The AB region of Nur77/NR4A1 mediates cell specificity and coactivator recruitment

The AF-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity and coactivator recruitment.

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ABSTRACT: Nur77/NGFI-B/NR4A1 is an ‘orphan member’ of the nuclear hormone receptor superfamily. Nur77 and its close relatives Nurr1 and NOR-1 bind as monomers to a consensus binding site [AAAGGTCA; NGFI-B binding response element (NBRE)]. The Nur77/NURR1/NOR1 nuclear receptors are classified as immediate early response genes which are induced through multiple signal transduction pathways. They have been implicated in cell proliferation, differentiation and apoptosis. Furthermore, the β-adrenergic, and calcium/calcineurin signalling pathways (which control muscle hypertrophy and fiber remodeling) regulate Nur 77 expression. However, the mechanism of coactivation and ligand independent trans-activation remains unclear. Hence we examined the molecular basis of Nur77-mediated cofactor recruitment and activation. We observed that Nur77 trans-activates gene expression in a cell specific manner. Nur77 operated in activation function–1 (AF-1) dependent manner. The AB region encodes an uncommonly potent N-terminal AF1 domain delimited to between amino acids 50 and 160, and is essential for the ligand independent activation of gene expression. SRC-2 (GRIP-1) modulates the activity of the N-terminal AF-1 domain. Moreover, SRC-2 dramatically potentiates the retinoid induced RXR dependent activation of the Nur77 LBD. Interestingly, the N-terminal AB region (not the LBD) facilitates coactivator recruitment and directly interacts with SRC, p300, PCAF, and DRIP205. Consistent with this, homology modeling indicated that the Nur77 LBD coactivator binding cleft was substantially different from that of RARγ, a closely related AF2 dependent receptor. In particular, the hydrophobic cleft characteristic of nuclear receptors was replaced with a much more hydrophilic surface with a distinct topology. This observation accounts for the inability of this nuclear receptor LBD to directly mediate cofactor recruitment. Furthermore, the AF-1 domain physically associates with the Nur77 C-terminal LBD, and synergizes with the RXR LBD. Thus, the AF-1 domain plays a major role in Nur77 mediated transcriptional activation, cofactor recruitment, and intra- and inter- molecular interactions.
INTRODUCTION: Nuclear hormone receptors (NRs) function as ligand activated transcription factors that regulate gene expression involved in reproduction, development and general metabolism (1). NRs function as the conduit between physiology and gene expression. The importance of NRs in human physiology is underscored by the extensive pharmacopeia that has been created to combat disorders associated with dysfunctional hormone signalling. These diseases affect every discipline of medicine (2). All members of the NR superfamily display a highly conserved structural organisation (1) with an amino terminal region AB (that encodes activation function 1, AF-1); followed by the C-region which encodes the DNA binding domain (DBD); a linker region D and the C-terminal E region. The DE region encodes the ligand binding domain (LBD), and a transcriptional domain, denoted as activation function 2 (AF-2)(1,2).

A decade ago, gene products were identified that appeared to belong to the nuclear receptor superfamily on the basis of their nucleic acid sequence identity. The endogenous signaling molecules which bound to these proteins were unknown and thus the term "orphan receptor" was coined. Based on members of the nuclear receptor superfamily that have been more fully characterized, the orphans forecast an enormous yet unexploited opportunity for the discovery of important new therapeutic agents. The potential impact of such a discovery cannot be overstated, since every known nuclear receptor has been implicated in human disease.

The Nur77, NURR1, and NOR1 (NR4A1-3 subgroup) family of orphan nuclear receptors (NRs) are well conserved in the DNA binding domain (~91-95%) and the C-terminal ligand binding domain (LBD, ~60%), but divergent in the N-terminal AB region. This subgroup of proteins functions as immediate early response genes which are induced by wide range of physiological signals (3-7). They have been implicated in proliferation, differentiation, apoptosis,
hypertrophy/remodelling, Parkinson’s disease, schizophrenia, manic depression, and autoimmune disease (8-19). In itself, the NR4A1-3 subgroup presents an exciting scientific challenge, unlocking the molecular mechanisms that mediate NR4A-dependent transcription will provide a potential platform for pharmacological intervention.

Nur77 has been shown to play a key role in regulating expression of various genes in the hypothalamic-pituitary-adrenal (HPA) axis related to inflammation and steroidogenesis (20-22). Specifically, NUR77 activate expression of Corticotropin-releasing hormone (CRH) (19). The Nur77 family can regulate steroid-21-hydroxylase, steroid-17-hydroxylase and the 20alpha-hydroxysteroid dehydrogenase promoters (21-22). As such, the NR4A family of proteins represents a potential target for therapeutic intervention to control inflammatory responses.

Nur77 is expressed in skeletal muscle (23), and mouse myoblasts in culture (24,26-27). Moreover, bFGF, TPA, and forskolin agonists induce Nur77 expression in mouse myoblasts. Early studies demonstrated that Nur77 expression in muscle appeared to be dependent to different extents on the activation of protein kinase A, C and MAP kinase. β-adrenergic agonists transiently induce Nur77 in mouse myoblasts (24,26-27). Chronic stimulation of muscle with adrenergic agonists induces hypertrophy, fast type II fibres, muscle remodeling; and reduces skeletal muscle wasting (25).

The NR4A family members can bind as monomers, and homodimers to single/tandem copies of the NR4A response element (NRE), AAAGGTCA, a variant NR half site and constitutively regulate transcription (28-30). Moreover, Nur77 and Nurr1 (but not NOR-1) heterodimerize with RXR, and mediate efficient transactivation in response to RXR-specific agonists from DR5.
The AB region of Nur77/NR4A1 mediates cell specificity and coactivator recruitment

motifs (31-33). The NR4A subgroup function as immediate early response genes which suggests cross talk between multiple classes of stimuli and retinoid-dependent signalling. Moreover, phosphorylation of Nur77 regulates retinoid signalling by controlling the subcellular localization and nucleo-cytoplasmic shuttling of RXR (7). In addition, serine and threonine residues in Nur77 have been demonstrated to be critical in the activity, function and localisation of the NR4A member (5, 7, 34, 35).

This receptor subgroup has proved refractory to the understanding of coactivator recruitment in the process of constitutive trans-activation and retinoid-RXR dependent regulation (31-33). Moreover, the Nur77 LBD lacks an intrinsic and classical activation domain, and the serine/threonine rich domain in the N-terminus has been implicated in the regulation of Nur77-dependent transcription (36). Furthermore, the N-terminal AB region has been implicated in growth factor dependent nucleo-cytoplasmic shuttling of this NR (7).

In summary, the N-terminal AF-1 region, the C-terminal LBD and AF2 domain of the Nur77 subgroup is very unusual. Nur77 lacks an archetypal AF-2 transcriptional activation domain and does not seem to interact with NR cofactors, notwithstanding these comments, this orphan modulates transcription. Currently, the molecular aspects of transcriptional activation remain obscure, furthermore, structural analysis of the LBD, and mechanistic analysis of Nur77 function has not been undertaken with respect to intra-molecular interactions, and cofactor recruitment.

We have demonstrated and analysed the functional role of AF-1 in mouse NR4A1 (Nur77) mediated trans-activation, further elucidated the molecular basis of RXR-retinoid dependent
trans-activation by Nur77, and provided a transcriptional, biochemical and structural analysis of coactivator function and recruitment.

**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 (FCS) respectively in 6% CO2. Cells grown in 12-well dishes to 60-70% confluence were transiently transfected with 0.33-1µg of pGL2b-tk-LUC (37) or pNBRE3-tk-LUC (31-33) reporter plasmid together with 0.33-0.5µg of pSG5-Nur-77-FL or pSG5-Nur77-ΔAB, or pSG5-Nur77-ΔLBD or pSG5 alone using a DOTAP/DOSPER (Boehringer-Mannheim) liposome mixture in HEBS (42mM HEPES, 275mM NaCl, 10mM KCl, 0.4mM Na2HPO4, 11mM Dextrose, pH 7.1) (total DNA 2-2.5µg/well). The DNA/DOTAP/DOSPER mixture was added to the cells in 1ml of fresh DMEM medium containing 10% FCS and incubated for 14hrs. Post transfection the medium was replaced and the cells grown a further 24-48hr. Cells were harvested and assayed for luciferase activity. Each experiment represented at least two sets of independent triplicates to overcome the variability inherent in transfection experiments (37-42).

**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 150µl of phenol red-free DMEM and 150µl of Luclite substrate buffer. Cell lysates were transferred to a 96 well plate and relative luciferase units were measured for 5 sec in a Wallac Trilux 1450 microbeta luminometer.
GAL4 hybrid assays: C2C12 and COS-1 cells were passaged into 12-well plates and transfected at 50-80% confluence with 0.33-1µg of reporter, G5E1b-LUC, and 0.33µg of GAL-Nur77 chimeric constructs (GAL-Nur77-FL, AB, DE, aa1-110, aa110-200, aa200-269, aa1-160, aa55-160, and the GAL-chimeric constructs containing mutations in the AB region) or the GAL4 DNA binding domain alone in the presence and absence of vectors pSG5-SRC-2 (0.33-0.66µg) and/or pSG5-RXRγ (0.66µg) expression plasmid per well, using a DOTAP/DOSPER liposome mixture in HEBS. Transfections were performed in DMEM containing 10% FCS, or 5% CST (DMEM containing Charcoal-stripped fetal calf serum was used to for transfections performed with GAL-con structs co-transfected with pSG5-RXRγ in the presence and absence of 9-cis-RA 1µM). After 16-24 hr the medium was replaced and the cells were harvested 24-48 hr 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 (37-42).

Plasmids, and Primer Sequences: The expression plasmids GALO (43), pSG5 (Stratagene), pSG5RXRγ, pSG5-RXRγ-ΔAB (CDE) (42), and the reporter plasmids pNBRE3-tk-LUC (31-33), and G5E1b-LUC (44) have been described elsewhere. Generation of full length mouse Nur77 was performed by RT-PCR from muscle RNA with Pfu DNA polymerase (Promega), using the manufacturers buffer. All PCR products were cloned into the EcoR1 site of pSG5 (Stratagene) and then isolated after EcoR1 digestion, and subsequently cloned into pGAL0 and pGEX4T1 (Pharmacia Biotech). The primers (GENEWORKS Australia) used for the synthesis of full length mouse Nur77 were GMUQ-713 5’ GCG GAA TTC ACC ATG CCC TGT ATT CAA GCT CAA, and GMUQ-714 3’ GCG GAA TTC TCA GAA AGA CAA TGT GTC CAT.
We then synthesised additional primers to subclone the DE, AB, ABC and CDE regions into pSG5 and GAL0. The primers used for the DE were GMUQ-715-5’ GCG GAA TTC ACC ATG CGG CGG AAC CGC TGC CAG, and GMUQ-714 3’. The primers used to synthesize the AB region were GMUQ-713 5’, and GMUQ-716 3’ GCG GAA TTC TCA CTC GCC ACC TGA AGC CCC. The primers used to synthesize the ABC region were GMUQ-713 5’ and GMUQ-717 3’ GCG GAA TTC TCA CAG GCG GAG GAT GAA GAG. The primers used to synthesize the CDE region were GMUQ-718 5’ GCG GAA TTC ACC ATG TGT GCA GTC TGT GGT GAC, and GMUQ-714 3’. We subsequently constructed various sub-domains of the AB region by PCR and cloned these segments into the EcoR1/SalI site of GAL4. The Nur77-AB-aa1-110 was PCRed and cloned into SV40-GAL0 using primers GMUQ-713 5’, and GMUQ-719 3’ GCG GTC GAC TCA CAC CTG GAA GTC CTC. The Nur77-AB-aa110-200 region was PCRed and cloned into SV40-GAL0 using primers GMUQ-720 5’ GCG GAA TTC ACC ATG TAC GGC TGC TAC CCG, GMUQ-721 3’ GCG GTC GAC TCA GCC AGT GGG AGG. The Nur77-AB-aa200-269 region was PCRed and cloned into SV40-GAL0 using primers GMUQ-722 5’ GCG GAA TTC ACC ATG CCC AGC CCC AGC, and GMUQ-723 3’ GCG GTC GAC TCA CTC GCT GCC ACC TGA AGC CCC. The Nur77-AB-aa1-160 region was PCRed and cloned into SV40-GAL0 using primers GMUQ-713 5’, and GMUQ-724 3’ GCG GTC GAC TCA AAA TGA GCC GTC CCA. The Nur77-AB-aa55-160 region was PCRed and cloned into SV40-GAL0 using primers GMUQ-725 5’ GCG GAA TTC ACC ATG TTC ATG GAC GGG, and GMUQ-724 3’.
We then synthesised several primers for site directed mutagenesis and used the Stratagene Quickchange multi-site directed mutagenesis kit as per manufacturers' instructions to produce GAL4-Nur77 AB chimeras that simultaneously carried double, triple and quadruple amino acid mutations. Nur77 AB mutant primers were synthesised and 5’ Phosphorylated by GENEWORKS Australia. The primers made were S54A T55A-GMUQ-726 5’ CTG CCC AGC TTC GCA GCC TTC ATG GAC GGG, S54P T55P-GMUQ-727 5’ CTG CCC AGC TTC CCC TTC ATG GAC GGG, S142A T145A -GMUQ-728 5’ TCA GCC CCC GCA CCA TCT GCA CCC AAC TTC C, S142P T145P -GMUQ -729 5’ TCA GCC CCC CCC CCA TCT CCA CCC AAC TTC C.

**GST Pulldowns:** GST and GST-fusion proteins were expressed in E-coli (BL21) and purified using glutathione-agarose affinity chromatography as described previously (37-42). The GST-fusion proteins were analysed on 10% SDS-PAGE gels for integrity and to normalise the amount of each protein. The Promega TNT-coupled transcription-translation system was used to produce 35S-methionine labelled Nur77, and coactivator proteins that were visualised by SDS-PAGE. *In vitro* binding assays were performed with glutathione-agarose beads (Sigma) coated with ~500ng of GST-fusion protein and 2-10 µl of 35S-methionine-labelled protein in 200 µl of binding buffer containing 100mM NaCl, 20 mM TRIS-HCL (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 5 µg ethidium bromide and 100 µg BSA. The reaction was allowed to proceed for 1-2 hours at 4°C with rocking. The affinity beads were then collected by centrifugation and washed five times with 1 ml of binding buffer without ethidium bromide and BSA. The beads were resuspended in 20 µl SDS-
PAGE sample buffer, boiled for 5 min. The eluted proteins were fractionated by SDS-PAGE, the gel was treated with Amersham Amplify fluor, dried at 70°C and autoradiographed.

**Molecular Modelling:** A model of the Nur77 LBD was constructed using the program Modeller 6.0 with the RARγ LBD as a template. The resulting model was subject to Ramachandran analysis and further quality checking with the WhatIf suite of programs (www.cmbi.kun.nl/swift/whatcheck/). Hydrophobic surface analysis was conducted in SCULPT 3.0. Initial peptide superimposition was achieved using SPDBV V3.7 and structures were raytraced with the freeware program "Persistence of Vision:POV-Ray" (http://www.povray.org) and the Macintosh patch "MacmegaPov" (http://users.skynet.be/cgi-bin/formd.cgi). Further analysis of peptide docking using spherical polar Fourier correlation docking simulation program was performed using the program HEX. (47-49)
RESULTS:

**Nur77 trans-activates gene expression in a cell specific manner:**

Nur77 is expressed in skeletal muscle tissue and cells (23-24, 26-27). We performed RT-PCR experiments using total RNA extracted from C2C12 skeletal muscle cells to isolate full length Nur77 cDNA. To verify the integrity of the cloned NR after full length sequencing we examined the ability of Nur77 to trans-activate a Nur77 dependent reporter gene, NBRE-3-tk-LUC (NGFI-B binding response element-NBRE derived from the monomeric consensus binding site AAAGGTCA). This plasmid contains three copies of a consensus binding site (AAAGGTCA) cloned upstream of the heterologous herpes simplex virus thymidine kinase (tk) promoter (31-33) linked to the luciferase (LUC) gene. We transfected both myogenic and non-muscle cell types to investigate cell specific aspects of NR4A1 function.

We investigated the ability of full length Nur77, and Nur77 lacking the AB region (Nur77ΔAB construct which encodes amino acids (aa) 269-601) to transactivate NBRE-3 in muscle and non-muscle cells in the absence of agonists. In control studies, C2C12 myogenic cells and COS-1 cells were transfected with the control reporter plasmid ptk-LUC (lacking an NBRE, in an pGL2basic backbone), Nur77 expression plasmids did not trans-activate the empty LUC reporter plasmid in either cell type (Figure 1A and B). However, when C2C12 muscle cells and COS-1 cells were cotransfected with the expression vectors Nur77, Nur77ΔAB and the reporter plasmid NBRE-3-tk-LUC, we observed muscle specific Nur77 dependent trans-activation of gene expression (Figures1C and D). Moreover, these experiments suggest the LBD (DE) region of Nur77 is not sufficient to mediate trans-activation of gene expression (figure 1C). In summary, the data suggest Nur77 trans-activated gene expression in a cell specific manner, and that efficient trans-activation requires the AB region, which encodes AF-1.
The AB (AF-1) region of Nur77 is necessary for agonist independent and Nur77 mediated transactivation: AF-1 functions in a cell specific manner.

The previous experiments suggested the Nur77 LBD unlike other orphan and classical nuclear receptors could not exclusively facilitate trans-activation, and that Nur77 operated in AF-1 dependent manner. We went on to examine the role of the AB region in Nur77 mediated transactivation from the NBRE x 3-tk-LUC plasmid. We investigated and compared the ability of full length Nur77, Nur77 lacking the AB region (Nur77∆AB construct contains aa 269-601) and Nur77 lacking the DE region (Nur77∆LBD construct encodes aa 1 to 356) to transactivate NBRE in muscle and non-muscle cells in the absence of agonists. Cell specificity has been found to play an important role in the activation functions (AFs) of the AB and DE domains in the estrogen (ER), glucocorticoid (GR), progesterone receptors (PR) and Retinoic Acid Receptors (RAR) (45,46). The ability of different AFs to function has been found to (i) vary in relation to the cell line used and (ii) depend on the spatio-temporal expression pattern of the specific receptor; indicating that cell specific activation mechanisms are involved in the functioning of the different AFs.

Nur77 is expressed in skeletal muscle (23-24, 26-27). Hence, we investigated whether the AB region which encodes the AF-1 domain of Nur77 independently activates gene expression in a cell specific fashion. C2C12 myogenic cells were co-transfected with receptor expression vectors Nur77, Nur77∆LBD, and Nur77∆AB, and the reporter plasmid pNBRE-tkLUC. As expected, Nur77 trans-activated gene expression ~10-fold in C2C12 cells, and Nur77∆AB did not activate transcription in C2C12 or COS-1 cells (Figure 1E and F, respectively). Surprisingly, the Nur77∆LBD construct trans-activated gene expression ~10-fold in a cell specific manner, as efficiently as the native receptor.
These experiments indicated that the AB region which encoded the AF-1 domain was necessary for optimal Nur77 dependant transactivation of NBRE dependent reporters in C2C12 muscle cells. Furthermore, and more importantly, these experiments clearly demonstrate that the AB region which encodes the AF-1 of Nur77 functions in a cell specific manner, operates in an AF-2/LBD independent manner.

**The AB region of Nur77 encodes a potent AF-1 domain located between amino acid positions 1 and 160.**

To identify and further characterise the domains of Nur77 involved in transcriptional activation, we utilised the GAL4 hybrid system, whereby putative activation domains are fused to the DBD of the yeast transcription factor GAL4 (43). 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 Luciferase gene (LUC) reporter. The system utilised an SV40 promoter expression vector pGAL4-DBD (43) that contains a multiple cloning site downstream of the GAL 4 DBD. We fused Nur77 and various domains (eg AB or DE regions etc) of Nur77 to the GAL 4 DNA binding domain and examined the ability of these chimeras to regulate the expression of the G5E1b-LUC reporter in C2C12 and COS-1 cells. The GAL4-Nur77 chimera containing the full ORF of Nur77 activated transcription ~100 fold above the control, pGALO (GAL4 DBD) in a cell specific manner (Figure 2A and B). The GAL-Nur77-AB plasmid, that contains only the AB regions of Nur77, with the DBD and LBD deleted, increased transcription of the reporter construct ~200 fold over the GAL-DBD alone, in myogenic cells, and >1000-fold in non-muscle cells (Figure 2A and B). This indicated the AB region of Nur77 contained a potent ligand independent AF-1 domain. Interestingly, in the absence of its native DNA binding and ligand binding domains, and outside the context of the native receptor in the GAL4 chimera, the activity of the AF-1 domain was not restricted to muscle.
The GAL-Nur77 DE which encodes the LBD of Nur77 with the AB and C regions deleted did not activate transcription (Figure 2A and B, which was consistent with the reporter analysis. This suggested that the LBD lacked an intrinsic and classical transcriptional domain.

The AB region is comprised of 269 amino acids which encodes a potent N-terminal AF-1 domain. In order to further characterise the AF-1 region of Nur77, we have constructed sub-regions of the AB domain by PCR and cloned these segments into the GAL4-DBD (Fig. 3A). These constructs were transfected into COS-1 cells and assayed in the GAL4 hybrid system (Figure 3A). The constructs GAL-Nur77-aa-1-110, GAL-Nur77-aa-110-200 and GAL-Nur77-aa-200-269 were created and assayed by transfection. These segments of the AB region of Nur77 increased activation 75-, 16-, and 2-fold relative to the control, GAL4-DBD alone. This suggested the AF-1 domain was located between aa positions 1 and 200 in the AB region. The plasmid, GAL-Nur77-aa-200-269, did not trans-activate gene expression in this assay system. This data demonstrated that aa 200-269 were not essential for the activity of the AF-1 domain and did not contain an activation function. We subsequently constructed GAL-Nur77-aa-1-160, GAL-Nur77-aa-55-160 and assayed these plasmids by transfection analysis. These segments of the AB region of Nur77 increased activation 280-, and 44-fold relative to the GAL4-DBD alone. This delimited the AF-1 domain to between aa positions 1 and 160 in the AB region, and that the region downstream of aa position 160 was not essential for the activity of the AB region.

This delimited domain has two S/T rich areas between aa 50-60, and aa140-150 that are highly conserved between the three member of the NR4A subgroup (Nur77, Nurr1, and NOR-1), and cross species (i.e. mouse and human). Moreover, phosphorylation of S142 has been implicated in the nucleocytoplasmic shuttling of Nur77 (7). Consequently, we investigated whether these regions played a role in AF-1 activity. This was investigated by site specific mutagenesis of S54, T55, S142 and T145 within the AF-1 domain of Nur77. The AB region carrying various combinations of mutants were cloned into the GAL4 hybrid system. We constructed several...
mutants that carry multiple mutations; GAL4-AB-S54P/T55P, GAL4-AB S142A/T145A, GAL4-AB-S54A/T55A/ T145A, and GAL4-AB S54A/T55A/S142A/T145A. We observed that these mutations had a minimal effect on the activity of the AB region in COS-1 cells (data not shown) and C2C12 cells (Figure 3B). However, we observed that simultaneous mutation of S142 and T145 moderately increased the activity of the AF-1.

**SRC-2 stimulates the activity of the Nur77 AF-1 domain, but not the putative LBD.**

The process of cofactor recruitment, and the interaction of coactivators with the NR4A subgroup has remained obscure, and has not been described to date. Since, Nur77 can activate transcription directly and/or by tethered protein-protein interactions with RXR (31-33) we examined the effect of SRC-2/GRIP-1 expression on Nur77 mediated transactivation in the GAL4 hybrid system (Figure 4). In these assays the activity of Nur77 is independent of its binding to its cognate binding motif, the NBRE. If SRC-2 regulates the transcriptional activity, then the potential of the GAL4-Nur77 fusions to trans-activate gene expression should be increased in this assay (Figure 4A).

Cells were cotransfected with GAL-Nur77-AB, GAL-Nur77-DE, GAL-Nur77-aa-110-200, and GAL-Nur77-aa-55-160, the G5E1b-LUC reporter, in the presence and absence of an SRC-2 expression vector. G5E1b-LUC contains five copies of the GAL4 binding site placed upstream of a minimal E1b promoter.

Transfection of Gal-Nur77AB and GAL-Nur77-55-160 as expected efficiently induced transcription relative to the GAL4 DBD, this level of activity was significantly stimulated by addition of SRC-2 by 4-fold. Moreover, GAL-Nur77-aa-110-200 was stimulated ~ 10-fold by co-expression of SRC-2. This suggested that SRC-2 modulates the activity of the N-terminal AF1
domain in Nur77, and this coactivation is dependent on the region between aa positions 110 and 160 in the AB region. SRC-2 did not modulate or increase the activity of the C-terminal LBD (Figure 4B).

**SRC-2 potentiates the retinoid induced RXR dependent activation of the Nur77 LBD:**

The NR4A family members can bind as monomers, and homodimers to single/tandem copies of the NBRE, a variant NR half site and constitutively regulate transcription (28-30). Moreover, Nur77 and Nurr1 (but not NOR-1) heterodimerize with the C-terminal RXR LBD, and mediate efficient transactivation in response to RXR-specific agonists from a DR5 motif (GGTCA n5 AGTTCA) (31-33). The Nur77 LBD lacks an intrinsic activation domain, however, mutations and deletions in the activation function-2 (AF-2) domain effect ligand independent function (32-33, 36, 50). Moreover, this receptor subgroup has proved refractory to the understanding of coactivator recruitment in the process of trans-activation (50).

Consequently, we examined the ability of SRC-2 to modulate the activity of the GAL4-Nur77 chimeras in the presence and absence of RXRγ (which is preferentially expressed in skeletal and cardiac muscle) (42), and the RXR agonist, 9-cis-retinoic acid (9-cis-RA) (Figure 5A). Cells were cotransfected with GAL-Nur77-DE, SRC-2 and RXRγ expression vectors, in the presence and absence of 9-cis-RA. As expected cotransfection of RXRγΔAB in the presence of 9-cis-RA increased the activity of the Nur77-DE >100-fold relative to the control GAL4-DBD alone (Figure 5B and C). Co-expression of SRC-2 potently and dramatically potentiated the retinoid induced RXR dependent activity of the Nur77 LBD from 73 fold (relative to the GAL4 DBD alone) to a dramatic 2610-fold (relative to the GAL4 DBD) which translates to a ~35-fold increase in the activity of the Nur77 LBD (Figure 5B). In contrast the activity of the GAL4 DBD alone was increased ~6 fold by retinoids in similar conditions (i.e in the presence of RXR and SRC-2)
The AB region of Nur77/NR4A1 mediates cell specificity and coactivator recruitment

(Figure 5C). This demonstrated that SRC-2 (GRIP-1) coactivates the efficient Nur77-mediated retinoid-RXR dependent transactivation.

**The steroid receptor coactivators directly interact with Nur77: the AF1 domain recruits SRC**

The N-terminal AF-1 region, the C-terminal LBD (and the imbedded AF2 domain) of the NR4A subgroup is very unusual and has not been demonstrated to directly interact with coactivators. However, we have demonstrated that SRC-2 modulates the activity of the Nur77 AF1 domain. Furthermore, activation of gene expression by the classical nuclear hormone receptors is linked to recruitment of the Steroid Receptor Coactivators (SRC-1, -2, and -3). These SRCs recruit p300/CBP and PCAF to synergistically activate transcription. SRC’s, CBP/p300 and PCAF posses intrinsic histone acetyltransferase (HAT) 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 localised nucleosome structure (as reviewed in 51).

Consequently, we examined the ability of the SRCs (-1, -2, and -3) to directly interact with Nur77. We tested this hypothesis using a biochemical approach, the *in vitro* GST pulldown assay. Glutathione agarose immobilised GST-Nur77, GST-Nur77-AB, and GST-Nur77-DE were tested for direct interaction with *in vitro* $^{35}$S-radiolabelled full length SRC-1, -2, and –3 (Figure 6A). We observed that SRC-1 and –2 efficiently interacted with Nur77. Surprisingly, SRC-1 and –2 directly interacted with the AB region of Nur77 that encoded the potent AF1 domain. In contrast to other nuclear hormone receptors the DE region that encodes the LBD, did not interact with the steroid receptor coactivators. (Figure 6A). This is consistent with the NBRE-reporter assays, and the GAL4 hybrid analysis.
P300 and PCAF directly interact with the N-terminal AF1 domain: the AB region mediates directs coactivator recruitment.

To further investigate cofactor recruitment, we examined the ability of p300 and PCAF to interact with Nur77. Glutathione agarose immobilised GST-Nur77, GST-Nur77-AB, and GST-Nur77-DE were tested for direct interaction with in vitro 35S-radiolabelled full length p300 and PCAF. We observed that p300 and PCAF efficiently interacted with Nur77 and Nur77 AB (Figure 6B). Surprisingly, p300 interacted very poorly with the C-terminal LBD of Nur77 (Figure 6B). We also investigated the interaction of DRIP205 with Nur77, however, we did not observe any significant binding (Figure 6C).

As described SRC, p300 and PCAF independently and directly interacted with GST-Nur77 and GST-Nur77-AB. However, we also observed that GST-Nur77, and GST-Nur77-AB could simultaneously and efficiently pull down SRC, p300 and PCAF (Figure 6D). In contrast the DE region that encodes the LBD poorly interacted with this complex. Moreover, we demonstrated that in the presence of SRC-2 and p300, that Nur77 and the Nur77 AF-1 now significantly interacts with DRIP205 (figure 6E)

These studies suggested that the AB region that encodes the AF-1 domain mediates cofactor recruitment in Nur77, and that the LBD poorly interacts with coactivators.

The Nur77 LBD coactivator binding cleft is unusually hydrophilic: coactivator derived peptide binds poorly in docking simulations.

We used molecular modelling as a tool to further investigate the inability of the atypical nuclear receptor LBD to interact with coactivators. The starting point for homology modelling was the crystal structure of the human retinoic acid receptor gamma (hRARγ) in a complex with the
retinoid Cd564 (PDBid 1FCY). Alignments were performed with ClustalV and adjusted manually thereafter. Homology modelling was performed by satisfaction of spatial restraints using the program Modeller6 (47). The resulting model was subjected to Ramachandran analysis and further quality checking with the WhatIf suite of programs. Hydrophobicity analysis was performed using the molecular modelling program SCULPT (48). An LXXLL containing peptide from a previously published thyroid hormone receptor/steroid receptor coactivator peptide complex was superimposed onto both the RARγ and Nur77 LBDs enabling us to delineate a hypothetical coactivator binding interface.

Figure 7 shows ribbon and surface views of the template structure from hRAR (A), and the modelled Nur77 (B). Examination of the molecular surface in region of the hypothetical coactivator interfaces shows startling differences in hydrophobicity; hRAR(C) shows the characteristic hydrophobic groove (blue) found in AF2 activating receptors whilst Nur77 (D) encoded an unusually hydrophilic surface (red shade). Additionally, there are conspicuous changes in local surface topography as shown in close up views (E and F). This prompted us to use the spherical polar fourier correlation docking simulation program HEX (49) to assess the ability of the Nur77 LBD to bind a coactivator derived peptide (E and F). When we performed this simulation using the LBD of RARγ we found that 6 of the 10 highest scoring solutions docked to within 2 Å RMS of a coactivator/receptor model based on superposition of the existing TR/GRIP-1(SRC-2) peptide crystal structure. In contrast, the best 100 solutions for Nur77 did not approach to within 5 Å of the equivalent position (data not shown). This is most likely due to the differences in hydrophobicity and topology between the two interfaces. Given the dominance of the SRC-2/GRIP1 type LXXLL coactivator peptide in receptor interaction, this observation...
may account for the inability of this nuclear receptor LBD to directly mediate cofactor recruitment.

The N-terminal AB region directly interacts with the C-terminal LBD: the interaction is potentiated by AF1 mediated recruitment of SRC.

It has been previously demonstrated that human SRC-2 concomitantly interacts with the ER AF1 and AF2 to synergistically regulate transcription (52-54). Our studies suggested that the Nur77 AF1, but not the C-terminal LBD interacts with SRC. We investigated whether the atypical role of the Nur77 LBD in transcriptional activation involved intramolecular interactions with the N-terminal AF1, and whether this process was modulated by SRC recruitment (Figure 8A).

We tested this hypothesis using a biochemical approach, the in vitro GST pulldown assay. Glutathione agarose immobilised GST-Nur77–AB was tested for direct interaction with in vitro 35S-radiolabelled full length SRC-2 and the Nur77 LBD (CDE)(Figure 8A). As expected from our studies above we observed that SRC-2 efficiently interacted with the Nur77-AB. Very interestingly, we observed that the N-terminal AB region that encodes the potent AF-1 domain directly interacts with the C-terminal LBD region, furthermore, the interaction is significantly potentiated by AF1-mediated recruitment of SRC (Figure 8B). This evidence clearly demonstrates intramolecular interactions between the N-and C-terminal regions of the Nur77, and provides evidence for the involvement of the Nur77 LBD in the absence of an intrinsic activation function. In summary this suggests SRC-2 potentiates the direct interaction between the N-terminal AF-1 domain and the C-terminal LBD region of Nur77.
The Nur77 AF-1 domain synergizes with the RXR LBD in a retinoid dependent manner:

In the context of the above data we hypothesised that the Nur77 AF-1 may mediate transcriptional synergy with RXR during retinoid dependent induced activation of Nur77 (Figure 9A). Consequently, we examined the ability of RXRγ to potentiate the activity of the GAL4-Nur77 AB plasmid (that encodes AF1) in the presence and absence of the RXR agonist, 9-cis-RA. The experiments were internally controlled with the GAL4 DBD, and GAL4-Nur77-DE, which has been well characterised in the literature and in this study with respect to retinoid-RXR specific modulation.

9-cis-RA increased the activity of the GAL-Nur77-DE chimera in the presence of RXR, approximately 728-fold (Figures 9B). In contrast the activity of the GAL4 DBD alone was increased ~4-fold by 9-cis-RA in similar conditions (i.e in the presence of RXRγΔAB) (Figure 9C).

9-cis-RA increased the activity of the GAL-Nur77-AB chimera in the presence of RXR, approximately 13-fold, respectively (Figure 9D). The GAL4 DBD alone was increased ~4-fold by 9-cis-RA in similar conditions (i.e in the presence of RXRγΔAB) (Figure 9C). This suggested that the Nur77 AF-1 very efficiently synergizes with RXRγ. We observed stimulation of the Nur77 AF-1 by 9-cis RA in the absence of exogenous RXR, which reflects the well characterised low endogenous expression of RXRs in this cell type (56, 57).

We verified the interaction of the Nur77 AF-1 with RXRγ using a biochemical approach, the in vitro GST pulldown assay. Glutathione agarose immobilised GST-Nur77–AB was tested for direct interaction with in vitro 35S-radiolabelled SRC-2 (as a control) and RXRγ (Figure 9E). As
expected from our previous experiments we observed that SRC-2 efficiently interacted with the Nur77-AB. Moreover, we observed that the N-terminal AB region that encodes the potent AF-1 domain directly interacts with RXRγ (Figure 9E), and is in agreement with the GAL4 analysis.
DISCUSSION: In this investigation we have provided compelling evidence that Nur77 mediated trans-activation operated in an activation function–1(AF1) dependent manner. The AB region encodes an uncommonly potent N-terminal AF1 domain delimited to between amino acids 50 and 160 which is essential for the ligand independent activation of gene expression. Interestingly, the N-terminal AB region (not the LBD) facilitates coactivator recruitment, directly interacts with SRC, p300, PCAF, and DRIP205, and synergizes with RXRγ in a retinoid dependent manner. Moreover, we demonstrate that SRC-2 (GRIP-1) (i) modulates the agonist independent activity of the N-terminal AF-1 domain; (ii) potentiates the retinoid induced RXRγ dependent activation of the Nur77 LBD; and (iii) facilitates the physical association of the N-terminal AF-1 with the C-terminal LBD of Nur77. The results imply that the AF-1 domain plays a major role in Nur77 mediated transcriptional activation, cofactor recruitment, and intra- and inter- molecular interactions.

Activation by nuclear hormone receptors is mediated by two distinct regions localised within the amino and carboxy terminal AF-1 and AF-2 regions, respectively. The AF-1 domain is structurally divergent, the AF-2 region is evolutionary conserved suggesting a general mechanism for transcriptional regulation (1). Accordingly, the majority of receptors regulate gene expression, and recruit coactivator complexes in an C-terminal AF-2 dependent manner (51). However, the rat Nur77 LBD (i.e. NGFI-B) does not encode an activation domain per se. Moreover, the N-terminal of NGFI-B encodes a domain important for transcriptional activity, and this receptor subgroup has proved refractory to the elucidation of coactivator recruitment (36, 50).

Specifically, in this study we demonstrate that Nur77 (mouse NR4A1) trans-activates gene expression in a cell specific manner, and in an AF-1 dependent manner. This is consistent with the cell specific gene expression of NR4A2/Nurr1, and the spatio-temporal specific patterns of expression associated with this gene family (33,58). This suggests that Nur77 activity is dependent on a cell specific signal transduction pathway, and or combination of cofactors.

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The Nur77 N-terminal AB region encodes an unusually potent AF-1 domain located between amino acids positions 1 and 160, and is modulated by SRC-2/GRIP-1. Coactivation by SRC-2 is dependent on the region between amino acids positions 110 and 160.

The N-terminal AF-1 region, the C-terminal LBD (and the imbedded AF2 domain) of the NR4A subgroup as discussed has not been demonstrated to directly interact with coactivators. However, we observed that SRC-2 modulates the activity of the Nur77 AF1 domain. Since, the activation of gene expression by the classical nuclear hormone receptors is linked to the binding of steroid receptor coactivators and the subsequent recruitment of p300/CBP and PCAF (51), we investigated coactivator interactions with the GST-pulldown assay. We observed that the steroid receptor coactivators, p300 and PCAF directly interacted with the AB region of Nur77 that encoded the potent AF1 domain. In contrast to other nuclear hormone receptors the DE region that encodes the LBD, did not interact with the coactivators, SRC-1, -2 and p300. This is consistent with the NBRE-reporter assays, and the GAL4 hybrid analysis, which suggested the Nur77 LBD did not contain an intrinsic activation domain. This demonstrated that the N-terminal AB region mediates coactivator recruitment in Nur77, and explains the atypical nature of NR4A mediated trans-activation. Furthermore, in a cofactor dependent manner, the AB region of Nur77 and not the LBD interacted with DRIP205 that is involved in the regulation of nucleosome structure.

Interestingly, we show that the AF-1 mediated recruitment of SRC-2 promotes intra molecular interactions with the LBD that may function to stabilise the receptor during agonist independent transcription.
Serine/threonine rich domains in the N-terminus have been implicated in the regulation of NGFI-B-dependent transcription (7, 35-36). Furthermore, the N-terminus and phosphorylation of amino acid residues in the AB region are required for the growth factor dependent nucleo-cytoplasmic shuttling, and regulation by erk2, Trk, Ras, and MAP kinase (7, 35-36). We mutated the serine and threonine residues at amino acid positions 54, 55, 142 and 145 that had previously been implicated as key kinase targets during growth factor dependent modulation (7, 35-36). However, these residues were not involved in the transcriptional modulation of this nuclear receptor in our cell culture systems, the literature suggests phosphorylation of these residues mediates nucleo-cytoplasmic transport of this orphan receptor (see below). We did consistently observe a modest increase in the activity of the AF-1 domain when S142 and T145 were simultaneously mutated.

NGFI-B (rat Nur77) and Nurr1 (but not NOR-1) heterodimerize with the C-terminal RXR LBD, and mediate efficient transactivation in response to RXR-specific agonists (31-33). Our study demonstrated that SRC-2 (GRIP-1) potently coactivates the retinoid-RXRγ dependent transactivation of Nur77-mediated transcription. Moreover, we provided evidence for transcriptional synergy between the Nur77 AF-1 and the RXRγ LBD in a 9-cis-RA dependent manner. These intermolecular interactions mediated by the Nur77 AF-1 with the LBD of RXRγ are consistent with the observations that growth factor dependent phosphorylation of the N-terminal AB region of Nur77 regulates nucleo-cytoplasmic translocation of this NR, and the modulation of retinoid signalling (7).

Our molecular modeling analysis of the NR4A1 (Nur77) C-terminal DE region structurally substantiates the inability of this orphan LBD to interact with coactivators (e.g. SRC-2/GRIP-1). Examination of the molecular surface in this regions shows an unusually hydrophilic surface which is, in contrast to the archetypal and characteristic hydrophobic groove found in AF2 activating receptors (e.g. RAR and TR). Computer modelling strongly suggests the Nur77 LBD
The AB region of Nur77/NR4A1 mediates cell specificity and coactivator recruitment

is unable to bind a coactivator-derived peptide due to the differences in hydrophobicity and topology between the Nur77 and classical NRs.

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 (50), in contrast the NR4A subgroup (Nur77, Nurr1 and NOR-1) have a conserved lysine.

In summary, we have characterised the functional role of AF-1 in mouse NR4A1 (Nur77) mediated trans-activation, further elucidated the molecular basis of RXR-retinoid dependent trans-activation by Nur77, and provided a transcriptional, biochemical and structural analysis of coactivator function and recruitment.
The AB region of Nur77/NR4A1 mediates cell specificity and coactivator recruitment

References:


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**Figure legends:**

**Figure 1.** Nur77 transactivates gene expression in a cell specific manner. (A, B, C and D) SG5-Nur77-FL, SG5-Nur77-ΔAB (0.33µg) were co-transfected into C2C12 proliferating myoblasts and COS-1 cells together with the reporters genes pGL2b-tk-Luc (1µg) (A&B) or NBRE-3-tk-Luc (C&D) respectively. The AB (AF-1) region of Nur77 is necessary for agonist independent and Nur77 mediated transactivation of gene expression: AF-1 functions in a cell specific manner. (E&F) SG5-Nur77-FL, SG5-Nur77-ΔAB and SG5-Nur77-ΔLBD (0.33µg) were co-transfected into C2C12 proliferating myoblasts (E) and COS-1 cells (F) together with the reporter gene NBRE-3-tk-Luc (1µg). Fold activation is expressed relative to luciferase activity obtained after cotransfection of the pSG5 alone, arbitrarily set at 1. The mean Luciferase fold activation values and standard deviations (bars) were derived from a minimum of 2-3 independent triplicate experiments.

**Figure 2.** The AB region of Nur77 encodes a potent AF-1 activation domain. (A&B). GAL-Nur77-FL, GAL-Nur77-AB and GAL-Nur77-DE (0.33µg) were co-transfected with the GAL reporter G5E1b-Luc (1µg) into C2C12 proliferating myoblasts (A) and into COS-1 cells (B). 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 standard deviations (bars) were derived from a minimum of 2-3 independent triplicate experiments.

**Figure 3.** The activation domain within the AB region is located between amino acid position 1-160. (A) GAL-Nur77-AB, GAL-Nur77-aa1-110, GAL-Nur77-aa110-200, GAL-Nur77-aa200-269, GAL-Nur77-aa1-160 and GAL-Nur77-aa55-160 chimera’s (0.33 µg) were co-transfected with the GAL4 dependent reporter G5E1B-Luc (1µg) into COS-1 cells. (B) GAL-Nur77-AB, GAL4-AB-S54P/T55P, GAL4-AB-S142A/T145A, GAL4-AB-S54A/T55A/T145A and GAL4-AB-S45A/T55A/S142A/T145A (GAL4-Nur77-AB chimera’s that carried double, triple and quadruple amino acid mutations) (0.33 µg) were co-transfected with the GAL4 dependent reporter G5E1B-Luc (1µg) into C2C12 cells. 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 standard deviations (bars) were derived from a minimum of 2-3 independent triplicate experiments.

**Figure 4. SRC-2 potentiates the activity of Nur77 AB region.** A diagrammatic representation of the Gal4 hybrid assay is shown. This assay was used to determine the effect of SRC-2/GRIP-1 expression on Nur77 mediated transactivation of gene expression. GAL-Nur77-AB, GAL-Nur77-DE, GAL-Nur77-aa110-200 and GAL-Nur77-aa55-160 (0.33 µg) were co-transfected with the GAL4 dependent reporter G5E1B-Luc (1 µg) into COS-1 cells in the presence and absence of the co-transfected expression plasmid encoding SG5-SRC-2 (0.66 µg). 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 standard deviations (bars) were derived from a minimum of 2-3 independent triplicate experiments.

**Figure 5. SRC-2 stimulates the retinoid induced RXR dependent activation of the Nur77 LBD.** (A) A diagrammatic representation of the Gal4 hybrid assay is shown. This assay was used to determine the effect of SRC-2/GRIP-1 expression on retinoid induced RXR dependent activation of the Nur77 LBD. (B) COS-1 Cells were co-transfected with GAL-Nur77-DE or GAL4-DBD (C) (0.33 µg), SG5-SRC-2 (0.33 µg), and SG5-RXRγ-ΔAB (0.66 µg), together with the GAL4 dependent reporter G5E1B-Luc (1 µg) in the presence and absence of 9-cis-retinoic acid (1 µM). 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 standard deviations (bars) were derived from a minimum of 2-3 independent triplicate experiments.

**Figure 6. SRC coactivators directly interact with Nur77 AF-1 domain.** (A) Glutathione-agarose-immobilized GST, GST-Nur77, GST-Nur77-AB and GST-Nur77-DE were incubated independently with in vitro ³⁵S-radiolabelled full-length SRC-1, SRC-2, and SRC-3 respectively. The input lane represents ~10% of the total protein. p300 and PCAF directly interact with Nur77 AF-1 domain (B) GST, GST-Nur77, GST-Nur77-AB and GST-Nur77-DE were
incubated independently with \textit{in vitro} $^3$S-radiolabelled full-length p300 and PCAF respectively. The input lane represents ~10% of the total protein. (C) GST, GST-Nur77-AB and were incubated independently with \textit{in vitro} $^3$S-radiolabelled full-length SRC-2, DRIP-205, p300 respectively. The input lane represents ~10% of the total protein. (D) GST, GST-Nur77, GST-Nur77-AB and GST-Nur77-DE were incubated simultaneously with \textit{in vitro} $^3$S-radiolabelled full-length p300, SRC-2 and PCAF. The input lane represents ~10% of the total protein. (E) GST, GST-Nur77, GST-Nur77-AB were incubated simultaneously with \textit{in vitro} $^3$S-radiolabelled full-length SRC-2, DRIP-205, and p300. The input lane represents ~5% of the total protein.

**Figure 7 Molecular modelling of the Nur77 LBD: comparison of the RAR$\gamma$ LBD with the LBD of Nur77.** RAR$\gamma$ from crystal structure 1FCY (A) and the Nur77 (B) LBD model and are shown in ribbon form. Helices 3-5 (signature motif) are highlighted in red whilst helix 12 is coloured blue. An LXXLL peptide (coloured pink) from a previously published TR/SRC-2(GRIP-1) coactivator complex has been superimposed on both structures to delineate a hypothetical coactivator interaction interface. Critical leucines within the coactivator peptide are depicted in stick form. The backbone conformations of the two LBDs is very similar. Hydrophobic analysis of RAR$\gamma$ (C) and Nur77 (D) molecular surfaces. Surfaces were defined by a probe with a radius of 1.4 Å using SCULPT 3.0. Hydrophobic areas are coloured blue whereas hydrophilic areas are coloured red. SRC-2 (GRIP-1) coactivator peptide from a previously published structure (55) has been superimposed to delineate an approximate coactivator binding area. Leucines from the coactivator peptide are depicted in stick form. A reduction in hydrophobicity is apparent in the Nur77 LBD (D) coactivator interface region when compared to a similar area of RAR$\gamma$ (C). Additionally, the surface topography is different in this region despite the similarity in backbone conformation. Close up view of hydrophobic analysis focusing on interaction of leucines from the coactivator peptide with the RAR$\gamma$ (E) and Nur77 LBD surfaces (F)

**Figure 8. AF-1 domain directly interacts with the LBD of Nur77: this interaction is potentiated by AF-1 mediated recruitment of SRC-2.** (A) A diagrammatic representation of the intra-molecular interaction between AF-1 and AF-2 of Nur77 that is potentiated by AF-1 mediated recruitment of SRC-2. (B) GST (lane3 and4) and GST-Nur77-AB (lane 6 and7) were
incubated independently with *in vitro* $^{35}$-S-radiolabelled SRC-2 and Nur77-CDE respectively and GST (lane5) and GST-Nur77-AB (lane8) were incubated simultaneously with *in vitro* $^{35}$-S-radiolabelled SRC-2 and Nur77-CDE.

**Figure 9.** AF-1 domain of Nur77 synergizes with RXR$\gamma$ LBD in a retinoid dependent manner. (A). A diagrammatic representation of the Gal4 hybrid assay is shown. This assay was used to determine the effect of retinoid induced RXR dependent activation of the Nur77 AB region. (B) Cos-1 Cells were co-transfected with GAL4- Nur77-DE (0.33$\mu$g), and SG5-RXR$\gamma$-ΔAB (0.66$\mu$g), together with the GAL4 dependent reporter G5E1B-Luc (1$\mu$g) in the presence and absence of 9-*cis*-retinoic acid (1$\mu$M). (C) COS-1 Cells were co-transfected with GAL4-DBD (0.33$\mu$g), and SG5-RXR$\gamma$-ΔAB (0.66$\mu$g), together with the GAL4 dependent reporter G5E1B-Luc (1$\mu$g) in the presence and absence of 9-*cis*-retinoic acid (μM). (D) Cos-1 Cells were co-transfected with GAL-Nur77-AB (0.33$\mu$g), and SG5-RXR$\gamma$-ΔAB (0.66$\mu$g), together with the GAL4 dependent reporter G5E1B-Luc (1$\mu$g) in the presence and absence 9-*cis*-retinoic acid (1$\mu$M). 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 standard deviations (bars) were derived from a minimum of 2-3 independent triplicate experiments. (E) GST (lane3 and 4) and GST-Nur77-AB (lane 5 and 6) were incubated independently with *in vitro* $^{35}$-S-radiolabelled SRC-2 and RXR$\gamma$ respectively.
A

Nur77/NR4A1

GAL4DBD

GAL-Nur77-FL

GAL-Nur77-AB

GAL-Nur77-DE

AF-1  DBD  LBD

AB   C    DE

1  269  356  601

C2C12:

FOLD ACTIVATION

B

G5E1b-LUC

GAL4DBD

GAL-Nur-77

GAL-Nur-77-AB

GAL-Nur-77-DE

FOLD ACTIVATION

Cos-1:

+  +  +  +  +

+  -  -  -  -

-  +  -  -  -

-  -  +  -  -

-  -  -  +  +
The AF-1 domain of Nur77/NR4A1 mediates trans-activation, cell specificity and coactivator recruitment

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