Identification of a minimal promoter sequence for the human *N*-acetyltransferase Type I gene that binds AP-1 (activator protein 1) and YY-1 (Yin and Yang 1)

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Human *N*-acetyltransferase Type I (NAT1) catalyses the acetylation of many aromatic amine and hydrazine compounds and it has been implicated in the catabolism of folic acid. The enzyme is widely expressed in the body, although there are considerable differences in the level of activity between tissues. A search of the mRNA databases revealed the presence of several NAT1 transcripts in human tissue that appear to be derived from different promoters. Because little is known about *NAT1* gene regulation, the present study was undertaken to characterize one of the putative promoter sequences of the *NAT1* gene located just upstream of the coding region. We show with reverse-transcriptase PCR that mRNA transcribed from this promoter (Promoter I) is present in a variety of human cell-lines, but not in quiescent peripheral blood mononuclear cells. Using deletion mutant constructs, we identified a 20 bp sequence located 245 bases upstream of the translation start site which was sufficient for basal NAT1 expression. It comprised an AP-1 (activator protein 1)-binding site, flanked on either side by a TCATT motif. Mutational analysis showed that the AP-1 site and the 3′ TCATT sequence were necessary for gene expression, whereas the 5′ TCATT appeared to attenuate promoter activity. Electromobility shift assays revealed two specific bands made up by complexes of c-Fos/Fra, c-Jun, YY-1 (Yin and Yang 1) and possibly Oct-1. PMA treatment enhanced expression from the NAT1 promoter via the AP-1-binding site. Furthermore, in peripheral blood mononuclear cells, PMA increased endogenous NAT1 activity and induced mRNA expression from Promoter I, suggesting that it is functional *in vivo*.

Key words: *N*-acetyltransferase, activator protein 1 (AP-1), gene induction, promoter, transcription factor.

INTRODUCTION

In humans, the acetylation of primary arylamines and hydrazines is catalysed by two cytoplasmic acetyltransferases (EC 2.3.1.5), *N*-acetyltransferase Type I (NAT1) and NAT Type II (NAT2) (also called arylamine *N*-acetyltransferases). The genes encoding these proteins were first identified by Grant et al. [1,2], and both have been mapped to chromosome 8p21.3–23.1 [3]. Sequencing of *NAT1* and *NAT2* revealed a number of allelic variants that affect activity of each gene *in vivo* [4,5]. This work provided a genetic understanding of the long known polymorphism in NAT1 and NAT2 activity [6–9].

NAT1 acetylates a number of exogenous substrates, as well as the folate catabolite *p*-aminobenzoylglutamate [10,11]. Whereas NAT1 is widely expressed in humans, there is a considerable heterogeneity in the levels of expression between tissues [12]. Two NAT1 mRNAs submitted to GenBank are homologous to the coding and 3′ non-coding region of the genomic sequence, but differ from each other at the 5′ non-coding region. The first mRNA (GenBank® accession number D90041) was isolated from human liver [13] and has a 5′ non-coding sequence identical with that of the published genomic sequence, indicating a lack of introns [2]. Several expressed sequence tags confirm the presence of this transcript in a variety of tissues. The predicted length of the message determined by Northern blots [2] suggests that the promoter of this NAT1 transcript resides approx. 250–300 bases upstream of the translational start site. We have tentatively called this Promoter I. A second transcript derived from ovarian adenocarcinoma cells (GenBank® accession number BC013732) contains a 5′ non-coding sequence that, when aligned to the genomic sequence of 8p21.3–23.1, indicates the presence of several introns in this region of the gene that extend 12 kb upstream of the coding sequence. This transcript indicates that a second promoter (Promoter II) for *NAT1* exists further upstream. Neither of these putative promoters have been characterized to date.

In the present study, we have investigated the structure and function of Promoter I and show that the transcript generated from this regulatory region is present in a variety of human cell lines. We identified a 20 bp sequence 245 bases upstream of the coding region that contains an AP-1 (activator protein 1)-binding site and is activated by PMA. The AP-1-binding site is flanked on either side by a TCATT motif which have a role in gene expression.

EXPERIMENTAL

Materials

All cell lines were originally obtained from the A.T.C.C. (Rockville, MD, U.S.A.). Donor PBMCs (peripheral blood mononuclear cells) were obtained by venipuncture and isolated as described previously [9]. Restriction enzymes, dNTPs, Dual Luciferase kit, Erase-A-Base kit, pRL-SV40 and pGL3-enhancer vectors were obtained from Promega (Madison, WI, U.S.A.).

Abbreviations used: AP-1, activator protein 1, CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic-mobility-shift assay; GM-CSF, granulocyte/macrophage colony-stimulating factor; NAT, *N*-acetyltransferase; NAT1-MP, NAT1 minimal promoter; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cell; YY-1, Yin and Yang 1.

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Table 1 Oligonucleotides used in this study

Bases that differed from the NAT1-MP sequence are underlined. For the EMSA experiments, complementary oligonucleotides (not shown) were used to generate double-stranded oligonucleotides. The non-specific oligonucleotide NS is from Naora et al. [17].

Oligonucleotide name	Sequence	Comment
PCR		
NAT1-F	5′-GGGAAATTGAGTGGGTCAGG-3′	Forward primer for initial cloning of the *NAT1* 5′ region
NAT1-R	5′-CCATGATCCCCTAAGCAAGG-3′	Reverse primer for initial cloning of the *NAT1* 5′ region
FP1	5′-TAGCCATAATTAGCCTACTC-3′	Forward primer for mapping of transcription start site*
FP2	5′-GGGACTATTAACTGAACTTATGT-3′	Forward primer for mapping of transcription start site*
FP3	5′-ATCATTTGACTCATCATTTA-3′	Forward primer for mapping of transcription start site*
FP4	5′-AAATTGAGTGGGTCAGG-3′	Forward primer for mapping of transcription start site*
RP1	5′-AAGGTGAATCATGCCAGTGC-3′	Reverse primer for mapping of transcription start site
97-F	5′-CCCAAGCTTATCTTGATTTCCAGC-3′	Forward primer for construction of pGL-97
M1-F	5′-CCCAAGCTTCCGATGTGACTCATCATTTAATCTTG-3′	Forward primer for construction of pGL-M1
M2-F	5′-CCCAAGCTTATCATTGCCGACTTCATTTAATCTTG-3′	Forward primer for construction of pGL-M2
M3-F	5′-CCCAAGCTTATCATTTGACTCAGCACTTCATCTTGATTTC-3′	Forward primer for construction of pGL-M3
PGLprimer2	5′-CTTTATGTTTTTGGCGTCTTCCA-3′	Reverse primer for construction of pGL-97, pGL-M1, GL-M2 and pGL-M3
Actin-F	5′-TCACCCACACTGTGCCCATCTACGA-3′	Forward primer for amplification of β-actin
Actin-R	5′-CAGCGGAACCGCTCATTGCCAATGG-3′	Reverse primer for amplification of β-actin
EMSA		
NAT1-MP	5′-ATCATTTGACTCATCATTTA-3′	Wild-type NAT1 oligonucleotide
NS	5′-CCGATGGCCGACTGCACTTCT-3′	Non-specific sequence used as control
AP-1	5′-CGCTTGATGACTCAGCCGGAA-3′	Consensus AP-1 oligo lacking both TCATT motifs
M1	5′-CCGATGTGACTCATCATTTA-3′	Mutations in 5′ TCATT motif of NAT1-MP
M2	5′-ATCATTGCCGACTTCATTTA-3′	Mutations in AP-1 like motif of NAT1-MP
M3	5′-ATCATTTGACTCAGCACTTC-3′	Mutations in 3′ TCATT motif of NAT1-MP

* See Figure 1 for location.

Primers and plasmid purification kits were purchased from GeneWorks (Adelaide, Australia), and Rneasy kit was obtained from Qiagen (Melbourne, Australia). Poly[d(I-C)] and High Pure RNA Isolation Kits were supplied by Roche (Castle Hill, Australia). RPMI 1640, DMEM (Dulbecco's modified Eagle's medium), DH5α bacteria and T4 polynucleotide kinase were obtained from Invitrogen (Melbourne, Australia), and antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). G-25 minispin columns and pUC18 were purchased from Amersham Pharmacia Biotech (Melbourne, Australia). Fetal bovine serum was supplied by the Commonwealth Serum Laboratories (Melbourne, Australia). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

All cell lines were cultured in RPMI 1640 (except PBMCs which were cultured in DMEM), supplemented with 10% fetal bovine serum, 80 units/ml benzylpenicillin and 50 μg/ml gentamicin, at 37 °C in an atmosphere of 5% CO_2 in air.

Isolation of genomic fragment by PCR

The -416 to $+1$ region (relative to the translational start site) of the human *NAT1* gene was generated by PCR using human genomic DNA and the primers NAT1-F and NAT1-R (Table 1). This fragment was blunt-end ligated into the *Sma*I site of *pUC18* and sequenced by the dideoxynucleotide chain termination method. A 247 bp *Kpn*I–*Eco*RI fragment from the plasmid was then cloned into the multiple cloning site of the pGL3-enhancer vector upstream of the firefly luciferase gene, creating the pGL-247 construct.

Amplification of NAT1 mRNA

Cytoplasmic RNA was isolated using a High Pure RNA Isolation Kit or the Rneasy kit, and 0.5 μg of RNA was reverse transcribed with 15 units of avian myeloblastosis virus reverse transcriptase (RT), 5 mM $MgC1_2$, 1 mM dNTPs, 1 units/μl rRNasin ribonuclease inhibitor and 0.5 μg of oligo$(dT)_{15}$ primer in buffer (10 mM Tris/HCl, pH 8.8/50 mM KCl/0.1% Triton X-100). Reactions were incubated at 42 °C for 30 min and then inactivated by heating at 98 °C for 5 min. NAT1 cDNA was amplified by PCR using the common reverse primer RP1 (Table 1), which is located in the open reading frame of the *NAT1* gene, and the forward primers FP1-4 (Table 1), which are located at various distances upstream of the NAT1 open reading frame (Figure 1). PCR reactions were carried out in PCR buffer containing 5 μl of cDNA mix, 2 mM $MgCl_2$, 0.5 unit of Taq DNA polymerase, and 12.5 pmol of each primer in a final volume of 20 μl. Samples were amplified using the following conditions: denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 45 s. As a control, PCR reactions were also performed to detect β-actin using primers Actin-F and Actin-R (Table 1). PCR conditions were as described above, except the annealing temperature was 58 °C and the number of cycles 25.

Promoter deletion constructs

A series of deletion mutants was generated from the pGL-247 construct using the Erase-A-Base system. In brief, the pGL-247 plasmid was linearized with *Sac*I to generate exonuclease III resistant 3′ overhangs on each end. The 5′ end of the NAT1 promoter fragment was then converted to an exonuclease III sensitive overhang by digestion with *Nhe*I. The resulting DNA was

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Figure 1 5′ Untranslated region of the human *NAT1* gene

The complete 441 bp sequence, originally reported by Blum et al. [2], is shown with the start codon at the 3′ end. The 247 bp *Kpn*I–*Eco*RI fragment used in the present study (**bold** type) was cloned into the pGL3-Enhancer vector to create pGL-247. A series of deletion mutants were then generated as outlined in the Experimental section. Progressively shorter clones (pGL-220, pGL-185, pGL-144, pGL-117, pGL-97, pGL-83 and pGL-54) were selected and the locations of their 5′ ends are shown in the Figure. The deletion constructs are named with numbers representing their length from the 3′ T of the *Eco*RI site. Also shown is a putative consensus initiator element (boxed) [16], as well as the position of the four forward primers (FP1–4, underlined) used to locate the approximate position of the transcription start site.

phenol–chloroform extracted and then treated with exonuclease II and S1 nuclease, according to the manufacturer's instructions. At 30 s intervals, the reaction was terminated, the DNA was ethanol precipitated, redissolved in water and treated with Klenow fragment. After religation, the resulting plasmids were used to transform DH5α cells. Clones were isolated and sequenced, and six constructs with promoter lengths of 54 bp (pGL-54), 83 bp (pGL-83), 117 bp (pGL-117), 144 bp (pGL-144), 185 bp (pGL-185) and 220 bp (pGL-220) were selected for use in transient transfection assays (see Figure 1 for location of each fragment).

The pGL-97, pGL-M1, pGL-M2 and pGL-M3 constructs were prepared by PCR using appropriate oligonucleotides containing a *Hin*dIII site (97-F, M1-F, M2-F and M3-F respectively, see Table 1) and the common reverse primer pGLprimer2 (Table 1). The fragments generated were *Hin*dIII digested and cloned into the *Hin*dIII site of pGL3-enhancer vector, purified from DH5α clones and used for transfection experiments. DNA concentrations were determined by a fluorimetric assay [14], and all constructs were sequenced by the dideoxynucleotide chain termination method.

Transient expression of reporter constructs

Transient cell transfection using the DEAE-dextran method was performed as follows. Transfection solutions were prepared using 0.5 μg of plasmid DNA, 0.1 μg of the co-reporter (pRL-SV40 containing the *Renilla* luciferase gene) and 0.5 mg/ml DEAE-dextran in RPMI/20 mM Hepes buffer (RPMI/Hepes), pH 7.3. The cells, at a density of 4×10^6 cells/ml, were treated for 90 min, after which time they were washed twice with RPMI/Hepes and the medium exchanged for RPMI-1640 containing 10 % fetal calf serum. Cells were plated at 4.5×10^5 cells/ml and cultured for 48 h. They were then harvested and extracts prepared for the Dual Luciferase Assay, according to the manufacturer's instructions. *Renilla* luciferase activities served to normalize firefly luciferase activities, and all results are reported as means $\pm$ S.E.M. for at least three experiments.

Preparation of nuclear extracts

THP-1 cells (2.5×10^7) were harvested by centrifugation at 1000 ***g*** for 5 min, washed once with ice-cold PBS and resuspended in 600 μl of ice-cold lysis buffer [10 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM DTT (dithiothreitol)/0.5 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin A]. On ice, the cells were disrupted by passage through a 27-gauge needle. The nuclei were collected by centrifugation in a microcentrifuge at 15000 ***g*** for 8 s. The supernatants were discarded and the cell pellets resuspended in 70 μl of ice-cold extraction buffer (lysis buffer containing 20 mM Hepes, pH 7.9, 420 mM KCl, 0.2 mM EDTA and 25 % glycerol). After 30 min on ice, 50 μl of storage buffer (20 mM Hepes, pH 7.9/0.5 mM DTT/0.2 mM EDTA/20 % glycerol/0.5 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin A) was added and the extracts centrifuged at 15000 ***g*** for 10 min (4 °C). Supernatants were stored at -20 °C. Protein concentrations (usually 3–6 μg/μl) were determined by the Bradford method [15], using BSA as a standard.

EMSA (electrophoretic-mobility-shift assay)

Nuclear extracts (5 μg of protein) were incubated for 30 min at 22 °C in binding buffer (2 % Ficoll 400/5 % glycerol/20 mM Hepes, pH 7.9/50 mM KCl/1 mM EDTA/2.5 mM DTT) containing 0.5 μg of poly[d(I-C)] and ^{32}P-labelled oligonucleotide probe (1×10^5 d.p.m.) in a final volume of 10 μl. Samples were then resolved by electrophoresis on 5 % polyacrylamide gels at 200 V in 0.25 × Tris/borate/EDTA buffer for 150 min at 4 °C. Gels were dried and placed on a phosphorimage screen for 12 h.

Oligonucleotides were purified by reverse-phase chromatography and freeze-dried. Double-stranded oligonucleotides were prepared by heating both sense and anti-sense strands at 65 °C for 5 min before slowly cooling to 22 °C. Oligonucleotides were labelled using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase. Labelled probes were purified from unincorporated [γ-^{32}P]ATP using MicroSpin G-25 columns.

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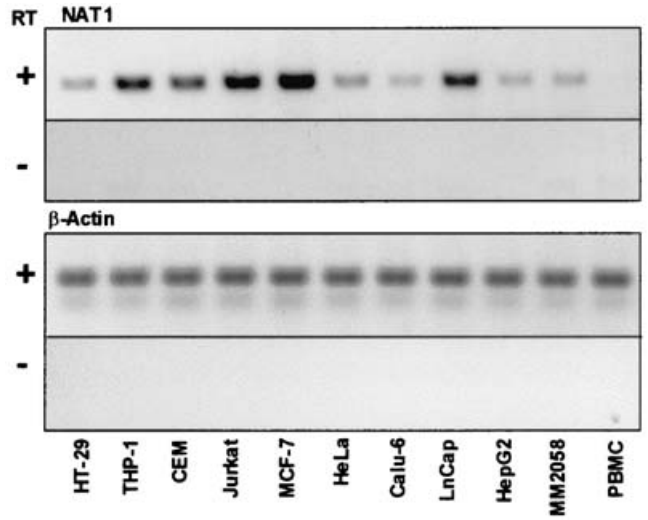

Figure 2 RT-PCR for message transcribed from the putative *NAT1* Promoter I

Cytosolic RNA from a variety of cell lines and PBMCs was reverse transcribed and then amplified using primers FP2 and RP1 (Table 1). β-Actin was also amplified as a positive control (lower panels). To ensure there was no DNA contamination, amplification was also performed on samples where RT was omitted from the reaction.

For competition assays, unlabelled oligonucleotides were added to the binding reactions at a 100-fold molar excess 10 min prior to the addition of radiolabelled probe. For antibody supershift analysis, binding reactions were incubated with 1 μg of antibody for 3 h at 4 °C before the addition of probe.

RESULTS

Identification of NAT1 mRNA in human cells

We used RT-PCR to detect NAT1 message transcribed from Promoter I (Figure 2). All cells except quiescent PBMCs expressed the transcript, although the amount of message appeared to vary between cell lines. Although the RT-PCR was not performed under quantitative conditions, there were clear qualitative differences between the various cell lines. The highest level of transcript was seen in breast carcinoma MCF7 cells and T-lymphotropic Jurkat cells. NAT1 cDNA from PBMCs did not amplify, despite amplification of β-actin gene.

Location of the NAT1 transcription start site

We attempted to locate the transcription start site for NAT1 mRNA by 5′-RACE (rapid amplication of cDNA ends), but were unsuccessful, possibly due to its low copy number. We then used a series of primers that hybridized upstream of the NAT1-coding sequence and RT-PCR to identify the approximate region of the transcription start site (Figure 3). The location of the primers used is shown in Figure 1. The results show that the NAT1 transcript commenced somewhere between the primers FP2 and FP3 or between -161 and -244 bases upstream of the translation start site, which is consistent with the size of human NAT1 mRNA reported by Ohsaka and Deguchi [13]. There is no evidence of a TATA box in this region, although at least one consensus initiator element (YYANWYY) [16] is present, located at -219 to -226

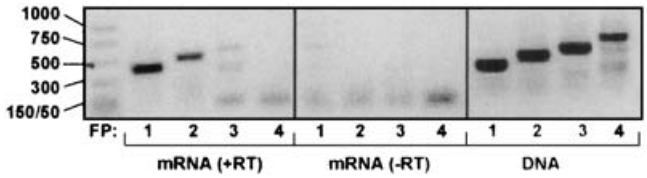

Figure 3 Approximate location of the *NAT1* transcription start site

To determine the approximate location of the start site for message transcribed from Promoter I, a series of forward primers (FP1–FP4; Table 1) and a common reverse primer (RP1) was used to amplify reverse-transcribed RNA isolated from Jurkat cells. Genomic DNA was used as a positive control to illustrate the expected PCR products (right-hand panel). Amplification of cDNA was only successful using FP1 and FP2 (left-hand panel). To ensure DNA contamination did not interfere with the RT-PCR, RT was omitted from each of the amplification reactions (middle panel).

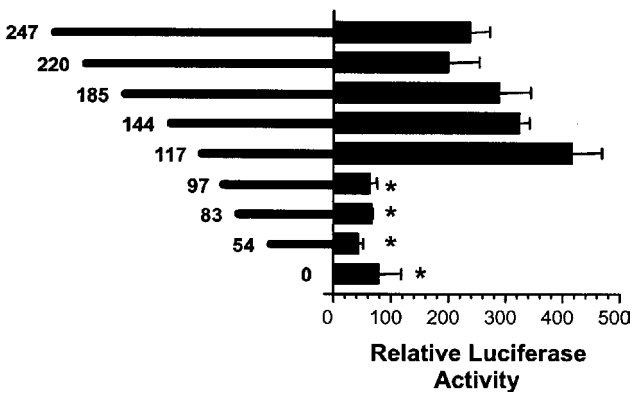

Figure 4 Location of promoter elements using deletion mutants

A series of deletion constructs was transiently transfected along with the co-reporter plasmid pRL-SV40 (internal control) into THP-1 cells. After 48 h, cell lysates were prepared for dual luciferase assays. Activity is expressed as relative luciferase activity (means $\pm$ S.E.M., $n = 3$). Asterisk indicates results significantly less ($P < 0.05$) than the full-length promoter (one-way analysis of variance with Dunnett's post-test comparison).

(Figure 1). Based on these findings, we investigated a 247 bp *Kpn*I–*Eco*RI fragment for promoter activity.

Identification of Promoter I

A series of deletion mutants of the *NAT1* 5′ non-coding region (-147 bp to -394 bp relative to the transcription start site) fused to a luciferase reporter gene was examined for basal promoter activity by transient transfection in the monocytic cell line THP-1 (Figure 4). The longest sequence (pGL-247, where the number represents the number of base pairs 5′ of the *Eco*RI site located in the 5′UTR; see Figure 1) produced a 3- to 4-fold increase in promoter activity compared with the pGL-enhancer vector. Activity was seen in all constructs containing at least the first 117 bp. Similar results were seen with CEM (T-cell leukaemia), HT-29 (colon carcinoma), MCF-7 (breast carcinoma) and Jurkat cells (results not shown). These results identify a promoter sequence (Promoter I, 5′-ATCATTTGACTCATC-ATTTA-3′) between -245 and -264 bases upstream of the coding region (shown as FP3 in Figure 1). Putative transcription factor binding sites are present within Promoter I, in particular an AP-1 consensus sequence (TGACTCA) and two TCATT motifs that have been identified in the regulatory sequences of several genes including the GM-CSF (granulocyte/macrophage

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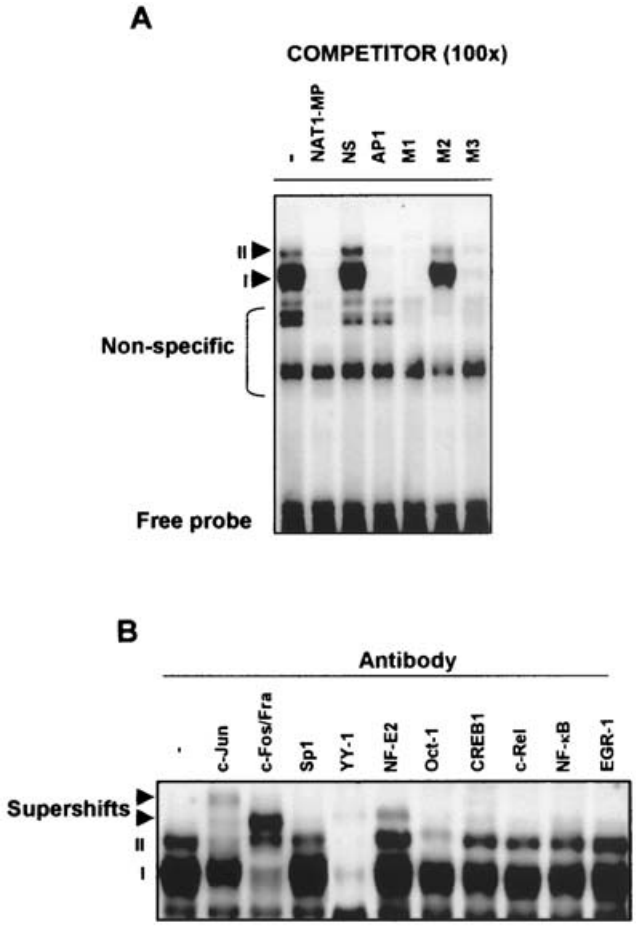

Figure 5 EMSAs with the Promoter I probe and THP-1 nuclear extracts

(**A**) Labelled oligonucleotide and nuclear extracts were resolved on 5 % polyacrylamide gels to show two specific bands (I and II) that were competed with 100-fold excess probe (NAT1-MP), but not with the non-specific oligonucleotide (NS). Both band I and band II were competed with the consensus AP-1 oligonucleotide, as well as the M1 and M3 oligonucleotides, which contain mutations in the 5′ and 3′ TCATT sequence respectively. Only band II was competed with M2 oligonucleotide which contained mutations in the AP-1-binding site. The sequence of each oligonucleotide used is shown in Table 1. (**B**) Supershifted EMSAs with the NAT1-MP probe and THP-1 nuclear extracts. Nuclear protein (5 μg) was incubated with antibody (1 μg) for 3 h at 4 °C before the addition of radiolabelled probe. The complexes were then resolved on 5 % polyacrylamide gels. The two specific bands identified in (**A**) are shown. Supershifted bands are indicated by arrow heads.

colony-stimulating factor) gene [17–22], interleukin-5 gene [17,23], CD35 gene [24], involucrin gene [25], glucagon gene [26], papillomavirus E6–E7 gene [27] and the angiotensin II type 1a receptor gene [28].

Binding of nuclear proteins to Promoter I

EMSAs were performed with nuclear extracts from THP-1 cells and the Promoter I oligonucleotide as a probe (Figure 5A). Two specific bands (I and II) were identified that could be competed with 100-fold molar excess of unlabelled probe, but not by a non-specific oligonucleotide (Figure 5A). There were three faster migrating bands that were also inhibited by NAT1-MP (NAT1 minimal promoter). However, since these bands were also partially or completely inhibited by the non-specific oligonucleotide, they were considered as non-specific bands for this study. The faster migrating specific complex (band I) was significantly more intense than band II and appeared to comprise multiple complexes with similar electromobilities. The formation of both bands were inhibited by the AP-1 consensus oligonucleotide, as well as M1 and M3 which have base changes in each of the flanking TCATT motifs, but not in the AP-1 sequence. M2, which has mutations in the AP-1-binding site only, had a slight effect on the formation of both bands. Collectively, these results suggest that the faster migrating complex was formed primarily through the interaction of nuclear proteins with the AP-1-binding site. The slower migrating complex is possibly formed by the binding of additional protein(s) to adjacent bases.

Identification of binding proteins in THP-1 nuclear extracts

The presence of the AP-1 consensus sequence in Promoter I suggested that transcription factors of the bZIP family bind to this sequence and possibly regulate NAT1 basal expression. We examined the binding of a number of transcription factors by supershift assays using the Promoter I oligonucleotide as a probe (Figure 5B). The anti-c-Jun antibody shifted band II and decreased the intensity of band I. In contrast, the anti-Fos/Fra antibody, which recognizes c-Fos, Fos B, Fra-1 and Fra-2, completely shifted band I. Antibodies directed against NF-E2 (nuclear factor E2), Sp1, CREB1 (cAMP-response-element-binding protein 1), c-rel, NF-κB (nuclear factor κB) and EGR-1 (early growth response gene product 1) had little or no effect. Anti-Oct-1 antibodies selectively disrupted band II, whereas antibodies directed against YY-1 (Yin and Yang 1) completely abrogated the formation of both complexes.

Transcriptional activity of mutant Promoter I

The mutant sequences (M1, M2 and M3) used for competition assays in Figure 5(A) were engineered into pGL-117 to produce the reporter plasmids pGL-M1, pGL-M2 and pGL-M3. These constructs were then used for transient transfection assays in THP-1, Jurkat and HT-29 cells (Figure 6). When compared with the wild-type sequence (pGL-117), mutations in the 5′ TCATT sequence (pGL-M1) enhanced activity by 2- to 3-fold in each cell line. In contrast, mutations in the AP-1-binding site (pGL-M2) decreased gene expression to that seen with the pGL-enhancer construct. Similarly, mutation of the 3′ TCATT sequence (pGL-M3) also resulted in a significant decrease in promoter activity. Thus both the AP-1-binding site and 3′ TCATT motif are required for optimum activity of Promoter I. In contrast, the 5′ TCATT motif appeared to act as an attenuator of activity.

Activation of AP-1 and *NAT1* expression by PMA

To determine whether Promoter I responds to AP-1 activation, the effect of PMA treatment on luciferase expression from the pGL-117, pGL-M1, pGL-M2, pGL-M3 and pGL-SV40 constructs was investigated in Jurkat cells. For these experiments *Renilla* control vector was not included, because the promoter (SV40) for this construct is inducible by PMA. Instead, cells were transfected with each construct and then divided into aliquots after 24 h. One aliquot of cells was treated with PMA and the second was treated with vehicle. Thus transfection efficiency within each experiment was constant. As a positive control for PMA activation, the pGL-SV40 construct was also included and showed a 6-fold increase in luciferase expression compared with untreated cells (Figure 7A).

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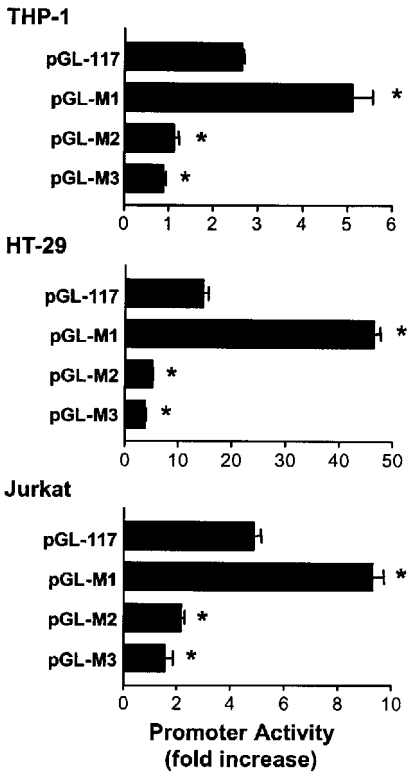

Figure 6 Effect of mutations in NAT1-MP on gene expression

Mutations were introduced into the pGL-117 reporter construct to alter the three motifs identified in the NAT1-MP sequence. Each construct was then transiently transfected into THP-1, Jurkat or HT-29 cells. Luciferase activities were measured 48 h later and are expressed relative to the *Renilla* luciferase activity. The results are expressed as the means ± S.E.M. for three experiments. Asterisk indicates results significantly different ($P < 0.05$) to pGL-117 (one-way analysis of variance with Dunnett's post-test comparison).

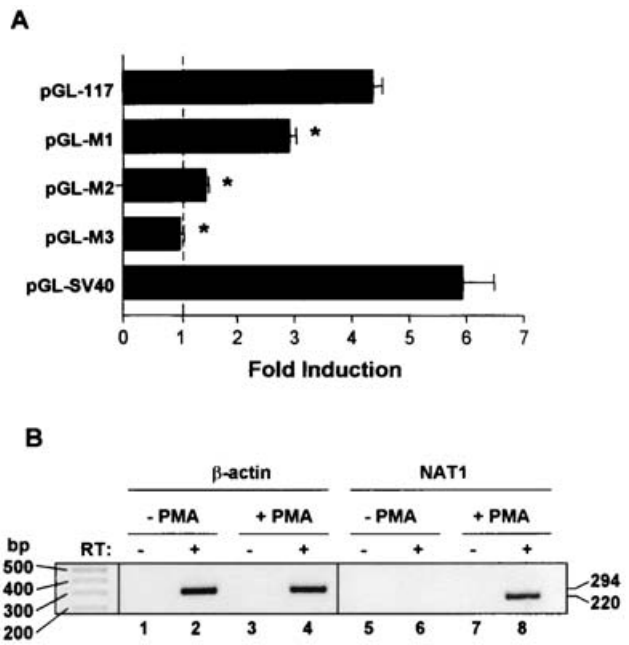

Figure 7 Effect of PMA on Promoter I activity and endogenous *NAT1* expression

(**A**) Jurkat cells were transiently transfected with pGL-117 and the respective mutant constructs as described in Figure 6. After 24 h, the transfected cells were divided in two and treated with PMA (100 ng/ml) or vehicle (0.1 % DMSO). Luciferase activity was measured 24 h later. The results are expressed as the fold increase in activity compared with vehicle-treated cells (means ± S.E.M., $n = 3$). Asterisk indicates results significantly different ($P < 0.05$) than pGL-117 (one-way analysis of variance with Dunnett's post-test comparison). (**B**) PBMCs were treated with PMA (100 ng/ml) for 24 h, following which cytosolic RNA was isolated and amplified by RT-PCR to detect the presence or absence of mRNA transcribed from Promoter I (lanes 5–8). To control for the integrity of the isolated RNA, β-actin was amplified from each RNA sample (lanes 1–4). Each PCR reaction was also performed using RNA without RT to ensure a lack of DNA contamination (lanes 1, 3, 5 and 7).

PMA enhanced expression from both pGL-117 and pGL-M1, but had little or no effect on pGL-M2 or pGL-M3, consistent with the AP-1-binding site and the 3′ TCATT motif being essential for gene expression.

We also examined the effects of PMA on endogenous *NAT1* activity in PBMC, the only cell type examined where there was no evidence of transcript produced from Promoter I (Figure 2). PMA significantly increased the activity of *NAT1* from 10.3 ± 0.7 to 16.0 ± 0.2 nmol/min per mg of protein (mean ± S.E.M., $n = 3$, $P < 0.05$) using *p*-aminobenzoic acid as substrate. RT-PCR showed NAT1 mRNA was generated in cells from Promoter I following PMA treatment, but not in untreated cells (Figure 7B), consistent with results shown in Figure 2. This could not be accounted for by DNA contamination, as there was no amplification in the absence of reverse transcriptase. Futhermore, the lack of a product for NAT1 in the untreated cells was not the result of little or no RNA, since β-actin cDNA amplified from these samples produced the predicted PCR product.

DISCUSSION

Human NAT1 is found in most tissues, although its level of activity varies markedly [12]. The genetic regulation of the *NAT1* gene has not been reported to date and there has been little study of its genomic organization. In the present study, we have identified a transcript that is generated from a regulatory region located 245 bases upstream of the translation start site. The transcript does not appear to contain introns and it is expressed in a range of human cell lines, but not in quiescent PMBCs. The promoter was mapped to a 20-bp fragment that contained an AP-1-like binding site, flanked on either side by a TCATT motif, and it was functional in several different human cell lines. Using mutagenesis and transient transfection assays, we found that both the AP-1 site and the 3′ TCATT sequence were essential for gene expression, whereas the 5′ TCATT appeared to act as an attenuator of promoter activity. We also identified several transcription factors, in particular members of the Fos/Fra family, capable of binding to this region of the gene. Antibodies to YY-1 and Oct-1 inhibited one or both of the specific complexes formed when nuclear extracts were incubated with the Promoter I oligonucleotide, suggesting that a complex of proteins bind to this sequence.

Promoter I is located 20 bases upstream of a putative consensus initiator element (Figure 1) that lies within the region of the transcription start site (Figure 3). The promoter is structurally similar to the rat glucagon gene promoter which contains a cAMP response element, flanked on either side by the TCATT repeat [26]. In the glucagon promoter, the flanking sequences are involved in the binding of accessory proteins as a complex with CREB, and the 3′ TCATT sequence attenuates stimulation of the promoter by protein kinase A. A similar CRE-TCATT motif is present in the VGF (vaccinia virus growth factor) gene promoter, although its role is yet to be fully defined [29]. The TCATT motif

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has also been found in the GM-CSF promoter, as described by several investigators [17–22], where it binds YY-1 [22]. YY-1 can display either activator or repressor activity depending upon the context of the promoter [22]. For some genes, YY-1 can suppress promoter activity, whereas for others it has been shown to activate transcription. Antibodies to YY-1 abrogated the specific interaction of nuclear proteins with the *NAT1* promoter I, a finding similar to that reported for the GM-CSF promoter [22].

PMA treatment induced gene expression from Promoter I, which is consistent with a central role for the AP-1-binding site. Interestingly, the pGL-M3 construct, which has an intact AP-1-binding site but lacks the 3′ TCATT motif, did not respond to PMA, suggesting that both motifs form a transcriptional complex that regulates promoter activity. The mRNA transcribed from Promoter I was present in a variety of cell lines, but not in quiescent PBMCs. PMA stimulation of PBMCs, which promotes cell proliferation, not only induced endogenous NAT1 activity, but also induced transcription from Promoter I. Although further work is required to fully understand the role of this region of the *NAT1* gene, it is possible that it is functional during the cell cycle or following stimuli that activate AP-1.

There has been little information about the regulation of the *N*-acetyltransferases published to date. Estrada-Rodgers et al. [30] have identified several putative regulatory elements, including a hormone-responsive element at −561, upstream of the mouse homologue *Nat2**. However, neither the TATA boxes nor the putative hormone-responsive element are evident in the human gene. A sequence similar to the human Promoter I described here is present in the mouse gene at −239 [30]. A more recent study by Mitchell and Warshawsky [31] examined regions of the *NAT1* and *NAT2* genes as far upstream as 1522 bases and reported that both genes were inducible by substrates selective for each enzyme. No specific sequence that endows inducibility was identified, and the activity of the different upstream regions in the absence of inducer was not reported. Nevertheless, both of these studies, along with the results of the present study, suggest that the regulation of *NAT1* may be a complex process that responds to several different external stimuli.

In conclusion, the present study has identified a regulatory region of the human *NAT1* gene with several consensus transcription factor binding sites. A number of studies have implied that NAT1 has a role in cells in addition to the acetylation of exogenous arylamine substrates [11,32–34]. The similarity of Promoter I with those from other functionally unrelated genes further supports this suggestion.

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