos-1, Hela, CV-1 and MCF-7) (data not shown). However, expressing NSD1 fragments fused to the ERα DBD [ERα(C)] (see above) in yeast PL3 specifically generated a strong stimulation of OMPdecase activity with DBD–NSD1(1–731) (Figure 9D; data not shown). Thus, the 1–731 segment of NSD1 appears to contain an efficient transcriptional activation domain, which can function in yeast but apparently not in mammalian cells, at least under the present cellular and promoter contexts.

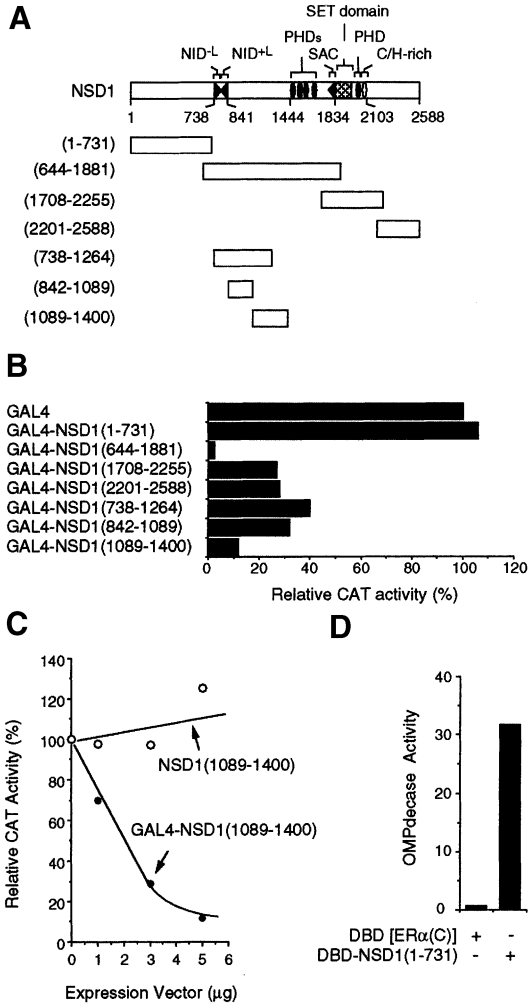


Fig. 9. NSD1 contains distinct repression and activation domains. (**A**) Schematic representation of the NSD1 regions linked to the DNA binding domain (DBD) of GAL4 (aa 1–147). (**B**) Analysis of the transcriptional activity of the GAL4–NSD1 fusion proteins. 2 μg of the 17M2-ERE-G-CAT reporter and 1 μg pCH110 were co-transfected into Cos-1 cells together with pSG5-based vectors expressing the activator ER(C)–VP16 (100 ng) and the GAL4 DBD (GAL4) either fused or unfused to the various regions of NSD1 (5 μg). CAT activities (±10%) resulting from activation by ER(C)–VP16 are expressed relative to that measured in the presence of the unfused GAL4 expression vector (taken as 100%). Values represent the averages of two independent triplicated transfections after normalization for the internal control β-galactosidase activity of pCH110. (**C**) Residues 1089–1400 of NSD1 repress transcription in a dose-dependent manner when tethered to DNA. The 17M2-ERE-G-CAT reporter (2 μg) was co-transfected into Cos-1 cells with ER(C)–VP16 expression vector (100 ng), together with increasing amounts of GAL4–NSD1(1089–1400) or NBSD1(1089–1400) expression vectors. CAT activities were expressed as in (B). (**D**) Residues 1–731 of NSD1 activate transcription in yeast. A yeast multicopy vector YEp90 expressing the 'unfused' DBD of ERα (aa 176–282; as a control) or DBD–NSD1(1–731) was introduced into the yeast reporter strain PL3. OMPdecase activity is expressed in nmol substrate/min/mg protein. Values (±10%) are the averages of three independent transformants.

Discussion

NSD1 contains conserved motifs found in proteins involved in the epigenetic control of transcription

NSD1 is a novel protein containing the evolutionarily conserved SET domain, that was first identified as a motif present in the *Drosophila* proteins Su(var)3-9, E(z) and Trx (Jones and Gelbart, 1993; Tschiersch *et al*., 1994), and then found in a number of eukaryotic proteins from yeast to mammals (Hobert *et al*., 1996). This family of proteins was subsequently divided into four subfamilies according to amino acid sequence identities within their SET domain (Laible *et al*., 1997; Prasad *et al*., 1997).

Su(var)3-9, the product of the *Drosophila suppressor of variegation (3) 9* gene (Tschiersch *et al*., 1994), its human homolog SUV39H (Laible *et al*., 1997), and the human proteins G9a and KG1T (Milner and Campbell, 1993), belong to one subfamily. E(z), the product of the *Drosophila Enhancer of zeste* gene (Jones and Gelbart, 1993), together with its two mammalian homologs EZH1 and EZH2 (Laible *et al*., 1997), constitutes another subfamily. The third subfamily comprises Trx, the product of the *Drosophila Trithorax* gene (Stassen *et al*., 1995), its mammalian homologs ALL-1/MLL/HRX (Tkachuk *et al*., 1992; Ma *et al*., 1993) and TRX2 (P.Angrand and F.Stewart, personal communication), the human ALL-1-related protein ALR (Prasad *et al*., 1997), and the *Saccharomyces cerevisiae* YHR9/SET1 protein (Nislow *et al*., 1998). The proteins encoded by the *Drosophila* gene *Ash1* (for *absent, small or homeotic disc 1* gene; LaJeunesse and Shearn, 1995; Tripoulas *et al*., 1996) and the *S.cerevisiae* gene *YJQ8* constitute the fourth subfamily. NSD1 belongs to this fourth subfamily as its SET domain is most similar to those of *Ash1* and *YJQ8*.

Each of the four SET domain subfamilies contains at least one member which was shown, or is presumed to be associated with chromatin and to function as a transcriptional repressor, a transcriptional activator or both. Su(var)3-9 functions in heterochromatinization (Tschiersch *et al*., 1994). dE(z), Trx and Ash1 are all involved in the heritable maintenance of repressive and active transcriptional states, and there is indirect evidence that these proteins function as components of complexes that affect chromatin structure (reviewed in Schumacher and Magnuson, 1997). Like other Polycomb group (Pc-G) members, E(z) may serve to maintain the target genes in a closed chromatin conformation (Jones and Gelbart, 1993), but at different developmental stages and in different tissues, E(z) may be involved in either repression or activation (LaJeunesse and Shearn, 1996). Trx and Ash1 are both activators of homeotic gene expression and, like other Trithorax group (Trx-G) members, they are supposed to antagonize the repressive activities of the Pc-G gene products by keeping chromatin in a transcriptionally active conformation (reviewed in Paro, 1990). It is noteworthy that some of the sites of Ash1 localization on polytene chromosomes are identical to sites reported to be binding sites for Trx and Pc-G members (Tripoulas *et al*., 1996). Recently, the yeast YHR9/SET1 protein has been shown to function both in telomeric silencing and in activation of transcription (Nislow *et al*., 1998). Interestingly, the SET domain of YHR9/SET1 on its own, as well as EZH2 but not SUV39H, can rescue the telomeric silencing defects of a set1-Δ mutant (Laible *et al*., 1997; Nislow *et al*., 1998). Thus, the SET domain may fulfill some conserved function in relation to chromatin organization and function.

Immediately preceding the SET domain, we have identified a Cys-rich region common to NSD1, Ash1, YJQ8, Su(var)3-9 and E(z), but not to Trx. This region, which is composed of different arrangements of three conserved motifs, corresponds to a novel protein domain, that we called SAC for SET-associated Cys-rich domain, as it is unique to proteins containing SET domains. As for the SET domain, evolution appears to have conserved the SAC domain; it is indeed present in YJQ8, one of the two yeast SET proteins. Interestingly, a thermosensitive mutant of E(z), E(z)^{61}C603Y, has been isolated and shown to contain a Cys to Tyr substitution at Cys603, a conserved cysteine residue of the SAC motif (Carrington and Jones, 1996). The existence of this mutation supports the suggestion that the SAC domain is functionally important, and the failure of this E(z) mutant to bind chromatin at restrictive temperature (Carrington and Jones, 1996) indicates that the SAC domain could be involved in chromosome binding.

In addition to the SET and SAC domains, NSD1 contains five PHD fingers belonging to the C4HC3 class, although three of them may form a subclass of PHD-H2 fingers. PHDs with C4HC3 motifs have been described in a number of proteins, some of which are known to be implicated in chromatin-mediated transcriptional regulation (Aasland *et al*. 1995). These include: (i) the Trx-G proteins Trx and Ash1, which in addition to a SET domain have, four PHDs and a single PHD, respectively; (ii) the Pc-G member, Polycomb-like (Pcl), which has two PHDs (Lonie *et al*., 1994); and (iii) the putative mediators of the ligand-dependent activation function AF-2 of nuclear receptors TIF1α and CBP/p300, which all have a PHD finger and a bromodomain (Glass *et al*., 1997 and references therein). TIF1α, as well as TIF1β which belong to the same gene family, interact specifically with the mammalian heterochromatinic proteins HP1α, MOD1 (HP1β) and MOD2 (HP1γ) (Le Douarin *et al*., 1996), whereas the CREB-binding protein CBP and p300 have an intrinsic histone acetyltransferase activity and interact with histone acetyltransferase (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996). Thus, proteins containing PHD fingers appear to also be involved in chromatin structure and function.

In view of its similarity to chromatin-related factors, it is therefore likely that NSD1 is involved in some aspects of transcriptional control at the chromatin level. This conclusion is supported by preliminary nuclear fractionation studies indicating that NSD1 is associated with chromatin (N.Huang and E.Remboutsika, unpublished data).

NSD1 contains both an interaction domain (NID^{-L}) to which RAR and TR can bind in the absence, but not in the presence, of ligand and an interaction domain (NID^{+L}) to which the binding of RXR, ER, TR and RAR is strictly ligand dependent

Our initial observations concerning the binding of NSD1 to RAR, TR, RXR and ER were puzzling in several respects. First, RAR and TR bound to NSD1 in the absence of ligand and this binding was decreased, but not abolished upon ligand addition, whereas the interaction between NSD1 and either RXR or ER was strictly ligand dependent. Secondly, point mutations in (or deletion of) the conserved residues of the AF-2 AD core (H12) that abolish transactivation by AF-2 in animal cells had different effects on NSD1–NR interaction. These mutations prevented NSD1 interaction with RXR and ER, whereas they did not affect NSD1 binding to unliganded RAR and TR, while impairing their release upon ligand addition.

These enigmatic observations were solved by the identification of two adjacent interaction domains in the NSD1–

NR interacting region, NID^{-L} and NID^{+L}, which exhibit distinct functional characteristics. NID^{-L} interacts with the unliganded apo-LBD of RAR and TR, but not with their liganded holo-LBDs, nor with either apo- or holo-LBDs of ER and RXR. The NID^{-L}–apo-LBD interaction requires the integrity of H1, whereas it is not prevented by alterations within H12, which includes the AF-2 AD core. In contrast, the lack of interaction between the NID^{-L} and the holo-LBD of RAR and TR requires the integrity of H12 which is indispensable for the LBD transconformation which occurs upon ligand binding. All of these requirements are similar to those which characterize the interactions of the RAR and TR apo- and holo-LBD with the corepressors N-CoR and SMRT. Strikingly, the same mutations, which in the CoR box of H1 prevent the binding of the apo-forms of TR and RAR to N-COR and SMRT (Hörlein *et al*., 1995; Kurokawa *et al*., 1995), also prevent their binding to NSD1. Moreover, the mutations in the AF-2 AD core which allow the liganded LBD of RARα to interact with the NID^{-L} of NSD1 are also those which allow the liganded RARα LBD to interact with N-CoR and SMRT. Thus, the same or very similar RAR and TR apo-LBD surfaces appear to interact with the N-CoR and SMRT corepressors and NSD1. Nevertheless, in spite of these similarities, we did not find any obvious conserved sequence motif in NSD1 NID^{-L} and the N-CoR or SMRT regions which are known to interact with the apo-forms of RAR and TR (Chen and Evans, 1995; Hörlein *et al*., 1995).

In marked contrast, NSD1 NID^{+L} interacts not only with the liganded holo-forms of TR and RAR (albeit weakly), but also efficiently with those of ER and RXR. These interactions, which do not require the integrity of the CoR box, are crucially dependent on the integrity of the AF-2 AD core (H12). In this respect, it is interesting to note that some mutations in the AF-2 AD core which are deleterious for the interaction between NSD1 NID^{+L} and the liganded RAR LBD, can nevertheless prevent the interaction between NID^{-L} and the liganded RAR holo-LBD (Figure 7C). This indicates that H12 is differentially involved in the ligand-induced transconformations of the RAR LBD which are required to disrupt the interaction surface with NID^{-L} and to generate the interaction surface with NID^{+L}. The ligand- and AF-2 AD core integrity-dependance of the binding of TR, RAR, RXR and ER to NSD1 NID^{+L} is reminiscent of the binding characteristics of these NRs to a number of putative NR AF-2 coactivators/mediators, e.g. TIF1α, RIP140, SRC-1, CBP/p300, pCIP/ACTR/RAC3/AIB1 and TIF2/GRIP1 (for references see Introduction). The NIDs of all of these putative coactivators contain single or multiple copies of the so-called NR box motif LxxLL, which was first identified in TIF1α (Le Douarin *et al*., 1996). A related α-helical motif FxxLL is present in NID^{+L}, and mutations within this motif abolish the ligand-dependent interactions between RAR, TR, RXR or ER and either the isolated motif or NSD1.

Thus, the present study clearly establishes that NSD1 possesses two independent NIDs, NID^{-L} and NID^{+L}, that bind the apo- and holo-form of the LBD of different subsets of NRs, with characteristics which have been previously ascribed to proteins functioning as corepressors and coactivators, respectively.

NSD1 may act as a bifunctional transcriptional intermediary factor both in the absence and presence of the ligand

As NSD1 appears to contain a silencing domain that can function autonomously in animal cells, it may act as a corepressor for unliganded TR and RAR bound to NID^{-L}, as well as for liganded TR, RAR, RXR and ER bound to NID^{+L}. The observation that, in addition NSD1 contains an activation domain that can function autonomously in yeast, suggests that NSD1 could also act as a coactivator for both unliganded and liganded TR and RAR, as well as for liganded RXR and ER. As RAR and TR are believed to function as heterodimers with RXR *in vivo* (Kastner *et al*., 1995, 1997; Chambon, 1996), NSD1 might conceivably interact through NID^{-L} with RAR or TR in the absence of ligand, and through NID^{+L} with RXR in the presence of ligand. Thus, NSD1 could be a highly versatile NR intermediary factor controlling transcription either negatively or positively, depending on both the promoter context of the target genes and the cell context with respect to other factors interacting with NSD1. As NSD1 is a nuclear protein tightly associated with chromatin (N.Huang and E.Remboutsika, unpublished data), it may participate to the epigenetic control of transcription, in a manner similar to that of *Drosophila* and yeast SET domain proteins which have been found to exert silencing and/or activation functions in a context-specific manner (see above). Because N-CoR and SMRT exert their silencing function through interactions within a multi-subunit complex that includes the SIN3A protein and an histone deacetylase (for references see Introduction), we tested whether NSD1 could also interact with SIN3 proteins (A and B) in a yeast two hybrid assay. However, we failed to detect such interactions under conditions where N-CoR strongly interacted with either SIN3A or B (data not shown).

In conclusion, NSD1 clearly possesses many of the properties expected for transcriptional intermediary factors which would be instrumental in the combinatorial mechanisms that act at the epigenetic level and must underlie the complex developmental and differentiation programs controlled by NR-mediated signaling pathways. Further molecular and genetic studies are obviously required to reveal the physiological function of NSD1.

Materials and methods

Plasmids

Receptor cDNAs used in this study correspond to human RARα1, ERα and VDR, chicken TRα and mouse RXRα (DDBJ/EMBL/GenBank database; Le Douarin *et al*., 1995a; vom Baur *et al*., 1996). RARα(DEF)–ER.CAS was expressed from the yeast HIS3 multicopy vector YEp90 (Heery *et al*., 1993). DBD and AAD fusion proteins were expressed from the yeast multicopy plasmids pBL1 and pASV3, respectively (Le Douarin *et al*., 1995b). pBL1 contains the HIS3 marker and directs the synthesis of epitope (F region of human ER)-tagged ER DBD fusion proteins. pASV3 contains the LEU2 marker and a cassette expressing a nuclear localized VP16 acidic activation domain (AAD), preceding a polylinker with cloning sites for the cDNA, and stop codons in all reading frames. LexA fusion proteins were expressed from a derivative of the yeast TRP1 multicopy plasmid pBTM116 (Vojtek *et al*., 1993). All inserts cloned into pBL1, pASV3 and pBTM116 were obtained by PCR and verified by sequencing. For transfection studies in mammalian cells, the indicated cDNAs were cloned into pSG5, the GAL4(1–147) chimeras were constructed by PCR amplification of the indicated regions of NSD1, followed by subcloning into pG4MpolyII (Tora *et al*., 1989).

The chimeric protein ER(C)–VP16, which encodes amino acids 176–280 of ER and amino acids 413–490 of VP16, has been described previously (Tora *et al*., 1989) as well as the reporter genes *17M5-TATA-CAT* (Voegel *et al*., 1998) and *17M2-ERE-G-CAT* (Le Douarin *et al*., 1996). For *in vitro* binding assays, the indicated cDNAs were fused to glutathione *S*-transferase (GST) in the pGEX-2T plasmid (Pharmacia; vom Baur *et al*., 1996). The HIS-NSD1(738–891) was cloned into the pET-15b plasmid (Novagen). Details concerning each construction are available upon request.

cDNA library screening

The construction of the mouse embryo cDNA library in the yeast AAD fusion vector pASV3 as well as the screening procedure were as described previously (vom Baur *et al*., 1996). To isolate the full-length NSD1 cDNA, a randomly primed mouse embryo cDNA library constructed in λZAPII was screened using the two-hybrid NSD1.6 cDNA insert (nucleotides 2194–3045; Figure 2A) as a probe. Several overlapping clones were obtained, including clones 6–12 (nucleotides –1099 to 3686) and 6–28 (nucleotides 1929–5766). To isolate clones containing the 3′ end of the NSD1 cDNA, a PCR product amplified from the C-terminal part of clone 6–28 was used to rescreen the library, which resulted in the isolation of clone 2–18 (nucleotides 4384–8920), among others. The 7764 bp full-length coding sequence of NSD1 was reconstituted in pBluescript from inserts 6–12, 6–28 and 2–18, using suitable restriction sites. The resulting construct was verified by sequencing.

Transactivation assays

Yeast PL3 transformants were grown exponentially for about five generations in minimal medium supplemented with uracil and the required amino acids. When necessary, medium was supplemented with the appropriate ligands. Yeast extracts were prepared and assayed for OMPdecase activity as described (Pierrat *et al*., 1992). L40 extracts were prepared and analyzed for β-galactosidase activity according to Rose *et al*. (1990). Transient transfection of mammalian cells and CAT assays were as described previously (Durand *et al*., 1994).

Antibodies and immunocytofluorescence

Monoclonal (mAbs) anti-NSD1 antibodies 1NW1A10 and 3NW3F8 were raised against amino acids 738–891 and 1715–1881 of NSD1, respectively. mAbs 2GV-3 and 2GV-4 are directed against the DBD of yeast GAL4 (amino acids 1–147) and VP16, respectively (Le Douarin *et al*., 1995a and references therein). RP(α)F is a rabbit polyclonal antibody against RARα (Gaub *et al*., 1992). mAbs B10 and F3 are directed against the B and F regions of ERα (Le Douarin *et al*., 1995a and references therein). Immunocytofluorescence studies were performed as described previously (Le Douarin *et al*., 1995a).

In vitro binding assays

Purified *E.coli*-expressed His-NSD1(738–891) (5 µg) was incubated with 5 µg of bacterially expressed GST fusion proteins bound on glutathione *S*–Sepharose beads in the presence of the cognate ligand or carrier (ethanol) for 1 h at 4°C in a final volume of 200 µl binding buffer (BB: 50 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.3 mM DTT, 10 mM $MgCl_2$, 0.1% NP40, 1 mM PMSF, 10% glycerol, containing protease inhibitors). The beads were washed four times with 1 ml of BB buffer, resuspended in a SDS-containing buffer, boiled for 10 min, and proteins were analyzed by SDS–PAGE. The ECL detection system was used for immunodetection as recommended by the supplier (Amersham).

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