Arylamine N-acetyltransferase 1 gene regulation by androgens requires a conserved heat shock element for heat shock factor-1

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Human arylamine N-acetyltransferase 1 (NAT1) is a widely distributed protein that has been implicated in a number of different cancers including breast and prostate. Previously, NAT1 gene expression was shown to be androgen dependent, although the effect of androgens was not due to direct activation of the NAT1 promoter. Here, we show that heat shock factor (HSF1) is induced by androgen in human cell lines. HSF1 also binds to a heat shock element (HSE) in the NAT1 promoter located 776 bp upstream of the putative transcription start site (3). Mutation of the HSE inhibited androgen responsiveness and prevented direct upregulation of the NAT1 promoter by HSF1. Although HSF2 also bound to the HSE, it did not increase promoter activity. HSF1 induced endogenous NAT1 activity in this cell line in the absence of androgen. This could be attenuated by pretreating cells with HSF1-directed transfections but not by scrambled transfections. Our results show that HSF1 is an important transcription factor for induction of NAT1 in human cells and is required for androgen activation of the NAT1 promoter.

Introduction

Human arylamine N-acetyltransferase 1 (NAT1; EC 2.3.1.5) is an important enzyme involved in the metabolism of many drugs and carcinogens (1,2). NAT1 and the closely related isozyme NAT2 are highly polymorphic and both have been studied extensively as risk factors for various diseases including cancer (3–5). Expression of NAT1 is subjected to genetic polymorphisms, it is also regulated by transcriptional and posttranscriptional mechanisms (6). The NAT1 promoter (NATb) appears to be functional in most tissues and is important modulators of this risk. Although the activity of NAT1 is subjected to genetic polymorphisms, it is also regulated by transcriptional and posttranscriptional events (10). NAT1 mutations found in the general population have been shown to destabilize the protein by promoting polyubiquitin-dependent degradation (11). Moreover, because the active site of the arylamine N-acetyltransferase consists of a cysteine-containing catalytic triad (12), electrophiles that modify thiol groups have been shown to inactivate the enzyme (15). The link between the arylamine N-acetyltransferases and disease risk suggests that environmental factors that increase or decrease enzyme activity may be important modulators of this risk.

Recently, we showed that human NAT1 expression is androgen responsive (19). This work was based on earlier observations in mice where the murine homolog of NAT1 was shown to be modulated by testosterone (20). We found that androgens increased NAT1 expression by upregulating transcription from the NATb promoter. Androgen responsiveness was mapped to a 157 bp sequence located ∼745 bp upstream of the transcription start site as previously reported by Barker et al. (8). However, we were unable to identify androgen receptor-binding elements in this region. The increase in NAT1 messenger RNA following treatment with the synthetic androgen methylenolone (R1881) was inhibited by cycloheximide suggesting that new protein synthesis was necessary for the effects of androgen on NAT1 expression. The results suggested that androgen responsiveness was not the consequence of direct promoter activation by the hormone-activated androgen receptor. However, the exact mechanism of gene activation was not identified.

In the present study, we have examined the 157 bp region of the NATb promoter to identify the sequences essential for the androgen responsiveness of the NAT1 gene. We isolated a 12 bp palindrome that contained two consensus nGAAn binding sites and formed part of a heat shock element (HSE). The results presented here provide new insight into NAT1 regulation that may be relevant to its role in activation or detoxification of arylamine carcinogens.

Materials and methods

Plasmid constructs

A 4228 bp fragment of the major NAT1 promoter NAT1b was amplified from 22Rv1 genomic DNA by polymerase chain reaction (PCR) using primers and conditions described previously (6). This fragment was digested with HindIII and cloned into HindIII-digested, shrimp alkaline phosphatase-treated pGL3-enhancer (Promega, New South Wales, Australia), yielding pGL3-ex4-3657, where the number indicates base pair upstream of exon 4. NAT1b construct pGL3-ex4-257 was made by PCR using pGL3-ex4-3657 as template and forward primer 5’-CTAAGGTACCCCATCACATGGG-3’, which contains an artificial KpnI restriction site, together with the reverse primer used to PCR the 4228 bp fragment (5’-CCCCCAAGAGTGGGACACACCACACTG-3’). This PCR fragment was digested with KpnI and HindIII and cloned into the same sites of pGL3-enhancer. A series of deletion constructs was made by PCR using the forward primers pGL3-ex4-902, 5’-CTGAATGCGACCTTCCCGAGC-3’ and the reverse primer 5’-CTAAGGTACCACTTCCCGAGC-3’. pGL3-ex4-804, 5’-CTAAGGTACCCCTGCTCAGG-3’; pGL3-ex4-790, 5’-CTAAGGTACCCACCCAATGCT-3’; pGL3-ex4-786, 5’-CTAAGGTACCCAGGACGCCAG-3’; pGL3-ex4-776, 5’-CTAAGGTACCCCTGCTCAGG-3’. All clones were verified by DNA sequencing.

Cell culture, transient transfection and luciferase assay

The human prostate cancer cell line 22Rv1 was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 supplemented with 5% charcoal-stripped fetal bovine serum. Cells were treated with up to 100 nM R1881 (Perkin Elmer, Victoria, Australia) or vehicle (dimethyl sulfoxide) for up to 48 h under androgen-free conditions.

Cells were seeded at a density of 2.5 × 105 cells/ml in 24-well plates and allowed to adhere overnight. Cells were transfected with 0.5 μg reporter plasmid DNA using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions and incubated for 24 h under androgen-free conditions. They were then treated with vehicle or 100 nM R1881 under androgen-free conditions for a further 24 h. Cells were then washed with phosphate-buffered saline (PBS) and lysed in passive lysis buffer, and firefly luciferase activity was measured using a luciferase assay system (Promega) as outlined in the manufacturer’s instructions. Luciferase activities were normalized to total protein.

Abbreviations

EDTA, ethylenediaminetetraacetic acid; EMSA, electrophoretic mobility shift assay; HSE, heat shock element; HSF, heat shock factor; NAT1, N-acetyltransferase 1; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PMSF, phenylmethylsulphonyl fluoride; siRNA, small interfering RNA.
For heat shock factor (HSF) overexpression studies, cells were transiently transfected as above with either pGL3-ex4-790 or pGL3-ex4-790m, along with pFLAG-EV, pFLAG-HSF1 or pFLAG-HSF2, and the internal control vector pSV-βgal. After transfection for 24 h, cells were treated with vehicle or 100 nM R1881 under androgen-free conditions for a further 24 h. Luciferase activities were measured by β-galactosidase activity.

To assess the effect of HSF overexpression on endogenous NAT1 activity, cells were transfected as above with either pFLAG-EV, pFLAG-HSF1 or pFLAG-HSF2 and incubated under androgen-free conditions for 48 h. Some cells were transfected with the pEGFP plasmid so that transfection efficiency could be determined. The number of viable cells expressing green fluorescent protein was assessed by flow cytometry (FACS Canto, BD Biosciences, North Ryde, New South Wales, Australia) and was typically 35–40%. This was then factored into the NAT1 activity calculation to account for non-transfected cells.

**Nuclear extracts**

Nuclear extracts of 22Rv1 cells were prepared as described previously (21). Briefly, cells (~5 × 10⁶) were trypsinized and harvested by centrifugation at 1000g for 5 min, washed once with ice-cold PBS and resuspended in 0.6 ml ice-cold lysis buffer [10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate and 5 mM sodium fluoride]. On ice, the cells were disrupted by passage through a 27-gauge needle. The nuclei were collected by centrifugation in a microcentrifuge at 15 000g for 8 s. The supernatants were discarded and the cell pellets were resuspended in 70 μl of ice-cold extraction buffer [lysate buffer containing 20 mM HEPES (pH 7.9), 420 mM KCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 25% glycerol]. After 30 min on ice, 50 μl of storage buffer [20 mM HEPES (pH 7.9), 0.5 mM dithiothreitol, 0.2 mM EDTA, 2% glycerol, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate and 5 mM sodium fluoride] was added and the extracts were centrifuged at 15 000g for 10 min (4°C). Nuclear extracts were stored as aliquots at −80°C. Protein concentrations were determined by the method of Bradford (22) using bovine serum albumin as a standard.

**Electrophoretic mobility shift assay**

Nuclear extracts (5 μg protein) were incubated for 30 min at room temperature in binding buffer [2% Ficoll 400, 5% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA and 2.5 mM dithiothreitol] containing 0.5 μg of poly(dI-dC) and 32P-labeled oligonucleotide probe (1 μl) in binding buffer [2% Ficoll 400, 5% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA and 2.5 mM dithiothreitol]. After 30 min on ice, 50 μl of storage buffer [20 mM HEPES (pH 7.9), 0.5 mM dithiothreitol, 0.2 mM EDTA, 2% glycerol, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 0.1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM sodium pyrophosphate and 5 mM sodium fluoride] was added and the extracts were centrifuged at 15 000g for 10 min (4°C). Nuclear extracts were stored as aliquots at −80°C. Protein concentrations were determined by the method of Bradford (22) using bovine serum albumin as a standard.

**Western blot analysis**

Equal amounts of nuclear extracts (20 μg) were electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gels, transferred to nitrocellulose membranes (350 mA, 1 h) and immunoblotted using polyclonal antibodies directed against HSF (sc-9144X) or HSF2 (sc-13056X) and loading control antibodies against α-tubulin (DM1A; Calbiochem, Darmstadt, Germany) or α-nucleolin (Sapphire Bioscience, New South Wales, Australia). Blots for FLAG-tagged proteins used anti-FLAG-M2-HP antibody (Sigma). Briefly, membranes were blocked for 1 h with 5% skim milk powder in PBS, washed with 0.05% Tween 20 in PBS (PBST) and then incubated for 1 h at room temperature with primary antibody diluted 1/5000 in PBST. Membranes were washed with PBST and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (GE Healthcare) diluted 1/5000 in PBST. After a final wash with PBST, membranes were developed using Immun-Star ECL reagent (Bio-Rad, New South Wales, Australia) and a ChemiDoc XRS system using Quantity One software V4.5.0 (Bio-Rad).

**siRNA synthesis and transfection**

The small interfering RNA (siRNA) directed against HSF1 was designed using software developed by Ambion (Austin, TX) and synthesized using a ‘Silencer’ siRNA construction kit (Ambion). According to the manufacturer’s instructions, three mutants of the sequence responsible for R1881 induction were designed using purified DNA as template with primers flanking the NAT1 promoter and sonicated for 3 s on ice. Following centrifugation, the supernatants were transferred to new tubes and diluted 1/10 with dilution buffer [20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin A]. A 50 μl aliquot of each supernatant was removed from each well and kept for positive PCR controls and the remainder was divided into three lots and immunoprecipitated with either HSF1 antibody (sc-9144X at 1/200), HSF2 antibody (sc-13056X at 1/200) or rabbit IgG (negative control) and Protein A-Sepharose beads (P9424; Sigma, New South Wales, Australia) overnight at 4°C. After extensive washing, immunoprecipitated DNA–protein complexes were eluted by a 10 min incubation in elution buffer (0.1 M NaHCO₃ and 1% sodium dodecyl sulfate) and then heated at 65°C overnight to reverse cross-linking DNA was purified using MiniElute PCR purification spin columns (Qiagen, Victoria, Australia). PCR was performed using purified DNA as template with primers flanking the NAT1 promoter region (sense 5'-ACAAAGATATGTTGCGCTTC-3' and anti-sense 5'-AGAA-CATTCCACGTAGTGG-3') or GAPDH as a negative control (sense 5'-ATGTTGGCACCCTGGGAATC-3' and anti-sense 5'-TGCCAAAGCTTACAAAAGGAG-3').

**Assay of NAT1 activity**

Cells were transfected with PBS, resuspended in buffer [20 mM Tris (pH 7.4), 1 mM EDTA and 1 mM dithiothreitol] and lysed on ice by sonication. Cell lysates were centrifuged at 4°C and the supernatant assayed for NAT1 activity as described previously (23). Protein concentrations were determined by the method of Bradford (22) using bovine serum albumin as a standard.

**Data analysis and statistics**

Data are expressed as mean ± SEM. Statistical comparisons between different treatments were assessed by Student’s t-tests or one-way analysis of variance assuming significance at a P value of 0.05 or less.

**Results**

The androgen responsive sequence of NATb maps to a 12 bp palindromic sequence (5'-TGTTCTAGAACA-3') that consists of two consensus nGAAn motifs (nTTCnnGAAn) that formed part of a putative HSE similar to that previously identified in several other genes (25–27). To confirm the essential role of this sequence in R1881 induction, three mutations were introduced into the HSE (5'-TGTTCTACCCCA-3', where mutated bases are underlined). The wild-type sequence showed a 2.4-fold induction following R1881 treatment, whereas the mutant sequence showed no significant effect suggesting that this core motif was essential for androgen induction of NAT1 transcription (Figure 1C).
The NAT1 HSE binds HSFs HSF1 and HSF2

To identify the transcription factors bound to the HSE in NATb, electrophoretic mobility shift assays (EMSAs) were performed with nuclear extracts from dimethyl sulfoxide (control) or R1881-treated 22Rv1 cells using the wild-type oligonucleotide sequence as a probe (Figure 2A). A single specific complex was observed that could be disrupted by addition of excess unlabeled wild-type probe (Figure 2B, lane 3). Addition of either M1, M2 or M1/M2 oligonucleotides, which have the nTTCn, nGAAn or both core motifs mutated, respectively, did not disrupt the specific complex (Figure 2B, lanes 4, 5, 6). Furthermore, an oligonucleotide containing the HSE from the HSP70 promoter (28) completely inhibited formation of the complex (Figure 2B, lane 7), suggesting that the HSE in NATb binds HSFs, at least in vitro.

To identify which HSF was present in the specific protein complex, antibody supershift assays were performed. Addition of antibodies directed against either HSF1 or HSF2 shifted the protein complex in both untreated (Figure 2C, lanes 1–3) and R1881-treated cells (Figure 2C, lanes 4–6). These results suggest that both HSF1 and HSF2 bind to the NATb HSE, possibly as heterotrimers. When nuclear extracts from heat-treated 22Rv1 cells were used in the EMSA, a large specific protein complex was observed (Figure 2C, lanes 7–9). The complex was completely shifted by anti-HSF1 antibody but not by anti-HSF2 antibody. This is consistent with previous reports that heat shock primarily upregulates HSF1 (29,30).

To determine if HSFs interacted with the NAT1 promoter in vivo, Chromatin immunoprecipitation (ChIP) assays were performed using chromatin from dimethyl sulfoxide or R1881-treated 22Rv1 cells. These results showed that both HSF1 and HSF2 interacted with the NAT1 promoter, both in the absence and presence of R1881 treatment (Figure 2D), which is in agreement with the EMSA results (Figure 2B).

Nuclear HSF1 and HSF2 levels increase following androgen treatment

Upon activation, HSFs trimerize and translocate from the cytosol to the nucleus (31). To determine if androgen treatment induced an increase in nuclear HSFs, 22Rv1 cells were treated with 100 nM R1881 (Figure 1A).
and nuclear extracts prepared at various times and analyzed by western blots using anti-HSF1 or anti-HSF2 antibodies. Both HSFs were present in the nucleus of 22Rv1 cells in the absence of R1881 (time 0), which is consistent with the EMSA and ChIP analyses (Figure 2B and D). R1881 treatment increased both HSF1 and HSF2 levels in the nucleus, by 2.6- and 2.2-fold, respectively (Figure 3). Maximal levels of both HSF1 and HSF2 were observed at ~16 h.

Overexpression of HSF1, but not HSF2, increases NAT1 promoter activity

We next investigated whether the HSFs were able to activate the NAT1 promoter in the absence of androgen. For this, 22Rv1 cells grown under androgen-free conditions were cotransfected with the NATb promoter construct pGL3-ex4-790, pSV-βGal (internal control) and either pFLAG-EV, pFLAG-HSF1, pFLAG-HSF2 or both pFLAG-HSF1 and pFLAG-HSF2. HSF1 significantly increased NATb promoter activity (1.5-fold), but this was considerably less than that seen following R1881 treatment alone (Figure 4A). The addition of R1881 to cells overexpressing HSF1 did not lead to a further increase in promoter activity compared with R1881 treatment alone. In contrast, promoter activity was not increased following overexpression of HSF2. In addition, coexpression of HSF2 with HSF1 had no effect on the increase in promoter activity seen with HSF1 overexpression alone. When mutations were introduced into the HSE of the reporter

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**Fig. 2.** EMSAs show that both HSFs 1 and 2 bind to the NATb HSE that is involved in the upregulation of NAT1 activity by androgen. (A) Sequences of the NATb oligonucleotides used in EMSAs. The 12 bp palindrome containing two consensus nGAAn motifs is underlined. (B) Labeled oligonucleotide (NATb–HSE) was incubated with nuclear extract from dimethyl sulfoxide (Control) or R1881 treated 22Rv1 cells and resolved on 5% polyacrylamide gels. A specific complex that was completely disrupted by addition of 100-fold molar excess of unlabeled NATb–HSE is indicated by an arrow. In competition studies, this complex was also completely disrupted by HSP70–HSE but not by NATb–HSE oligonucleotides where the HSE was mutated or by a non-specific oligonucleotide. (C) In antibody supershift assays, antibodies to either HSF1 or HSF2 completely shifted the specific complex (denoted by asterisk) when nuclear extracts from either Control or R1881 treated cells were used. Nuclear extract from 22Rv1 cells that had been heat shocked at 42°C for 30 min, which primarily activates HSF1, was used to confirm specificity of the HSF antibodies. (D) ChIP assays show that both HSF1 and HSF2 bind to the NATb promoter both in the absence and presence of androgen. ChIP was performed on dimethyl sulfoxide (−) or R1881 (+) treated 22Rv1 cells using specific antibodies against HSF1 or HSF2. PCRs were performed on extracts before (input) or after immunoprecipitation using primers flanking the NATb HSE or a region of the GAPDH promoter as a negative control.
Discussion

The role of arylamine N-acetyltransferases in the development of cancers through the bioactivation of carcinogens is well established (32,33), and several studies have reported associations between N-acetyltransferase activity levels and cancer risk (3–5). Recently, we showed that NAT1 activity can be modulated by androgens (19), which could therefore potentially modify susceptibility to those cancers where the enzyme may play a role.

Androgens appear to regulate expression of the NAT1 gene indirectly. Previously, we showed that cycloheximide prevents the androgen-induced increase in NAT1 messenger RNA levels, indicating that prior protein synthesis is required, possibly of a transcription factor that then acts on the NAT1 promoter NATb. The lack of a classical androgen response element located in the androgen-responsive region of NATb also supports an indirect effect (19). In the present study, we identified a 12 bp palindrome located 776 bp upstream of exon 4 in NATb that contains two consensus NGAn motifs that form part of a functional HSE. These motifs appear to be essential for the androgen responsiveness of the NAT1 gene. Moreover, a similar sequence can be found in the mouse homolog NAT2 gene promoter, although 2743 bp upstream of the transcription start site.

The HSE in NATb contains five NGAn motifs, two of which are consensus and contained within the 12 bp palindrome. Therefore, the synergistic binding of two HSF trimers to the HSE in NATb is possible (34,35). However, because mutation of the central consensus NGAn motif results in the complete loss of androgen responsiveness, a single HSF trimer most probably binds to the promoter. The palindromic sequence in NATb is similar to the HSE found in the clusterin promoter, which was reported to bind HSF1 and HSF2 as heterotrimers (36). The formation of trimers is a crucial step in the activation process of HSFs, resulting in increased DNA binding affinity (37). The DNA binding ability of HSF1 appears to be modulated by HSF2 and vice versa, and it has been suggested that interplay between the HSF’s may modulate their ability to drive gene transcription (38). The results presented here suggest that HSF1–HSF2 heterotrimers occupy the HSE in NATb, since specific antibodies against HSF1 or HSF2 completely disrupted complexes formed in EMSA. In support of this, ChIP analysis indicated that both HSFs bind to the NATb HSE in vitro. Recent evidence suggests that heterotrimerization may be a transcriptional switch at the interface of activation by either HSF1 or HSF2 (39). However, in the case of the NATb promoter, HSF2 does not appear to affect the ability of HSF1 to activate transcription, as evidenced by HSF overexpression studies.

The activation process of HSF’s involves several distinct steps, including trimerization, translocation to the nucleus and modifications such as phosphorylation and sumoylation, which regulate promoter transactivation ability. As a result, HSE occupancy by HSF trimers does not necessarily lead to efficient target gene transactivation. In other words, HSF activation can be uncoupled from transcriptional activation (40). Results from EMSA showed that both HSF1 and HSF2 bound to the HSE in NATb in the absence of androgen, and this was confirmed in ChIP analysis, suggesting that HSFs occupy the HSE constitutively. Although EMSA provided no evidence of increased HSF binding in response to androgen, it is possible that the transcriptional competence of the already bound HSF trimers is enhanced by androgen or that they are displaced by HSF trimers of greater transcriptional competence.

Western blot analysis showed a gradual and prolonged increase in both HSF1 and HSF2 in the nuclei of cells treated with androgen. Several other laboratories have reported that steroid receptor activation can affect HSF1 activity both in vitro and in vivo (41,42). We also observed that exogenous expression of HSF1, but not HSF2, was sufficient to increase NAT1 promoter activity, although the increase was considerably less than that seen with androgen treatment. Furthermore, HSF1 overexpression also resulted in higher endogenous NAT1 enzyme activity in the absence of androgen. Although heat shock is known to activate HSF1, it had no effect on NATb promoter activity or endogenous NAT1 activity (data not shown). However, western blot showed that HSF1 levels in the nucleus returned to basal levels within 30 min following heat shock (data not shown), as opposed to the prolonged increase caused by androgen treatment. This may explain the lack of effect on NAT1 and suggests that NAT1 expression is not part of the cellular stress response.

Although HSF1 is best known as the master regulator of the heat shock response, it is becoming increasingly evident that it also plays an important role in supporting tumor development (43). It has been suggested that HSF1 supports malignant transformation by coordinating a diverse array of cellular functions including cell proliferation, survival, protein synthesis and glucose metabolism (43). NAT1 overexpression in breast cancer cells promotes cell proliferation and resistance to etoposide (44), and several studies suggest that it may play...
a role in cellular metabolism (10). How or if this role of NAT1 is related to its regulation by HSF1 remains to be determined.

The potential role of NAT1 in prostate cancer, a disease that is often dependent on androgen receptor activity, has been widely investigated. Prostate tissue possesses N-acetylation capacity and can O-acetylate carcinogens such as N-hydroxy-PhIP, which is commonly found in cooked meat (45). However, studies investigating the link between NAT1 genotypes and prostate cancer have been equivocal. Although some have suggested that the ‘rapid’ NAT1 genotype (NAT1/C310) is a risk factor (46,47), others have not seen an association (48). However, NAT1 activity is not only under the control of genetic polymorphisms (10) and environmental factors that increase or decrease enzyme activity may influence pharmacogenetic studies. In humans, NAT1 activity is highest in the peripheral zone of the prostate (49), a region where most carcinomas arise (50). Both NAT1 and the androgen receptor are colocalized to the epithelium of prostate tissue (19), which is consistent with our findings that androgens can regulate NAT1 expression.

In summary, we have identified a HSE in the human NAT1 gene promoter that is essential for androgen induction of NAT1 activity. Our data suggest that NAT1 activity may vary with differential expression of HSF1, such as during embryonic development where both HSF1 and HSF2 have been proposed as important regulators of developmental events (51). However, the exact physiological role of the HSE in the NAT1 gene remains to be elucidated.

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Overexpression of HSF1, but not HSF2, upregulates NATb promoter reporter activity and endogenous NAT1 activity. To assess the effect of HSF1 and HSF2 on NATb promoter reporter activity, 22Rv1 cells were transiently transfected with either the NATb promoter construct (A) pGL-ex4-790 (intact HSE) or (B) pGL-ex4-790m (mutated HSE) together with pFLAG-EV (empty vector), pFLAG-HSF1, pFLAG-HSF2 or both HSF constructs. All transfections contained the pSV-βGal plasmid (internal control). Following transfection, cells were treated with either dimethyl sulfoxide (DMSO; closed columns) or 100 nM R1881 (open columns) for 24 h and then lysed and luciferase and β-galactosidase assays were performed. Asterisk indicates significant increase compared with paired DMSO treatments (Student’s t-test, P < 0.05). Double asterisks indicate significant increase compared with DMSO-treated EV (Student’s t-test, P < 0.05). Above each graph is a representative FLAG western blot showing expression of FLAG-tagged HSF1 and HSF2 from DMSO-treated cells. (C) Effect of HSF1 and HSF2 on endogenous NAT1 activity. 22Rv1 cells were transfected with either pFLAG-EV, pFLAG-HSF1 or pFLAG-HSF2 constructs for 24 h and then lysed and NAT1 activity assayed. Some non-transfected cells were treated with 100 nM R1881 for 24 h for comparison. Transfection efficiency was assessed by FACS of cells transfected with pEGFP. A representative FLAG western blot showing expression of FLAG-tagged HSF1 and HSF2 is shown above the graph. Asterisk indicates significant increase compared with control (Student’s t-test, P < 0.05).

![Figure 5](https://example.com/fig5.png)

**Fig. 5.** HSF1 siRNA downregulates HSF1 and attenuates R1881 induction of NAT1. Prostate 22Rv1 cells were treated with siRNA directed against HSF1 (closed columns) or a scrambled sequence (Scr; open columns), for 48 h followed by R1881 (100 nM) treatment for a further 24 h. The effect of treatment on HSF1 protein levels (A), NAT1 messenger RNA (mRNA) levels (B) and NAT1 enzyme activity (C) is shown. Asterisk indicates significant decrease in HSF1 siRNA-treated cells compared with scrambled siRNA-treated cells (Student’s t-test, P < 0.05).
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