The nuclear receptors NUR77 and SF1 play additive roles with c-JUN through distinct elements on the mouse *Star* promoter

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Abstract

The steroidogenic acute regulatory protein plays an essential role in steroid biosynthesis in steroidogenic cells. It is involved in the transport of cholesterol through the mitochondrial membrane where the first step of steroidogenesis occurs. *Star* gene expression in testicular Leydig cells is regulated by the pituitary LH through the cAMP signaling pathway. So far, several transcription factors have been implicated in the regulation of *Star* promoter activity in these cells. These include the nuclear receptors NUR77 and SF1, AP-1 family members (particularly c-JUN), GATA4, C/EBPβ, DLX5/6, and CREB. Some of these factors were also shown to act in a cooperative manner to further enhance *Star* promoter activity. Here, we report that NUR77 and c-JUN have additive effects on the *Star* promoter. These effects were abolished only when both elements, NUR77 at −95 bp and AP-1 at −78 bp, were mutated. Consistent with this, *in vitro* co-immunoprecipitation revealed that NUR77 and c-JUN interact and that this interaction is mediated through part of the ligand binding domain of NUR77. Furthermore, we found that SF1 could cooperate with c-JUN on the mouse *Star* promoter but this cooperation involved different regulatory elements. Collectively, our data not only provide new insights into the molecular mechanisms that control mouse *Star* transcription in Leydig cells but also reveal a novel mechanism for the regulation of NR4A1-dependent genes in tissues where NUR77 and c-JUN factors are co-expressed.

Journal of Molecular Endocrinology (2009) **42,** 119–129

Introduction

The steroidogenic acute regulatory protein (STAR) plays a crucial role in the regulation of steroid biosynthesis by transporting cholesterol from the outer to the inner membrane of the mitochondria (reviewed in Manna *et al.* 2003*a*), an essential step in the initiation of steroidogenesis. In Leydig cells, the main steroidogenic cell within the mammalian testis, *Star* gene expression and steroidogenesis are mainly regulated by the pituitary LH that binds to its G-protein coupled receptor leading to production of cAMP and activation of cAMP-regulated pathways ultimately resulting in transcriptional activation.

Transcriptional regulation of the *Star* gene in steroidogenic cells was shown to require two groups of transcription factors: some already present in the cell activated by post-translational modifications, and others that must be *de novo* synthesized. In Leydig cells, *Star* transcription was shown to rely on several transcription factors belonging to the first group. These include SF1, LRH1, GATA4, C/EBPβ, SREBP, SP1, DLX5/6, CREB/CREM, members of the AP-1 family (c-FOS and c-JUN), and DAX-1 (reviewed in Manna *et al.* 2003*a*). Regarding transcription factors belonging to the second group, we have recently identified the orphan nuclear receptor NUR77 (NR4A1) as a rapidly and strongly induced transcription factor that contributes to mouse *Star* transcription in Leydig cells in response to cAMP/forskolin stimulation (Martin *et al.* 2008).

The orphan nuclear receptor NUR77 (NGFI-B, NR4A1) is the founding member of a family that also comprises NURR1 (NR4A2) and NOR1 (NR4A3), all of which are characterized as immediate early response genes expressed in various tissues (Maxwell & Muscat 2005), including in hormonally stimulated steroidogenic cells (Davis & Lau 1994, Park *et al.* 2001, 2003, Song *et al.* 2001, Li *et al.* 2004, Martin & Tremblay 2005, Martin *et al.* 2008). NUR77 transactivation potential is also enhanced by post-translational modifications (Davis *et al.* 1993, Hirata *et al.* 1993, Li & Lau 1997, Martin *et al.* 2008) and/or modulation of its intracellular localization in response to certain stimuli (Klopotowska *et al.* 2005). NUR77 binds to DNA as a monomer to a regulatory element called NGFI-B response element (NBRE) similar to that recognized by the nuclear receptor steroidogenic factor 1 (SF1, Ad4BP, NR5A1; Wilson *et al.* 1993), a critical regulator of steroidogenic cell development and of many steroidogenic genes (Parker *et al.* 2002). Consistent with this, NUR77 and SF1 have been found to regulate a common set of genes involved in steroidogenesis in

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DOI: 10.1677/JME-08-0095
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Leydig cells (Zhang & Mellon 1997, Bassett *et al.* 2004, Hong *et al.* 2004, Havelock *et al.* 2005, Martin & Tremblay 2005), including *Star* (Martin *et al.* 2008). As for many other transcription factors regulating *Star* transcription, NUR77 is also expressed in non-steroidogenic tissues (Maxwell & Muscat 2005). The tissue- and cell-specific expression as well as hormonal regulation of the *Star* gene in steroidogenic cells is believed to be the result of cooperations between transcription factors. Indeed, various studies of the *Star* promoter have reported protein–protein interactions between GATA4 and C/EBPβ (Tremblay *et al.* 2002), CREB, and SF1 (Manna *et al.* 2003*b*), SF1 and C/EBPβ (Reinhart *et al.* 1999), SP1 and SF1 (Sugawara *et al.* 2000), DLX5/6 and GATA4 (Nishida *et al.* 2008), and between c-JUN and SF1, GATA4, and C/EBPβ (Manna *et al.* 2004).

Transcription factors belonging to the AP-1 family are ubiquitously expressed and can be divided into two groups based on their amino acid similarity; the FOS (c-FOS, FRA-1, FRA-2, and FOSB) and the JUN (c-JUN, JUNB, and JUND) subfamilies (O'Shea *et al.* 1992). AP-1 family members have been shown to bind as dimers to specific DNA sequences located in the promoter of target genes (O'Shea *et al.* 1992). The FOS subfamily members must heterodimerize with JUN proteins, whereas JUN members can form either homo- or heterodimers with any other AP-1 members.

Since the DNA binding elements for NUR77 and AP-1 are only 12 bp apart in the mouse *Star* promoter from various species, we hypothesized that these two transcription factors might work together to regulate *Star* transcription in Leydig cells. Here, we report that all three NR4A family members (NUR77, NURR1, and NOR1) and c-JUN have additive effects on mouse *Star* transcription. Although, SF1 and LRH1 (NR5A family members) can also cooperate with c-JUN, we found that different regulatory elements are involved in the NR4A/c-JUN and the NR5A/c-JUN cooperation. Thus, our results provide new insights into the mechanisms of action of two closely related families of nuclear receptors in the regulation of the mouse *Star* promoter in Leydig cells.

Materials and methods

Plasmids

Luciferase reporter constructs harboring the −902 bp murine *Star* promoter, deletions of the *Star* promoter to −193, −144, −121, −104, and −71 bp, and the −902 bp *Star* reporter construct with a mutation inactivating the SF1/NBRE element at −95 bp (CAT*CC*TTGA to CAT*AA*TTGA) have been described previously (Tremblay & Viger 2001, Martin *et al.* 2008). The −902 bp *Star* reporters containing either a mutation of the AP-1 element at −78 bp or a double mutation of the AP-1 and SF1/NBRE elements were generated by site-directed mutagenesis using the QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA, USA) and the wild-type and mutated at SF1/NBRE *Star* reporter constructs as template and the following pair of oligos (mutations are italicized): AP-1 element at −78: sense: 5′-CCT TGA CCC TCT GCA CAA TGA *GAG T*TG ACT TTT TTA TCT CAA GTG-3′, antisense: 5′-CAC TTG AGA TAA AAA AGT CA*A C*T*C* TCA TTG TGC AGA GGG TCA AGG-3′. The −104 bp *Star* reporter containing a mutation in the −95 bp SF1/NBRE element was generated by PCR using the mutated −902 bp as template. The −104 bp *Star* reporter containing mutations in both the −95 bp SF1/NBRE and −45 bp SF1 elements was generated by site-directed mutagenesis (QuikChange XL mutagenesis kit, Stratagene) using the −104 bp *Star* reporter mutated in the −95 bp SF1/NBRE and the following oligos (mutations are italicized): sense: 5′-CTT TTT TAT CTC AAG TGA TGA TGC A*T*A *CGT A*TC CAC GGG AAG CAT TTA AGG CAG C-3′, antisense: 5′-GCT GCC TTA AAT GCT TCC CGT GGA *TACG*T*A* TGC ATC ATC ACT TGA GAT AAA AAA G-3′. The mouse SF1 expression vector has been described previously (Tremblay & Viger 2001). Rat NUR77, NOR1, and NURR1 expression vectors (Philips *et al.* 1997) were provided by Dr Jacques Drouin (Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de Montréal, Montréal, Canada). The c-JUN, c-FOS, JUNB, JUND, FRA-1, and FRA-2 expression vectors (Teyssier *et al.* 2001) were obtained from Dr Dany Chalbos (Institut National de la Santé et de la Recherche Médicale, Endocrinologie Moléculaire et Cellulaire des Cancers, Montpellier, France).

Expression vectors for *in vitro* co-immunoprecipitation were generated by inserting the coding sequences (CDS) of the various transcription factors in the pRSETb vector (Invitrogen). For c-JUN, the CDS was obtained by PCR using mouse cDNA as template and the forward primer (5′-GC*TCTAGA*A TGA CTG CAA AGA TGG AAA CG-3′) containing an *Xba* I cloning site (italicized) and the reverse primer (5′-CG*GGTACC*T CAA AAC GTT TGC AAC TGC TG-3′) containing a *Kpn* I cloning site. The full-length NUR77 and a truncated protein lacking amino acids (aa) 1–225 were generated by PCR using rat cDNA as template, along with a common reverse primer containing a *Bam* HI (italicized) cloning site (5′-CG*GGATCC*T CAG AAA GAC AAG GTG TCC AT-3′) and the following forward primers containing a *Xba* I cloning site: full length NUR77, 5′-GC*TCTAGA*A TGG ACC TGG CCA GCC CCG AG-3′; and 226 to 577 aa NUR77, 5′-GC*TCTA GA*G TGA CCT CCA CCA AGT CCC GG-3′. Vectors for other NUR77 deletion variants have been generated by restriction enzyme digestions followed by purification

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and ligation. NUR77 lacking aa 389 to 467 was obtained by internal deletion using *Bsg* I. NUR77 lacking aa 1–225 and 389–467, aa 1–225 and 426 to C-terminal, or aa 1–225 and 454 to C-terminal were obtained by internal deletion of the truncated NUR77 (lacking aa 1–225) using *Bsg* I, *Sac* I, and *Pst* I respectively.

Cell culture and transfections

Mouse MA-10 Leydig cells (Ascoli 1981), provided by Dr Mario Ascoli (University of Iowa, Iowa City, IA, USA), were grown and transfected as described in Martin *et al.* (2008).

Protein purification and western blots

Mouse MA-10 Leydig cells were incubated in serum-free medium containing 0·5 mM $(Bu)_2$cAMP for times ranging from 0 to 6 h. MA-10 cells were then rinsed twice with ice cold PBS and harvested for nuclear protein extractions. Nuclear proteins were prepared by the procedure outlined by Schreiber *et al.* (1989). Protein concentrations were estimated using standard Bradford assay. Twenty microgram nuclear proteins were boiled for 10 min in a denaturing loading buffer, fractionated by SDS-PAGE, and transferred onto Polyvinylidene Fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Immunodetection was performed using an avidin–biotin approach according to the manufacturer's instructions (Vector Laboratories Inc., Ontario, Canada). Detections of NUR77, SF1, LRH1, c-JUN, c-FOS, STAR, and α-TUBULIN were performed using a monoclonal anti-NUR77 antibody (1:500 dilution; BD Biosciences Pharmingen, San Diego, CA, USA), an anti-SF1 polyclonal antiserum (1:5000 dilution; kindly provided by Ken-Ichirou Morohashi, National Institute for Basic Biology, Japan), an anti-LRH1 polyclonal antiserum (H-75X, 1:2000 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), an anti-c-JUN polyclonal antiserum (N, 1:200 dilution; Santa Cruz Biotechnologies), an anti-c-FOS polyclonal antiserum (4, 1:200 dilution; Santa Cruz Biotechnologies), an anti-STAR polyclonal antiserum (FL-285, 1:200 dilution; Santa Cruz Biotechnologies), and a monoclonal anti-α-TUBULIN antibody (1:50 000 dilution; Sigma–Aldrich) respectively.

Protein–protein interaction assays

In vitro interactions were analyzed using NUR77, c-FOS or c-JUN full-length proteins and either ^{35}S-labeled full-length SF1, c-FOS, c-JUN, or truncated NUR77 proteins. All proteins were obtained using the Quick Coupled TnT *in vitro* transcription/translation kit (Promega Corp, Madison, WI, USA). Proteins were incubated in 500 µl binding buffer (Tremblay *et al.* 2002) with 4 µg NUR77, c-FOS, or c-JUN antisera for 24 h at 4 °C with agitation, followed by an additional 24 h in the presence of 20 µl protein G-Sepharose beads (GE Healthcare, Baie d'Urfe, QC, Canada). Bound immunocomplexes were washed five times in binding buffer, resuspended in 20 µl 2× SDS-loading buffer, and subjected to SDS-PAGE. Gel was soaked in 10% glycerol for 5 min, and dried at 80 °C for 90 min. Proteins were visualized by autoradiography.

Statistical analyses

To identify significant differences between multiple groups, statistical analyses were done using a non-parametric Kruskal–Wallis one-way ANOVA followed by Mann–Whitney *U*-tests. Single comparisons between two experimental groups were done using the Mann–Whitney *U*-test. For all statistical analyses, $P<0·05$ was considered significant. All statistical analyses were done using the SigmaStat software package (Systat Software Inc., San Jose, CA, USA).

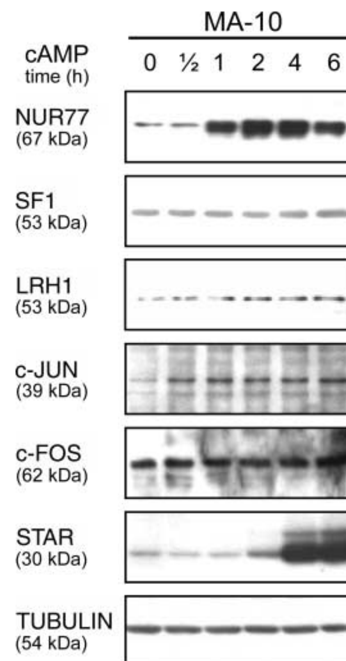

Figure 1 c-JUN and c-FOS are expressed in MA-10 Leydig cells. MA-10 Leydig cells were treated with $(Bu)_2$cAMP (0·5 mM) for the indicated times. For detection of c-JUN, c-FOS, SF1, LRH1, and NUR77, nuclear extracts (since located in the nucleus) were prepared while whole cell extracts were used for STAR (since located in mitochondria). TUBULIN was used as a loading control. Western blots were done as described in Materials and methods. All experiments were repeated at least three times and produced identical results.

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Results

MA-10 Leydig cells have been shown to express c-JUN and c-FOS (Li *et al.* 1997, Manna *et al.* 2004). We tested whether expression of these two proteins in MA-10 Leydig cells is influenced by $(Bu)_2cAMP$ treatment. As shown in Fig. 1, we found that only c-JUN was upregulated in response to cAMP treatment and that this increase occurred within 30 min. As previously reported (Martin *et al.* 2008), NUR77 and STAR were both strongly upregulated in response to cAMP in MA-10 Leydig cells (Fig. 1). The same treatment led to a weak increase in the nuclear receptors SF1 and LRH1 (Fig. 1).

NUR77 and c-JUN have additive effects on the mouse *Star* promoter

Although, chromatin immunoprecipitation assay revealed that NUR77 is significantly recruited to the proximal region of the *Star* promoter (Martin & Tremblay 2008, Martin *et al.* 2008), *in vitro* approaches such as EMSA showed a weak binding of NUR77 to the −95 bp SF1/NBRE element (Martin *et al.* 2008), suggesting that recruitment of NUR77 to the *Star* promoter might be mediated by interactions with other DNA-bound transcription factors. Moreover, the close proximity of the AP-1 (−78 bp) and SF1/NBRE (−95 bp) elements on the *Star* promoter (Fig. 2A) combined with the fact that AP-1 and NUR77 can individually regulate *Star* promoter activity in Leydig cells (Manna *et al.* 2004, Martin *et al.* 2008) raised the possibility that NUR77 and AP-1 family members might work together on the *Star* promoter. To test this, expression vectors encoding NUR77 and various AP-1 family members were transiently transfected in MA-10 Leydig cells along with a −902 bp mouse *Star* reporter. Consistent with previous reports (Wooton-Kee & Clark 2000, Manna *et al.* 2004, Martin *et al.* 2008), both NUR77 and c-JUN individually activated the *Star* promoter 3·5 and 9 fold respectively (Fig. 2B). An

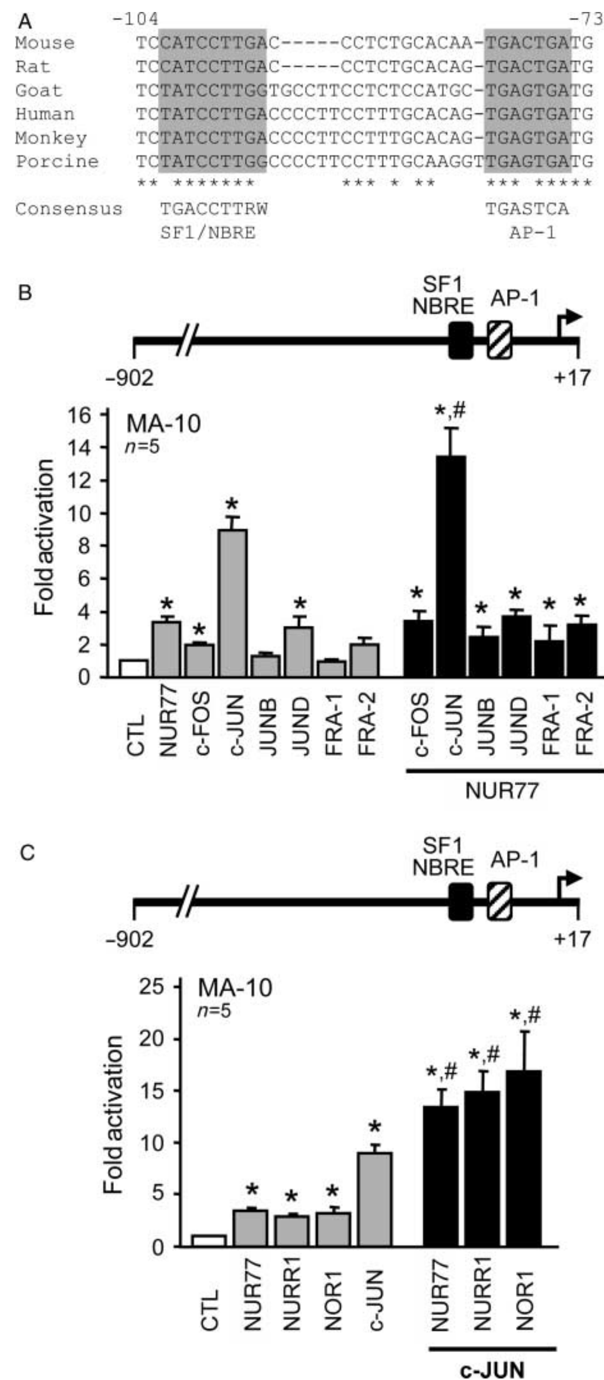

Figure 2 Additive effects of NR4A family members and c-JUN on the mouse *Star* promoter. (A) DNA sequence alignment of the −104 to −61 bp region of the *Star* promoter from different species highlighting the SF1/NBRE and AP-1 elements (grey shaded box). The sequence of the consensus SF1/NBRE and AP-1 elements is also shown. W=A or T, R=G or A, S=G or C. (B) c-JUN and NUR77 activate the *Star* promoter in an additive manner. MA-10 Leydig cells were co-transfected with a −902 to +17 bp mouse *Star* promoter construct along with an empty expression vector (CTL; open bar) or expression vectors for various AP-1 family members as indicated in the absence (grey bars) or presence (black bars) of an expression vector encoding NUR77. (C) NR4A family members and c-JUN have additive effects. MA-10 Leydig cells were co-transfected with a −902 to +17 bp mouse *Star* promoter construct along with an empty expression vector (CTL; open bar) or expression vector for NR4A family members (NUR77, NURR1, NOR1 as indicated) in the absence (grey bars) or presence (black bars) of an expression vector encoding c-JUN. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control (±S.E.M). An asterisk (*) indicates a statistically significant difference from control (empty expression vector). # indicates that the effect observed with a combination of transcription factors is statistically different than the effect of each factor individually.

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additive effect reaching 14 fold was observed when both factors were combined (Fig. 2B). Addition of c-FOS did not further enhance *Star* promoter activity (data not shown), suggesting that c-JUN homodimers are prevalent in *Star* transcriptional activation or that c-FOS is already sufficiently abundant in MA-10 Leydig cells (Fig. 1). As shown in Fig. 2C, other NR4A family members (NURR1 and NOR1) also had additive effects with c-JUN on the *Star* promoter.

To identify the binding elements required for these additive effects, transfections were performed using *Star* reporter constructs harboring mutations known to prevent NUR77 (Martin *et al.* 2008) and/or c-JUN (Manna *et al.* 2004) binding/transactivation. The NUR77/c-JUN additive effect was still observed on *Star* reporters harboring mutations in either the SF1/NBRE (at −95 bp) or AP-1 (at −78 bp) element (Fig. 3). The absolute fold activation, however, was lower when the AP-1 element was mutated (Fig. 3). The NUR77/c-JUN additive effect was only abolished when both elements were mutated (Fig. 3). Residual c-JUN-dependent activation of the *Star* promoter constructs containing a mutated AP-1 element at −78 bp might be attributed to the presence of other functional AP-1 elements within the −902 bp *Star* promoter fragment (Shea-Eaton *et al.* 2002). Therefore, the additive effect of NUR77/c-JUN requires an intact binding site for one of the factors.

NUR77 physically interacts with c-JUN *in vitro*

The fact that an intact SF1/NBRE or AP-1 element is sufficient to sustain the NUR77/AP-1 additive effect suggests that the two factors might interact. To assess this possibility, co-immunoprecipitation using ^{35}S-labeled *in vitro* translated proteins was performed. As shown in Fig. 4A, NUR77 physically interacts with c-JUN (lane 6) but not with c-FOS (lane 4). Co-immunoprecipitation of NUR77 and SF1 (Fig. 4A, lane 2) was used as a negative control since these nuclear receptors were

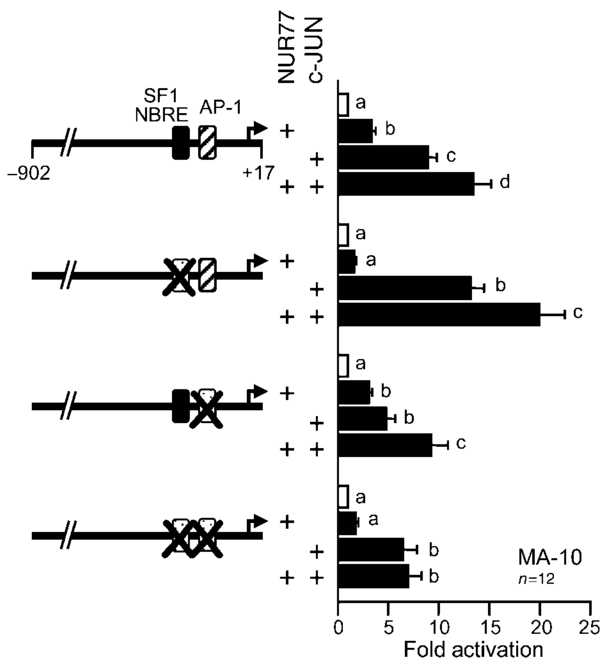

Figure 3 The NUR77/c-JUN additive effect requires either the SF1/NBRE element at −95 bp or the AP-1 element at −78 bp. MA-10 Leydig cells were co-transfected with expression vectors for NUR77 and c-JUN as indicated by the plus sign, along with a wild type −902 to +17 bp *Star* reporter, a reporter harboring a two nucleotide mutation in the SF1/NBRE element at −95 bp (CCAT*CC*TTGA to CCAT*AA*TTGA), a reporter containing a three-nucleotide mutation in the AP-1 element at −78 bp (TGA*CT*G*A*TG to TGA*GAG*T*T*G), or a reporter containing mutations in both elements (−95 bp and −78 bp). Mutated elements are represented by a large X. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control (±S.E.M). A different letter indicates a statistically significant difference.

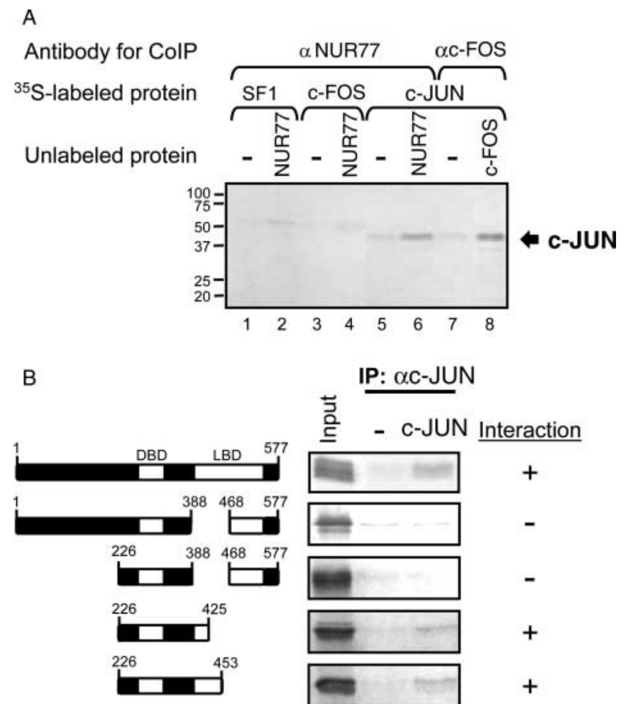

Figure 4 NUR77 physically interacts with c-JUN through its LBD. (A) NUR77 physically interacts with c-JUN in *in vitro* co-immunoprecipitation. *In vitro* produced ^{35}S-labeled and unlabeled proteins were mixed, immunoprecipitated using a NUR77 or c-FOS antibody as indicated, and subjected to SDS-PAGE followed by proteins visualization by autoradiography. (B) Protein–protein interaction between NUR77 and c-JUN is mediated by NUR77 LBD. *In vitro* co-immunoprecipitation using various deletion constructs of NUR77 were performed as described above. Positive c-JUN/NUR77 interactions are indicated on the right (plus and minus signs). Results are representative of three independent experiments.

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previously shown not to directly interact (Hong *et al.* 2004). Interaction between c-FOS and c-JUN (Fig. 4A, lane 8) served as a positive control (Kouzarides & Ziff 1988). Taken together, our results indicate that NUR77 physically interacts with c-JUN in a protein complex that contributes to *Star* transcriptional regulation.

The same approach was next used with various truncated NUR77 proteins and the full length c-JUN to map the region of NUR77 involved in the interaction. As reported in Fig. 4B, we found that the region between aa 388 and 425 of NUR77 which contains the N-terminal portion of the ligand binding domain (LBD) was essential for the interaction with c-JUN in this *in vitro* protein–protein interaction assay.

SF1 and LRH1 cooperate with c-JUN on the *Star* promoter

In addition to NUR77 (this study), c-JUN has recently been reported to cooperate with the nuclear receptor SF1 to regulate human *CYP11A1* promoter activity in steroidogenic cells (Guo *et al.* 2007). SF1 was also found to directly interact with c-JUN in a mammalian double-hybrid system (Manna *et al.* 2004). Since SF1 is an essential regulator of *Star* expression in Leydig cells, we tested the possibility that SF1 and c-JUN might work together on the mouse *Star* promoter. As shown in Fig. 5A, we found that SF1 and c-JUN can each activate the mouse *Star* promoter (2 and 11 fold respectively) and that the combination of both factors resulted in a transcriptional cooperation (20 fold). Similar results were obtained with LRH1 (FTF, NR5A2), another member of the NR5A family that has identical DNA binding properties to SF1 and is expressed in Leydig cells (Pezzi *et al.* 2004; Fig. 5B). Thus, although SF1 and LRH1 are weak activators of the *Star* promoter in MA-10 Leydig cells, they can also cooperate with c-JUN as do NR4A members (NUR77, NURR1, NOR1). The weak activation by SF1 and LRH1 may be explained by the already high levels of these factors in MA-10 cells (Zhang & Mellon 1997, Daggett *et al.* 2000, Aesoy *et al.* 2002) since in heterologous cells that do not express SF1, SF1 can activate the *Star* promoter (Sugawara *et al.* 1996, Rust *et al.* 1998, Reinhart *et al.* 1999, and our unpublished data).

NUR77 and SF1 have different site requirements for the cooperation with c-JUN

The −95 bp element in the mouse *Star* promoter, that we identified as essential for NUR77 responsiveness (Martin *et al.* 2008) and important for the additive effects of NUR77 and c-JUN (Fig. 3), can also be bound by SF1/LRH1. In fact, NR5A members (SF1 and LRH1)

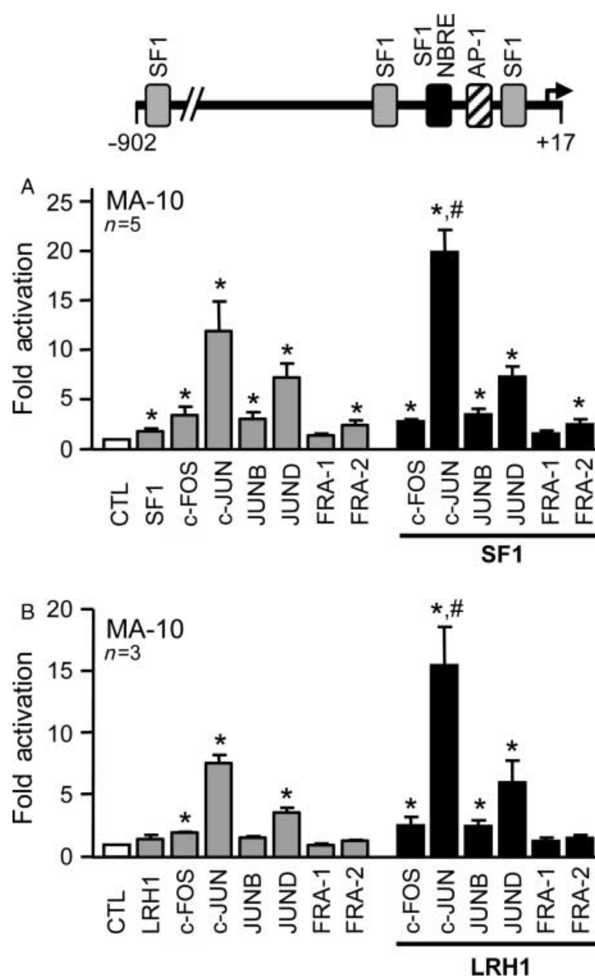

Figure 5 NR5A family members cooperate with c-JUN on the mouse *Star* promoter. MA-10 Leydig cells were co-transfected with a −902 to +17 bp mouse *Star* promoter construct along with an empty expression vector (CTL; open bar) or expression vectors for various AP-1 family members as indicated in the absence (grey bars) or presence (black bars) of an expression vector encoding SF1 (A) or LRH1 (B). The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control (±S.E.M). An asterisk (*) indicates a statistically significant difference from control (empty expression vector). # indicates that the effect observed with a combination of transcription factors is statistically different than the effect of each factor individually.

have a better affinity for this site than NR4A members (NUR77, NURR1, NOR1; Martin and Tremblay, unpublished data). We therefore tested whether this −95 bp element, along with the −78 bp AP-1 element, were required for the cooperation between c-JUN and NR5A family members. As shown in Fig. 6, a double mutation (−95 bp SF1/NBRE and −78 bp AP-1), which completely abrogated the NUR77/c-JUN additive effect (Fig. 3), had no impact on the SF1/c-JUN and LRH1/c-JUN cooperations on the mouse *Star* promoter. This

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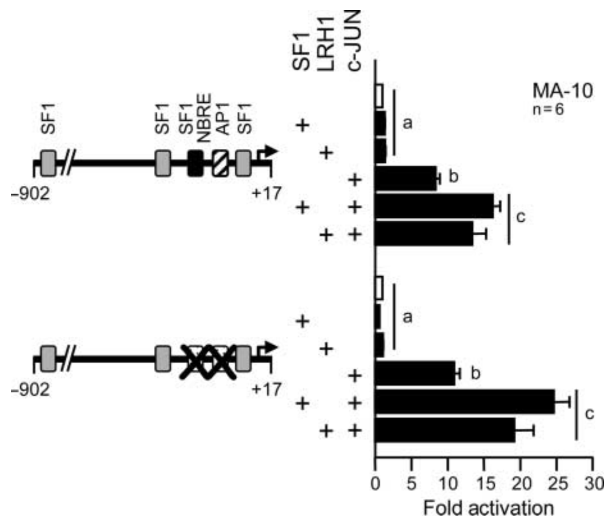

Figure 6 Distinct site requirements for the cooperation between c-JUN and NR5A members on the mouse *Star* promoter. MA-10 Leydig cells were co-transfected with an empty expression vector (open bars) or expression vectors for NR5A family members (SF1, LRH1) and c-JUN as indicated by the plus signs, along with a wild-type −902 to +17 bp *Star* reporter or a reporter containing mutations in both the −95 and −78 bp elements as described in Fig. 3. Mutated elements are represented by a large X. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control (±S.E.M). A different letter indicates a statistically significant difference.

indicates that another SF1/LRH1 element within the mouse *Star* promoter is involved in the NR5A/c-JUN cooperation.

In addition to the −95 bp element, the mouse *Star* promoter contains three other SF1 elements at −890, −135, and −45 bp (Caron *et al.* 1997). To identify which of these elements is sufficient for the cooperation with c-JUN, 5′-progressive deletion constructs of the *Star* promoter were transiently transfected in MA-10 Leydig cells. Deletion constructs from −902 to −104 bp which remove two SF1/LRH1 elements did not impair the SF1/c-JUN cooperation (Fig. 7A). Further deletion to −71 bp which removes the −95 bp SF1/NBRE and −78 bp AP-1 elements completely abrogated the synergy between SF1 and c-JUN. This indicates that the -45 bp SF1 element alone is not sufficient and that elements located within the −104 bp promoter fragment are required and sufficient for the SF1/c-JUN cooperation. To assess whether the −95 bp SF1/NBRE element is implicated in this cooperation, the −104 bp *Star* reporter construct harboring a mutation in the −95 bp SF1/NBRE element was tested and found to still be cooperatively activated by SF1 and c-JUN (Fig. 7B). Collectively, these results indicate that the −95 bp SF1/NBRE element is not essential for the SF1/c-JUN cooperation and that the −78 bp AP-1 and −45 bp SF1 elements are both required and/or that

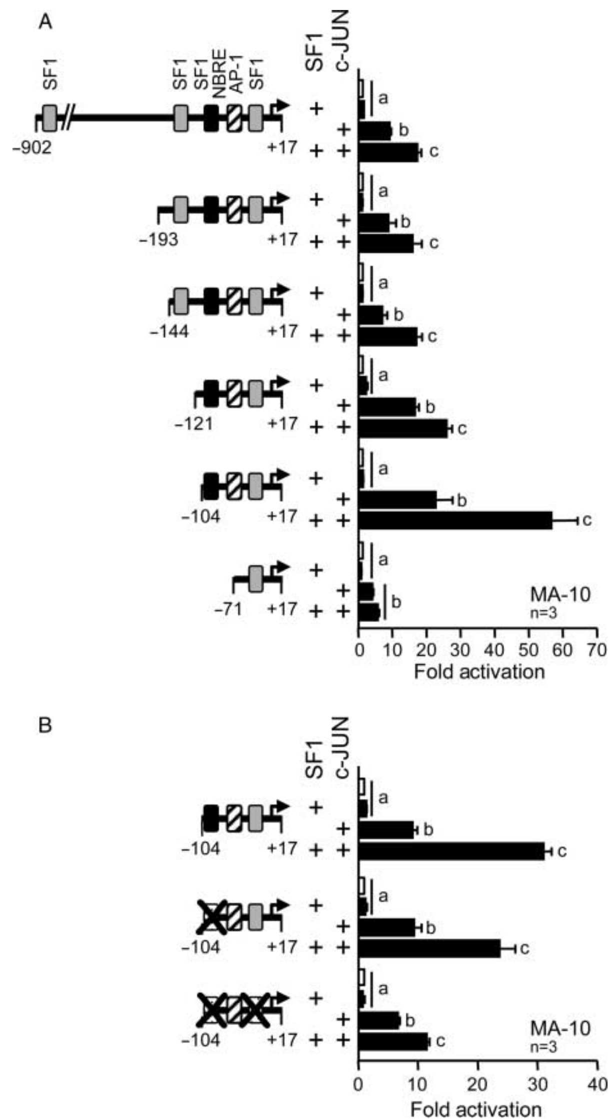

Figure 7 Mapping of the elements required for the SF1/c-JUN cooperation on the mouse *Star* promoter. MA-10 Leydig cells were co-transfected with an empty expression vector (open bars) or expression vectors for SF1 and c-JUN as indicated by the plus signs, along with (A) various 5′ progressive deletions of the mouse *Star* promoter or (B) −104 bp *Star* reporter constructs harboring mutations in the SF1/NBRE element at −95 bp (CCAT*CC*TTGA to CCAT*AA*TTGA) or in both the −95 bp SF1/NBRE, and the −45 bp SF1 (A*AGGC*T*G* to A*TACG*T*A*) elements. The mutated elements are depicted by large X. The number of experiments, each performed in duplicate, is indicated. Results are shown as fold activation over control (±S.E.M). A different letter indicates a statistically significant difference.

the −78 bp AP-1 element alone is sufficient. To test this possibility, mutations were introduced in both the SF1/NBRE at −95 bp and the SF1 at −45 bp elements in the context of the −104 bp *Star* reporter keeping only the −78 bp AP-1 element intact. This reporter was still cooperatively activated by SF1 and c-JUN (Fig. 7B).

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Discussion

Transcriptional regulation of the mouse *Star* gene is complex and yet the most important regulatory elements responsible for *Star* basal and hormone-induced expression are located within a relatively short promoter region encompassing the first 150 bp upstream of the transcription start site. More specifically, a 40 bp region located between −105 and −65 bp is perhaps the most intricate since it was shown to contain overlapping binding sites for several transcription factors. For instance, an element at −95 bp of the mouse *Star* promoter was found to be important for basal and cAMP-responsiveness and shown to bind SF1 (Caron *et al.* 1997, Sugawara *et al.* 1997, Wooton-Kee & Clark 2000). We recently reported that this −95 bp element is also essential to confer NUR77 responsiveness to the *Star* promoter (Martin *et al.* 2008). Less than 20 bp away, at −78 bp, is another species-conserved sequence (TGACTGA) found to be important for basal and cAMP stimulation of the mouse *Star* promoter (Wooton-Kee & Clark 2000, Manna *et al.* 2004). This element closely resembles an AP-1 element (consensus: TGA(C/G)TCA, O'Shea *et al.* 1992) as well as a cAMP response element (CRE) for the binding of CREB/CREM family members (consensus: TGACGTCA Montminy *et al.* 1986). AP-1 family members and CREB were both found to bind this element (Wooton-Kee & Clark 2000, Manna *et al.* 2002, 2004, Hiroi *et al.* 2004, Clem *et al.* 2005, Manna & Stocco 2007).

How these transcription factors (SF1, NUR77, CREB, c-JUN, c-FOS) are recruited to this region of the *Star* promoter and how they contribute to the basal and cAMP responsiveness of *Star* expression remains to be fully elucidated. Recent studies, however, have provided some answers. For instance, it was reported that some transcription factors, such as CREB and AP-1, are constitutively associated with the proximal *Star* promoter in mouse Leydig cells and upon cAMP stimulation, CREB becomes rapidly phosphorylated leading to the recruitment of the CBP co-activator and to acetylation of histones (Hiroi *et al.* 2004, Clem *et al.* 2005). Furthermore, dominant negative forms of c-FOS, c-JUN, and CREB blunted cAMP responsiveness of the *Star* promoter confirming their implication in this process (Manna *et al.* 2002, 2004). On the other hand, binding of other transcription factors such as SF1 and GATA4 to the proximal *Star* promoter was shown to be increased following cAMP stimulation even though their protein levels remained unchanged (Hiroi *et al.* 2004, Martin & Tremblay 2008). Finally, we found that cAMP stimulation of Leydig cells results in a rapid and strong induction in NUR77 protein levels that correlates with an increased association of NUR77 with the proximal *Star* promoter in an intact chromatin environment in primary Leydig cells and in MA-10 Leydig cells (Martin & Tremblay 2008, Martin *et al.* 2008).

Because the binding motifs for NUR77, CREB, and AP-1 within the −105 to −65 bp region of the *Star* promoter are not perfect consensus sequences for these factors, it has been suggested that increased DNA binding and stability of these transcription factors might be achieved through protein–protein interactions. Consistent with this, there have been numerous reports of direct protein–protein interactions involving these transcription factors on the *Star* promoter in Leydig cells (Reinhart *et al.* 1999, Sugawara *et al.* 2000, Tremblay *et al.* 2002, Manna *et al.* 2003*b*, 2004). In the present study, we have found that NUR77 and c-JUN have additive effects on the mouse *Star* promoter in Leydig cells. This finding may also have broader implications since NUR77 and c-JUN are co-expressed in a number of tissues where they could potentially act together to regulate gene transcription.

In vitro interaction between NUR77 and c-JUN

Using an *in vitro* co-immunoprecipitation approach, we provide evidence that NUR77 can physically interact with c-JUN. So far, *in vivo* co-immunoprecipitation assays using nuclear extracts from MA-10 Leydig cells to detect the presence of endogenous NUR77 and c-JUN together in a protein complex have been inconclusive (our unpublished data). This could be explained by inadequate epitope accessibility for the antisera tested in *in vivo* co-IP. Alternatively, the interaction between NUR77 and c-JUN might be weak and may be better observed with overexpressed proteins. Although, the NUR77/c-JUN protein interaction data need to be validated in a *in vivo* system, there is nonetheless evidence in the literature reporting that both NUR77 and c-JUN are associated with the proximal *Star* promoter region *in vivo* in Leydig cells (Manna & Stocco 2008, Martin & Tremblay 2008, Martin *et al.* 2008). Through our *in vitro* approach, we found that the region of NUR77 involved in the interaction with c-JUN was located within the LBD. This is unusual for NUR77 since most of its intra- and intermolecular interactions, including co-factor recruitment, described so far are mediated through the AF-1 domain (aa 50–160; Wansa *et al.* 2002). It is therefore possible that the interaction of c-JUN with NUR77 LBD might alleviate the need for a ligand. In agreement with this, ligand-independent activation of nuclear receptors mediated by post-translational modifications (phosphorylation) or protein–protein interactions have been previously described (Tremblay *et al.* 1999*a*,*b*).

In addition to the c-JUN/NUR77 interaction reported herein, there has been only one other report of a protein–protein interaction involving NUR77 LBD

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and it is with protein kinase C (PKC; Kim *et al.* 2006). This is relevant to the present study since AP-1 factors, including c-JUN, typically mediate PKC signaling induced by phorbol-12-myristate-13-acetate (PMA; Angel *et al.* 1987). Furthermore, Leydig cells respond to PMA/PKC with increased phosphorylation of CREB and upregulation of *Star* transcription through regulatory elements located within the proximal *Star* promoter (Jo *et al.* 2005). Interestingly, CREB, which can bind to the −78 bp element of the *Star* promoter (Manna *et al.* 2002, Clem *et al.* 2005), was also reported to physically interact with NUR77 in another system (Mynard *et al.* 2004) raising the possibility that NUR77 and CREB might also interact in Leydig cells to activate *Star* transcription. We did not, however, observe any cooperation between CREB and NUR77 on the *Star* promoter in MA-10 Leydig cells (data not shown).

SF1/c-JUN and NUR77/c-JUN cooperations involve different regulatory elements

SF1, which is known to directly interact with c-JUN (Li *et al.* 1999), was recently reported to transcriptionally cooperate with c-JUN on the human *CYP11A1* promoter in steroidogenic cells (Guo *et al.* 2007). In the present study, we found that SF1, as well as LRH1, can cooperate with c-JUN to synergistically activate the *Star* promoter in Leydig cells. Consistent with the fact that the *Star* promoter contains multiple SF1 elements, we found that the SF1/c-JUN cooperation does not require the −95 bp SF1/NBRE element. Mutation of this element was previously reported to impair SF1- and NUR77-responsiveness/binding (Wooton-Kee & Clark 2000, Martin *et al.* 2008). Here, we also found that this element also contributes to the additive effects between NUR77 and c-JUN. Thus, the NUR77/c-JUN and SF1/c-JUN effects on the mouse *Star* promoter involve different regulatory elements. This suggests that the collaborations between c-JUN and NR5A (SF1 and LRH1) or NR4A (NUR77, NURR1, and NOR1) nuclear receptors are not mutually exclusive on the mouse *Star* promoter and may play complementary roles in *Star* basal and hormonally regulated expression.

Another mechanism by which NUR77 might contribute to *Star* transcription is by removing a transcriptional inhibitor. Indeed, NUR77 is known to directly interact with DAX-1 (Song *et al.* 2004), a nuclear receptor involved in the transcriptional repression of the *Star* gene (Zazopoulos *et al.* 1997). Therefore, the implication of NUR77 in *Star* promoter activation might happen via distinct mechanisms that are not mutually exclusive. These include direct recruitment to the promoter (Martin & Tremblay 2008, Martin *et al.* 2008), interaction with other transcriptional activators such as c-JUN (this study), and removal of repressors such as DAX-1 from the *Star* promoter region. Supporting this is the fact that the DAX-1-mediated repression of *Star* transcription is associated with NUR77 and SF1 (Manna *et al.* 2008).

In conclusion, our current findings along with data from the literature support the existence of a multi-protein complex containing NUR77, SF1, AP-1, PKC, GATA4, DLX5/6, C/EBPβ, and CREB along with co-activators assembled on the proximal *Star* promoter. Because more than one factor can bind to the same element (e.g., NUR77 and SF1 at −95 bp and CREB and AP-1 at −78 bp), this complex would be variable in composition depending on the absence or presence of a stimulus and on the nature of the stimulus itself. This would allow for a rapid integration of various signals ultimately leading to a fine-tuned regulation of *Star* transcription in Leydig cells.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice their impartiality.

Funding

L J Martin is supported by doctoral studentships from the Natural Sciences and Engineering Research Council of Canada and from Fonds de la recherche en santé du Québec. J J Tremblay holds a New Investigator scholarship from the Canadian Institutes of Health Research (CIHR). This work was funded by grants from NSERC (262224-2003) and CIHR (MOP-81387) to J J Tremblay.

Acknowledgements

We would like to thank Drs Jacques Drouin, Dany Chalbos, and Mario Ascoli for generously providing expression plasmids and cell line used in this study.

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Received in final form 8 October 2008
Accepted 5 November 2008
Made available online as an Accepted Preprint 5 November 2008

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