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Genes Immun. Author manuscript; available in PMC 2014 June 01.

Published in final edited form as:
Genes Immun. 2013 December ; 14(8): . doi:10.1038/gene.2013.41.

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HNRNPA1 interacts with a 5′ flanking distal element of interleukin 6 and up-regulates its basal transcription

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Abstract

Interleukin-6 (IL-6) is an important pro-inflammatory cytokine involved in many autoimmune and inflammatory diseases. We have shown previously that a region from −5307 to −5202 bp upstream of the IL-6 transcriptional start site is responsible for basal IL-6 gene expression and that there were DNA binding proteins involved from EMSA and transient expression experiments. Here we have combined surface plasmon resonance technology with mass spectrometry analysis and identified nuclear proteins bound to this region. HNRNPA1 and HNRNPA2B1 were found consistently. EMSA supershift and chromatin immunoprecipitation assays confirmed the involvement of HNRNPA1, but not HNRNPA2B1. Knocking down HNRNPA1 expression by siRNA resulted in reduced IL-6 transcriptional activity as assessed from transfection experiments using reporter constructs, mRNA and protein measurements. Overexpression of HNRNPA1 cDNA increased IL-6 mRNA expression. This regulation was dependent on the presence of the sequence from −5307 to −5202 bp of the IL-6 gene. Thus, HNRNPA1 is a novel transcriptional regulator of IL-6 expression, acting via the 5′ flanking sequence of the gene.

Keywords

IL-6; cis-regulation; Surface Plasmon Resonance; HNRNP; transcriptional regulator

INTRODUCTION

Interleukin-6 (IL-6) is a key cytokine in both innate and adaptive immune responses. Dysregulation of IL-6 signalling is implicated in many disease processes characterised by chronic inflammation and autoimmunity.[1] It is a pleiotropic cytokine produced in numerous cell types, but the primary sources are cells of the myeloid lineage (such as monocytes, macrophages, B cells), epithelial, endothelial, and muscle cells. Its function includes promotion of inflammation by induction of chemokines and adhesion molecules, but it also produces the interleukin 1 receptor antagonist (IL-1ra) and inhibitor of metalloproteinases. It is in addition a growth factor for a diverse population of cells and tissues including B-cells, T cells, endothelial cells, cardiac and skeletal muscle cells. It is also referred to as a myokine in the literature on muscle function and exercise.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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IL-6 gene transcription can be induced by other pro-inflammatory cytokines such as IL-1 and tumour necrosis factor alpha (TNFα) in addition to other stimuli such as bacterial lipopolysaccharide (LPS). Functional cis-regulatory elements described to date are transcription factor binding sites for NFκB (Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 1),[2] IRF-1 (interferon regulatory factor 1),[3] AP-1 (jun proto-oncogene),[4] C/EBP (CCAAT/enhancer-binding protein)[5] and SP1 (Sp1 transcription factor).[6] These cis-acting elements are all located within 1.2 kb upstream of the transcription start site (TSS) of the IL-6 gene. A functional SNP at −174 bp upstream of the IL-6 TSS (rs1800795) is the most extensively studied SNP and showed association with diseases including systemic onset juvenile arthritis (sJIA),[7, 8] Systemic Lupus Erythematosus (SLE),[9] and cardiovascular disease.[10, 11]

Previously we have reported that IL-6 gene transcription could be regulated beyond the 1 kb 5′ flanking region and identified cis-acting sequences as far as −5 kb upstream of the IL-6 TSS to be important for basal IL-6 gene transcription.[12] A specific region from −5307 bp to −5152 bp was found to bind nuclear proteins and reporter assays in HeLa cells showed higher IL-6 basal transcription activity. In this report, we have applied surface plasmon resonance (SPR) technology and mass spectrometry analysis to identify the nuclear proteins that bind to this region. Further experiments confirmed the presence of HNRNPA1 which was found to have a cis-regulatory role in IL-6 transcription in cultured cells.

RESULTS

Identification of proteins bound to the region −5307 bp to −5152 bp of the 5′flanking region of IL-6

To identify proteins bound to the IL-6 5′-flanking region at −5307 bp to −5152 bp (referred to as IL6-155 in the following text), we utilised SPR technology and mass spectrometry. The SPR approach consists of immobilising ligands to a surface and then observing changes in the refractive index at the surface in real-time as molecules bind, and has long been used to study protein-DNA interactions.[13] Biotinylated probes of IL6-155 were immobilised onto a Streptavidin-coated sensorchip. Approximately 1,500 RU was achieved per flow cell, equivalent to 1.14 ng or 11.4 fmol of DNA.

Nuclear proteins extracted from HeLa cells were passed across this surface and protein-DNA interaction was recorded in real time as a sensorgram shown in Fig. 1A. On the DNA-immobilised surface, RU increased with time during injection, while no change was observed on the DNA-free surface, indicating that there were proteins specifically binding to the DNA sequence. Further, the nuclear proteins bound to the DNA surface in a concentration-dependent manner (Fig. 1B). Since concentrations higher than 100 ng/μL did not lead to an appreciably higher signal, this concentration of nuclear extract was chosen for protein recovery experiments. Regeneration of the DNA surface enabled multiple capture/recovery cycles to increase the overall yield for mass spectrometric analysis.

Analysis of eluted material by SDS-PAGE and silver staining showed several protein bands between 35 and 45 kDa that were not seen in BSA buffer alone (Fig. 1C). Sufficient material was collected from repeat injections to reveal visible bands by colloidal Coomassie Blue staining. These were excised, the protein digested with trypsin and extracted peptides analysed by LC-MS/MS. The majority of identified proteins belonged to the family of heterogeneous nuclear ribonucleoproteins (HNRNPs) and included isoforms of HNRNPA0, A1, A/B, A2/B1, C1/C2 and D0 (see Supplemental Table 1). These could all be unambiguously identified by the presence of isoform-specific peptide sequences. The highest scoring proteins in the four most intense bands were isoform A1-B of HNRNPA1 and isoform B1 of HNRNPA2B1. In a repeat experiment, both of these proteins were again

identified. The presence of these bound proteins is notable in that they are generally considered as RNA binding proteins involved in pre-mRNA splicing. However, previous reports have hinted at their DNA binding capacity in promoter regions of the KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog), APOE (apolipoprotein E) and IL-10 genes.[14-16]

Interaction of HNRNPA1 and A2/B1 with IL6-155 region

To confirm the presence of the two HNRNP molecules in the complex with IL6-155 *in vitro*, EMSA supershift assays were performed using specific antibodies to the two HNRNPs. The nuclear protein-DNA complex was further 'shifted' by the addition of antibody against HNRNPA1, but not by non-specific mouse IgG or antibody against HNRNPA2B1 (Fig. 2A). These results confirmed the involvement of HNRNPA1 in complex with the IL6-155 sequence *in vitro*. The binding of HNRNPA2B1 to the DNA sequence was not confirmed by this method.

To test if HNRNPA1 interacts with this region *in vivo*, we performed chromatin immunoprecipitation (ChIP) assays on paraformaldehyde cross-linked HeLa cells. The result showed binding of HNRNPA1 to the region between −5368 and −5152 bp, which contains the IL6-155 sequence, but not to a downstream region between +691 and +896 bp (Fig. 2B). On the other hand, when immunoprecipitated with anti-HNRNPA2B1, a faint band was observed for the region −5368 to −5152 but not confirmed by qPCR.

Regulation of IL-6 gene expression by HNRNPA1

To investigate the influence of HNRNPA1 on IL-6 transcription, we measured luciferase activities of IL-6 promoter constructs in the presence of siRNA to HNRNPA1. Specific siRNAs (siHNRNPA1 or siHNRNPA2B1) or non-specific control siRNA (siNeg#2) were co-transfected into HeLa cells with IL-6 promoter-luciferase constructs, containing promoter sequence up to −5202 or to −5307 (namely pGL3-IL6-5202 or pGL3-IL6-5307). An average of 46% reduction in HNRNPA1 protein level and 88% in HNRNPA2B1 protein expression were achieved by their specific siRNAs after 48 hours of transfection, but both their mRNA levels decreased by an average of 90% (Fig. 3A&B). Despite the presence of a level of ~50% HNRNPA1 protein, significant inhibition of luciferase activity from pGL3-IL6-5307 was found. The presence of siRNA to HNRNPA1 had the opposite effect on pGL3-IL6-5202 where the cis-acting sequence was absent although all reporter activities were much less than that seen in experiments with the complete construct (pGL3-IL6-5307), suggesting these observations with the pGL3-IL6-5202 could be background variations (Fig. 3C). Overall transcriptional activity of the pGL3-IL6-5202 construct was very much diminished without the 155 bp region, as previously published. The suppression of IL-6 reporter activity by siHNRNPA1 suggests that HNRNPA1 positively regulates IL-6 transcription and this function is dependent on the IL6-155 region. In contrast, knockdown of HNRNPA2B1 by specific siRNA increased activities of both reporter constructs. Knockdown of either protein had little effect on the activity of the pGL3-control vector.

The physiological function of HNRNPA1 and HNRNPA2B1 on IL-6 gene expression was further examined by measuring IL-6 mRNA levels in HeLa cells transfected with the specific siRNAs. In comparison with siNeg#2-transfected control cells, siHNRNPA1-transfected cells had significantly lower IL-6 mRNA expression, further supporting the positive regulation of basal IL-6 transcription by HNRNPA1 (Fig. 3D). Knockdown of HNRNPA2B1 had no effect on IL-6 mRNA expression. The positive regulation of IL-6 expression by HNRNPA1 was also demonstrated in HeLa cells overexpressing HNRNPA1. The expression of IL-6 mRNA was increased by nearly 2-fold in cells transfected with

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expression vector pCMV6-HNRNPA1 compared with the control empty vector pCMV6 (Fig. 3E).

The 5′ end of the IL6-155 sequence is crucial for protein binding

In our previous publication, we designed five short DNA probes spanning the IL6-155 sequence in an attempt to identify sequences necessary for protein binding. Four out of five probes (except probe −5249 to −5210) showed protein binding with probe −5307 to −5268 giving the strongest signal.[12] Here we challenged the protein binding of the IL6-155 probe with a 100-fold molar excess of unlabelled short probes in EMSA assays. The results showed that only probe −5307 to −5268 completely abolished protein binding to IL6-155, with two probes −5220 to −5181 and −5191 to −5152 exhibiting partial competition (Fig. 4A). This indicates that region −5307 to −5268 is essential for protein binding. In addition, the addition of HNRNPA1 antibody was able to partially retard the protein-DNA band of probe −5307 to −5268 (Fig. 4B). Interestingly, a motif 5′-TACAGA-3′, located in the middle of the region (−5285 to −5280 bp), is similar to the sequence found to be responsible for HNRNPA1 binding to the IL10 promoter (5′-TACACA-3′; Fig. 4C).[16] To investigate whether this motif also contributes to binding with HNRNPA1 in IL6-155, we designed mutant probes bearing mutations at the 5′-TACAGA-3′ motif (mut1) or a sequence close by (−5302 to −5297 bp, mut2) and tested their ability to bind nuclear proteins using EMSA. Fig. 4D shows that mut1 had little protein-DNA binding, while mut2 could still form a protein-DNA complex like the wild-type probe. Moreover, protein binding to the wild-type probe was competed by unlabelled mut2 and wild-type probe, but not by the mut1 probe.

DISCUSSION

In this study, using a combination of SPR technology and mass spectrometry we have identified some of the nuclear proteins bound to a distal promoter sequence of the IL-6 gene that we had previously defined to be important for basal IL-6 transcription. SPR enables real-time monitoring interactions occurring on the sensor surface and provides a sensitive and specific approach to detect subtle differences in binding kinetics and binding affinity between target molecules and their ligands. Recovery of material from the surface and identification by mass spectrometry provides a powerful means to screen for unknown partners to a protein or DNA immobilised on the sensor surface. Its use has been well demonstrated.[17-19]

The limitation of the approach is that the amount of DNA that can be immobilised onto the surface is limited (normally fmol), and so this limits the amount of material recovered. Regeneration of the chip surface however, allows multiple runs to be performed and accumulation of sufficient material for MS analysis, as demonstrated here. Careful consideration was given to the use of a biologically relevant cell type. Primary cultures usually do not last in culture long enough for all the different experiments to be done on the same cells, and are often activated by isolation and culture methods. Moreover the number of cells needed for experiments such as those requiring nuclear extracts would be extremely challenging. Elutriated monocytes from whole blood from a blood bank may provide sufficient numbers but repeat experiments can be difficult to control for. Commercial supplies of cryopreserved human monocytes are possible sources but in addition to the above drawbacks, freeze and thaw will alter their activation status, and so not likely to reflect better the true *in vivo* state. Therefore transformed cell lines were considered to be a practical option for this particular type of investigation. Epithelial cells have been extensively described to secrete IL-6 in response to an inflammatory stimulus, and so is biologically relevant. Since HeLa cell is a papilloma transformed epithelial cell line *in vivo*, and is easy to transfect in tissue culture, we have continued to use this cell line

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experimentally. More importantly, HeLa cells have always been shown to produce IL-6 in response to biologic stimuli that are similarly effective in PBMC cultures, and like healthy PBMCs, do not have high levels of constitutive expression.

Our results have established that HNRNPA1 is at least one of the regulators of IL-6 basal transcription by interacting with sequence between –5307 bp to –5152 bp of the IL-6 promoter. First, the interaction was confirmed by EMSA supershift. Second, ChIP assays showed that HNRNPA1 interacted with the region of genomic DNA in cell culture. Third, reduction of HNRNPA1 protein levels by 50% in cells by siRNA significantly inhibited IL-6 promoter activity and mRNA expression. The relatively small effect size of transcriptional reduction of reporter constructs (~20%) and IL-6 mRNA is likely due to the presence of about 50% of the HNRNPA1 protein. Finally supporting evidence is provided by the fact that overexpression of HNRNPA1 increased IL-6 expression up to two-fold.

The HNRNPs are among the most abundant proteins in the eukaryotic cell nucleus and are involved in many aspects of mRNA processing. HNRNPA1 is the most studied and has primary roles in packaging of pre-mRNA into HNRNP particles, nuclear export of mature mRNA and selection of splice sites.[20-24] The observed regulation of IL-6 mRNA by HNRNPA1 may be due to its action on pre-mRNA. However, our DNA binding and reporter assay data would argue against this. Moreover, HNRNPA1 was reported to bind single-stranded telomeric DNA through its RNA recognition motifs (RRMs).[25, 26] The fact that only one of two RRMs is sufficient to bind telomeric DNA raises the potential for HNRNPA1 to interact with double-stranded DNA (dsDNA).

Transcriptional regulatory roles of HNRNPs (including HNRNPA1) via binding to dsDNA were reported more than a decade ago.[27-29] HNRNPA1 has since been shown to interact with promoter regions of the thymidine kinase gene,[29] APOE,[15] KRAS[14] and IL-10.[16] How HNRNPA1 binds to dsDNA to modulate gene expression is still unclear. The binding motifs identified from different promoters are not conserved. HNRNPA1 has been shown to bind an ‘ATTT’ sequence with high affinity in the thymidine kinase gene. An ‘AGGGT’ sequence was found to be important for HNRNPA1 binding to the APOE promoter. A parallel G-quaduplex DNA structure, similar to telomeric repeats, was shown to be responsible for binding in the KRAS promoter. The study on the IL-10 promoter and our own study suggest that the sequence ‘TACACA’ is critical for binding. Donev *et al*. showed that HNRNPA1 bound to DNA within a 36 bp sequence and this sequence was found widely dispersed throughout the genome.[30] These observations imply that HNRNPA1 may play a structural role and/or behave as a co-activator.

HNRNPA2B1 was also isolated as an IL6-155 binding protein, but we were unable to confirm any functional role this may have in regulating IL6 transcription; the ChIP assay suggested a weak, but discernible interaction, knockdown of HNRNPA2B1 led to increased reporter activity, whilst the EMSA was negative, and knockdown of HNRNPA2B1 in HeLa cells had no effect on IL-6 mRNA expression. It is possible that the antibodies used were not appropriate or that there are cell specific effects. Despite this, recent work by Guha *et al*. has implied that HNRNPA2B1 is a common transcriptional co-activator, functioning in response to mitochondrial respiratory stress,[31, 32] and may yet have a role in the protein complex binding to this region of the IL-6 gene.

The importance of HNRNPA1 in IL-6 transcription may have clinical implications since autoantibodies to the HNRNP family have been detected in systemic rheumatic diseases, such as SLE,[33, 34] in which IL-6 levels are raised.[35] Moreover, the mRNA expression of HNRNPA1, E1 and K were shown to be significantly increased in myocardial samples from patients with aortic stenosis or ischemic cardiomyopathy,[36] which is also characterised by

elevated IL-6 levels. Our data demonstrate that HNRNPA1 can upregulate IL-6 expression through interacting with its DNA. We propose that HNRNPA1 acts as part of a complex that alters chromatin structure to enhance basal expression. To fully investigate this possibility, we will need to use large scale preparative methods to isolate the co-factors and then rebuild the transcriptional apparatus, and this is currently beyond the scope of this report.

MATERIALS AND METHODS

Cell culture and nuclear extraction

HeLa cells were maintained in Dulbeco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37°C. Nuclear extracts were prepared from exponentially growing HeLa cells following the method described previously.[12] The resuspension buffer contained 10 mM HEPES-KOH pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 0.1 mM EGTA, 25% v/v glycerol, 1 mM DTT and protease and phosphatase inhibitors. Protein concentration was quantified using Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Surface Plasmon Resonance (SPR)

The SPR experiment was carried out using a BIAcore T100 with Sensor chip SA (GE Healthcare, Little Chalfont, UK). The DNA fragment containing sequence from −5307 to −5152 bp upstream of IL-6 TSS (IL6-155) was amplified using a forward primer 5′-biotin-TGGCTCAGACATAGACCACTG-3′ and a reverse primer 5′-TATTGTTCCAAGGGGTGCTG-3′, and purified on Qiagen PCR columns (Qiagen, Hilden, Germany). The SA chip surface was activated by injection of 1 M NaCl in 50 mM NaOH for 1 min three times. Biotin-labelled PCR product at 2 ng/mL in 0.5 M NaCl was injected onto the chip surface at a flow rate of 5 μL/min for 30 min. Approximately 1,500 RU (arbitrary resonance units) was achieved per flow cell. Empirically in the BIAcore technology, 1 ng of a globular protein or 0.78 ng of a DNA molecule bound at the surface gives a response of 1000 RU.[37]

For protein-DNA interaction analyses, 20 to 200 ng/μL nuclear proteins were first incubated with 10 ng/μL of poly [dI-dC], as a competitor for non-specific protein binding to the DNA chip, in binding buffer containing 20 mM HEPES pH 7.6, 50 mM KCl, 5mM $MgCl_2$, 1 mM EDTA, 40 ng/μL BSA and 0.05% P20 surfactant and then applied to the DNA-immobilised chip at a rate of 1 μL/min. The sensorgrams were recorded automatically and adjusted by subtracting the baseline response recorded immediately before the injection of each sample when only buffer plus BSA was applied. The usage of BSA was to block non-specific binding sites on the DNA surface. A flow cell without immobilised DNA served as a non-specific binding control. At the end of each cycle, bound proteins were eluted by two-pulse injections of 0.05% SDS to regenerate the chip. For protein recovery, all four flow cells on the chip were immobilised with IL6-155 DNA and the bound proteins recovered to collection tubes by using the 'Injection and Recovery' function of the BIAcore T100. Multiple cycles and repeats were applied to obtain sufficient protein for mass spectrometry analysis. The recovered samples were concentrated using a vacuum dryer and then resolved on a 10% Bis-Tris NuPAGE gel (Life Technologies, Carlsbad, CA, USA) followed by silver staining using SilverQuest Silver Staining Kit or colloidal Coomassie Blue (Life Technologies).

Mass Spectrometry analysis

Gel bands (stained with colloidal Coomassie Blue) were excised and washed three times in 50% (v/v) acetonitrile, dried in a vacuum centrifuge, reduced in 10 mM DTT in 5 mM ammonium bicarbonate pH 8.0 for 45 min at 50°C and alkylated with 50 mM iodoacetamide

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in ammonium bicarbonate for 1 hr at room temperature in the dark. Gel pieces were washed twice in 50% acetonitrile, vacuum dried, and then 50 ng sequence grade modified trypsin (Promega, Southampton, UK) in 5 mM ammonium bicarbonate was added to each dried gel piece. After allowing gel pieces to re-swell for 5 min, 5 μL of 5 mM ammonium bicarbonate was added and gel pieces were incubated at 37°C for 16 hr. Tryptic peptides were extracted three times with 50% (v/v) acetonitrile containing 5% (v/v) trifluoroactetic acid. Extracts from each gel piece were pooled and vacuum centrifuged to dryness. Peptides were finally resuspended in 5 μL of 0.1% (v/v) formic acid and stored at −20°C prior to mass spectrometric analysis.

Analysis of tryptic peptides from digested bands was performed by nanoflow reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA). Sample (5 μL) was injected onto a 300 μm i.d. × 5 mm C18 PepMap guard column (5 μm bead size, 100 Å pore size, LC Packings, Amsterdan, Netherlands) and washed for 3 min with 95% solvent A (water + 0.1% FA) at a flow rate of 25 mL/min using an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA). Reversed-phase chromatographic separation was then carried out on a 75 mm i.d. × 250 mm C18 PepMap nano LC column (3 μm bead size, 100 Å pore size; LC Packings) with a linear gradient of 5-50% solvent B (water/ACN 20%:80% v/v + 0.1% FA). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (*m/z* 400 to 2000) were acquired in the Orbitrap with a resolution of 60,000 at *m/z* 400. The top 6 most intense ions were selected for collision induced dissociation. Target ions that had been selected for MS/MS were dynamically excluded for 60 sec. For accurate mass measurement, the lock mass option was enabled using the polydimethylcyclosiloxane ion (*m/z* 455.12003) as an internal calibrant. For peptide identification, raw data files produced in Xcalibur software (Thermo Scientific) were processed in Mascot Distiller (V2.2) and searched against the IPI human database (version 20100213; 87,130 sequences). For searching, the MS tolerance was set to ±10 ppm and the MS/MS tolerance to 0.8 Da. One missed cleavage was allowed and carbamidomethylation (C) was set as a fixed modification. Methionine oxidation, acetylation (protein N-terminal), Glu->pyro-Glu (N-term Q) and deamidation (NQ) were set as variable modifications. Only peptides with ion scores >30 were accepted using a significance threshold of 0.05 and protein identifications had to have at least 2 unique peptides matched per protein.

Electrophoretic mobility shift assay (EMSA) and supershift

EMSA was performed using the non-radioactive LightShift Chemiluminescent EMSA kit (Pierce, Thermo Scientific). The biotinylated IL6-155 probe was the same as that used for the SPR experiments. Short 39 bp probes, used previously[12] or with mutations, were made by annealing primer pairs and labelled at the 3′ end using terminal deoxynucleotidyl transferase and biotin-11-dUTP (Fermentas, Thermo Scientific). EMSAs were performed using 10 nM biotinylated probes incubated with 2 μg of nuclear proteins in 1× binding buffer (8% Ficoll, 20 mM HEPES, 50 mM KCl, 1mM EDTA, 0.5 mM DTT, 40 ng/μL of poly [dI-dC] and 40 ng/μL BSA) for 30 min at 25°C. In experiments where competitor unlabelled probes were added, reactions were pre-incubated with unlabelled probes in 100-fold molar excess of the labelled probe at 25°C for 15 min prior to the addition of the labelled probe. For supershift, nuclear proteins were pre-incubated with 2 μg of antibody for 30 min at 25°C. The reaction mixture was loaded and run on a 5% native polyacrylamide gel. Gels were transferred to Hybond-N^+ nylon membrane (GE Healthcare) and immediately UV cross-linked. Streptavidin-horseradish peroxidase conjugate and the LightShift chemiluminescent substrate were used to detect biotin-labelled DNA. The nylon membranes were then visualised by exposing to X-ray film. Antibodies to HNRNPA1 (4B10),

HNRNPA2B1 (DP3B3) and normal mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Pure HNRNPA1 protein was obtained from Origene Technologies and 200 ng used in EMSA reactions as a positive control.

Chromatin Immunoprecipitation Assay (ChIP)

The ChIP procedure was performed using MAGnify ChIP kit (Life Technologies) following the manufacturer's instructions. Briefly, HeLa cells grown to 90-100% confluence were cross-linked by treatment with 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 125 mM for 5 min to quench the cross-linking reaction. Cells were rinsed 3 times with cold PBS, collected and resuspended in kit Lysis buffer supplemented with protease inhibitors. Chromatin was sheared by sonication using a Bioruptor sonicator (Wolflabs, York, UK) and diluted in the kit Dilution buffer. Antibodies against HNRNPA1, HNRNPA2B1 or normal mouse IgG (negative control) was coupled to protein A/G Dynabeads and then incubated with diluted chromatin. Chromatin from ~2×10^5 cells was used in each reaction. The beads were washed several times with kit IP buffers 1 and 2 and then protein-DNA crosslinks were reversed in the appropriate kit buffer at 55°C for 15 min, followed by 65°C for 15 min. The DNA was purified using DNA Purification Magnetic Beads. One tenth of input chromatin was also treated in the same way and purified. DNA aliquots were analysed by PCR with a primer pair, 5′-TATGGCTTCCAGGGTGAGA G-3′ and 5′-TATTGTTCCAAGGGGTGCTG-3′, encompassing −5368 to −5152 bp. The PCR conditions were as follows: 95°C for 5 min, 30 cycles of 95°C 30 s, 57°C for 30 s, and 72°C for 30 s, followed by 72°C for 7 min extension step. The amplified DNA was electrophoresed on a 2% agarose gel and visualised by staining with ethidium bromide. As a negative control, a separate region of the IL-6 gene located between +641 and +896 bp relative to TSS was amplified using the primers: 5′-AATGGGTCTGAAATCC ATGC-3′, and 5′-GGTGGGCTCTGAGGTATG AA-3′. The level of enrichment of the target DNA sequence was determined by real-time PCR using above primers. The enriched DNA fragments were presented as percentage of input chromatin.

Transfection and luciferase reporter assay

HeLa cells were seeded into 24-well plates and were transiently transfected with 10 nM HNRNPA1 or HNRNPA2B1 siRNA duplex (Life Technologies *Silenser*® Select siRNA) complexed with Lipofectamine RNAiMAX (Life Technologies) in serum-free medium, according to the manufacturer's instructions. For control siRNA, *Silencer*® Select Negative Control #2 was used. The cells were collected at 48 h after transfection for RNA and protein analysis. The sense and antisense sequences of HNRNPA1 siRNA were 5′-GAAUGGUUAUAAAGUGAUtt-3′ and 5′-AUCACUUUUAUAACCAUUCca-3′, and those of HNRNPA2B1 siRNA were 5′-GCAACCUUCUAACUACGGUtt-3′ and 5′-ACCGUAGUUAGAAGGUUGCtg-3′.

For co-transfection experiments, 100 ng of IL-6 promoter-luciferase constructs, bearing IL-6 promoter sequence up to −5307 bp or −5202 bp upstream of a luciferase gene in pGL3-basic vector,[12] were transfected into HeLa cells in a 96-well plate using Lipofectamine LTX (Life Technologies). Six hours later, the media was replaced and cells subjected to siRNA transfection as described above. After a further 48 h, cell extracts were prepared and the luciferase activity was measured with the Luciferase Assay System (Promega) on a TR71 Microplate Luminometer (Life Technologies). Total protein was measured with Bradford assay to correct for differences in cell number. The luciferase activity assays were measured in triplicate in each experiment and shown as fold-change relative to pGL3-control vector. To overexpress the HNRNPA1 protein, HeLa cells were transfected with 100 ng or 200 ng of vector pCMV6-XL5 harbouring human HNRNPA1 cDNA or the empty vector (OriGene,

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Rockville, MD, USA) using Lipofectamine LTX. Cells were harvested 48 hrs post-transfection.

Western blotting

Protein from whole cell lysates was resolved on 10% NuPAGE Bis-Tris gels (Life Technologies) and transferred to PVDF membrane (GE Healthcare). The membrane was blocked with 5% bovine serum albumin (Sigma, St Louis, MO, USA) in tris-buffered saline and 0.1% Tween 20 and then incubated with primary antibodies overnight at 4°C. The membranes were then washed and incubated with secondary HRP-conjugated antibody at room temperature for 1 hr, washed again and proteins of interest visualised using an enhanced chemiluminescence detection system (Thermo Scientific). Membranes were reprobed with antibody against TBP (TATA box binding protein) to control for loading. The corresponding bands were quantified by image processing software, Image J (NIH, http://rsb.info.nih.gov/ij/), on underexposed images and normalised to those of TBP bands before compared to those measured in siNeg#2-transfected cells. Antibodies against HNRNPA1, HNRNPA2B1 and TBP were purchased from Santa Cruz Biotechnology.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Life Technologies) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed to cDNA using Qantitect reverse transcription kit (Qiagen) and qPCR was performed using Taqman gene expression assay (Life Technologies) and Solaris qPCR Gene Expression Master Mix (Thermal Scientific) for IL6, HNRNPA2B1 and RPLP0. The HNRNPA1 transcripts were detected using Quantitect SYBR green PCR kit with a pair of primer, 5′-GCTTGGGTGGAGAAGCCAT-3′ and 5′-GAACTTCAGGTCACAACAGTAAC-3′. All qPCR reactions were performed on a Mastercycler ep realplex PCR system (Eppendorf, Hamberg, Germany) with cycling conditions as follows: 15 minutes of denaturation at 95°C and then 40 cycles of 95°C for 15s, 60°C for 1 minute. Relative levels of mRNA expression were calculated according to the ΔΔCT method[38] and normalised by comparison to RPLP0 mRNA expression.

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean ± SD. The significance of differences between experimental groups was determined with a two-tailed unpaired Student's *t*-test with $p<0.05$ considered as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by Arthritis Research UK (no. 17287). The work was undertaken at UCLH/UCL who received a proportion of funding from the Department of Health's NIHR Biomedical Research Centres funding scheme. We thank Dr. John Sinclair from Dr. Timms' group for processing preliminary samples, and Professor Steve Humphries for discussion of results.

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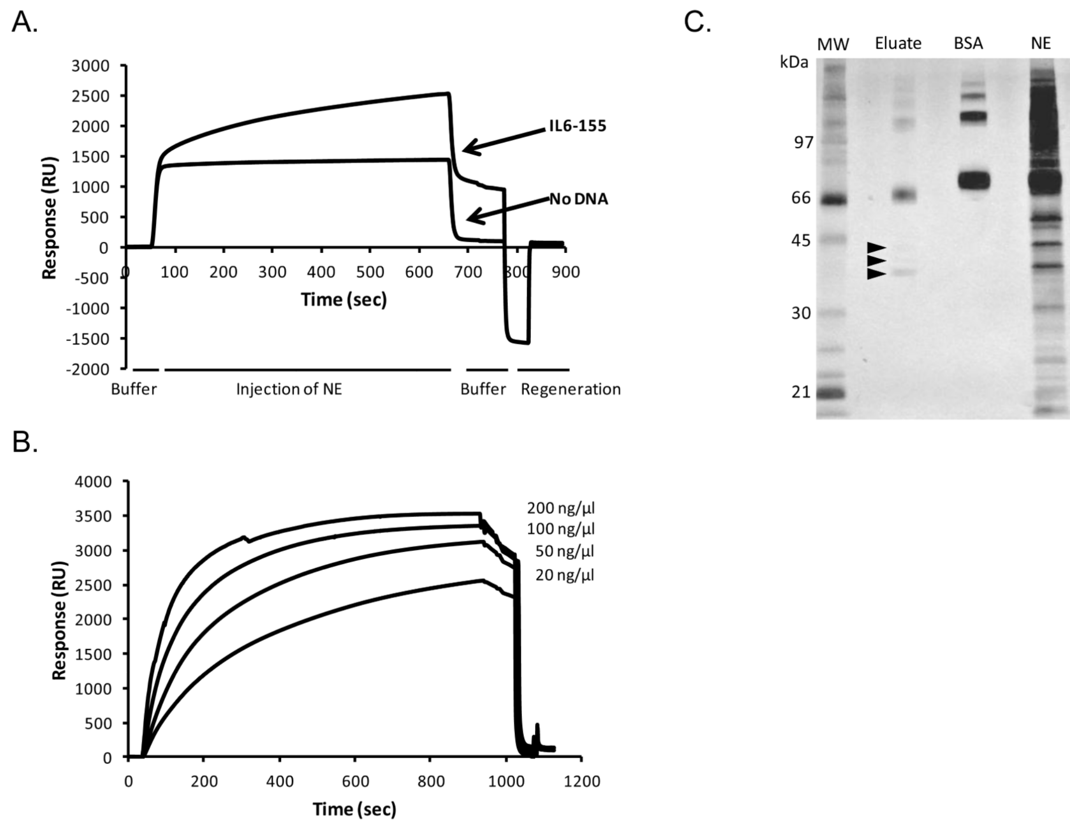

Figure 1.

Isolation of nuclear proteins binding to IL-6-155. A) Sensorgrams of SPR depicting binding of nuclear proteins to the immobilised IL6-155 DNA fragment. Nuclear proteins were injected at 20 ng/μL in binding buffer onto the chip surface with no DNA or with immobilised DNA at a rate of 1 μL/min for 10 min. B) Sensorgrams of SPR depicting binding of nuclear proteins to the DNA surface at various concentrations. To regenerate the chip between each injection, 0.05% SDS was injected at a rate of 5 μL/min for 30 sec. Sensorgrams were adjusted to zero baseline level before injections. Nuclear proteins were injected at 20, 50, 100 and 200 ng/μL in binding buffer and sensorgrams were adjusted by subtracting the response recorded on the surface without DNA. C) Separation of recovered samples by SDS-PAGE. Recovered protein from multiple SPR injections was loaded onto a 10% NuPAGE Bis-Tris gel along with protein molecular weight marker and buffer control and the gel was silver-stained. Lanes: MW, molecular weight markers; Eluate, sample recovered from BIAcore (contains BSA from binding/elution buffer); BSA, running buffer with BSA; NE: nuclear extract (NE) in binding buffer with BSA. The bands marked by black arrows were clearly not present in the lane containing BSA alone, and were therefore excised for LC-MS/MS analysis.

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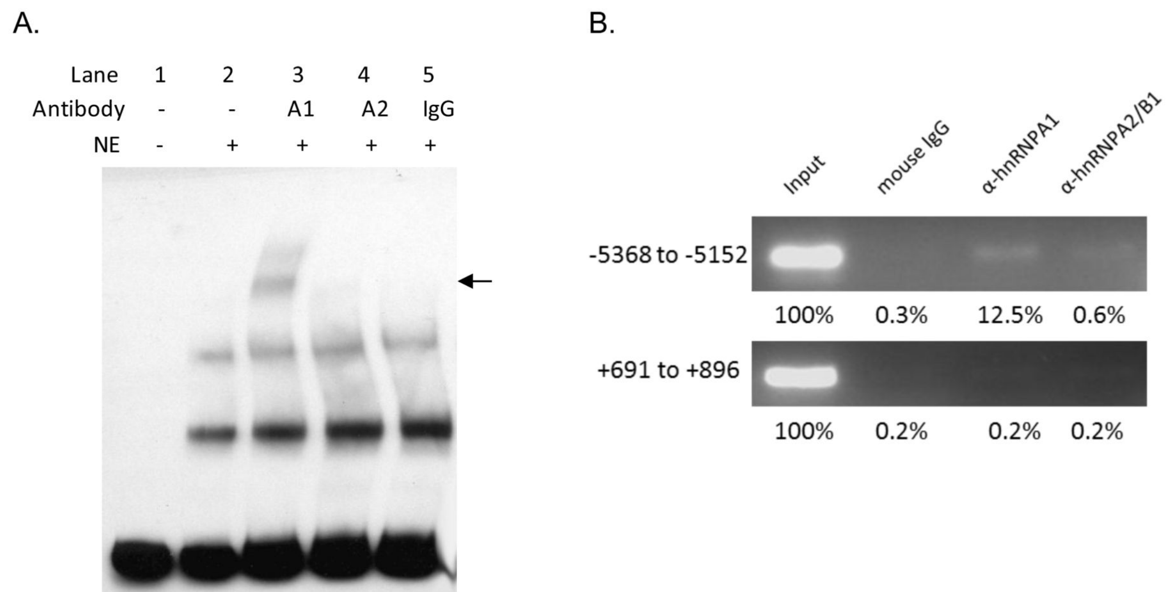

Figure 2.

EMSA supershift assay and chromatin immunoprecipitation. A) EMSA supershift with antibodies against HNRNPA1 and HNRNPA2B1. Nuclear extracts were pre-incubated with 2 μg of antibody for 30 min at 25°C before adding to biotin-labelled IL6-155 probe. The position of the supershifted band is indicated by a black arrow. A1, anti-HNRNPA1; A2, anti-HNRNPA2B1; IgG, mouse IgG. B) ChIP of HeLa cells with anti-HNRNPA1, anti-HNRNPA2B1 or control mouse IgG, followed by PCR amplification of IL-6 promoter regions between positions −5368 and −5152 bp or positions +691 and +896 bp (negative control region), in input DNA or immunoprecipitated DNA samples. The amplification from input DNA reflects the amount of starting DNA and was used to determine the level of enrichment of the target DNA sequence. The gel image is representative of three separate experiments. The enrichment of the two regions co-immunoprecipitated with various antibodies was quantified by real-time PCR, and the result is shown as percentage of input DNA under the corresponding bands.

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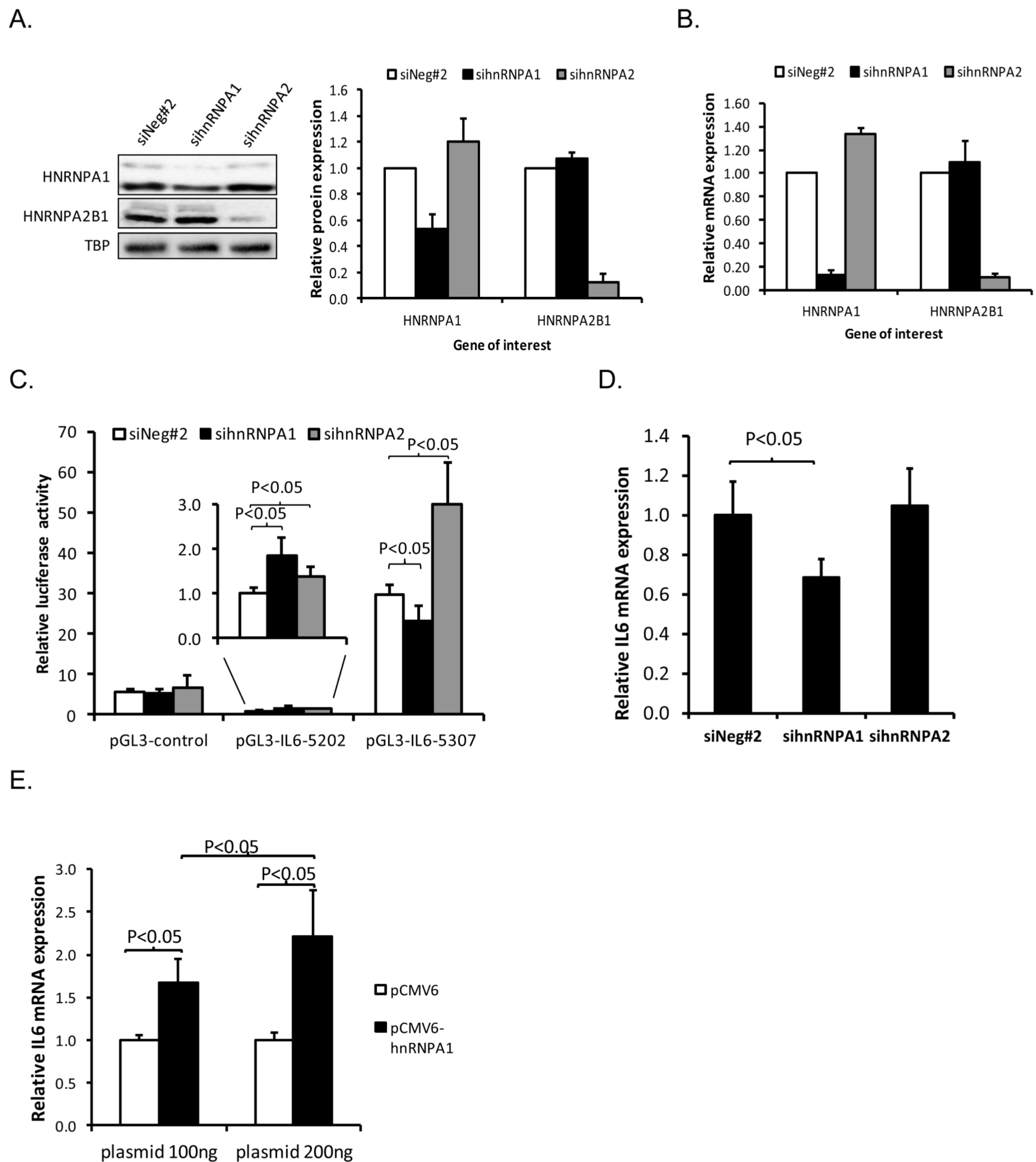

Figure 3.

Effect of HNRNPA1 siRNA knockdown and overexpression on IL-6 promoter reporter activity and mRNA expression. A) The effect of specific siRNAs on protein expression of HNRNPA1 and HNRNPA2B1 by western blot. Both the specific and negative control siRNAs (10 nM) were transfected into HeLa cells using Lipofectamine RNAiMAX for 48 hrs before cells were collected for western blotting analysis. The image shown is representative of five independent experiments. The corresponding bands were quantified by Image J on underexposed images from 5 experiments and normalised to those of TBP bands before compared to those measured in siNeg#2-transfected cells (=1.0). The mean and SD from five experiments are shown in the bar chart. B) Relative mRNA expression of

HNRNPA1 and HNRNPA2B1 in siRNA-transfected HeLa cells. C) Effect of siRNAs for HNRNPA1 or HNRNPA2B1 on luciferase activities of IL-6 promoter reporter constructs. One hundred nanogram of IL-6 promoter luciferase constructs, bearing IL-6 5′ flanking sequence up to −5307 bp or −5202 bp (pGL3-IL6-5307 or pGL3-IL6-5202), were transfected into HeLa cells using Lipofectamine LTX. Six hours later, cells were subject to siRNA transfection as described. Cell extracts were prepared 48 hrs later and the luciferase activities were measured and shown as fold-change relative to pGL3-control vector. D) IL-6 mRNA expression in HeLa cells transfected with siRNAs. The data shown are relative to cells transfected with siNeg#2 control. E) IL-6 mRNA expression in HeLa cells transfected with vector encoding HNRNPA1 (pCMV6-HNRNPA1) or empty vector (pCMV6). The data shown are relative to cells transfected with empty vector. All experiments were performed at least three times. The error bars represent the standard deviation (SD) of the replicate experiments and $p<0.05$ was regarded as significant.

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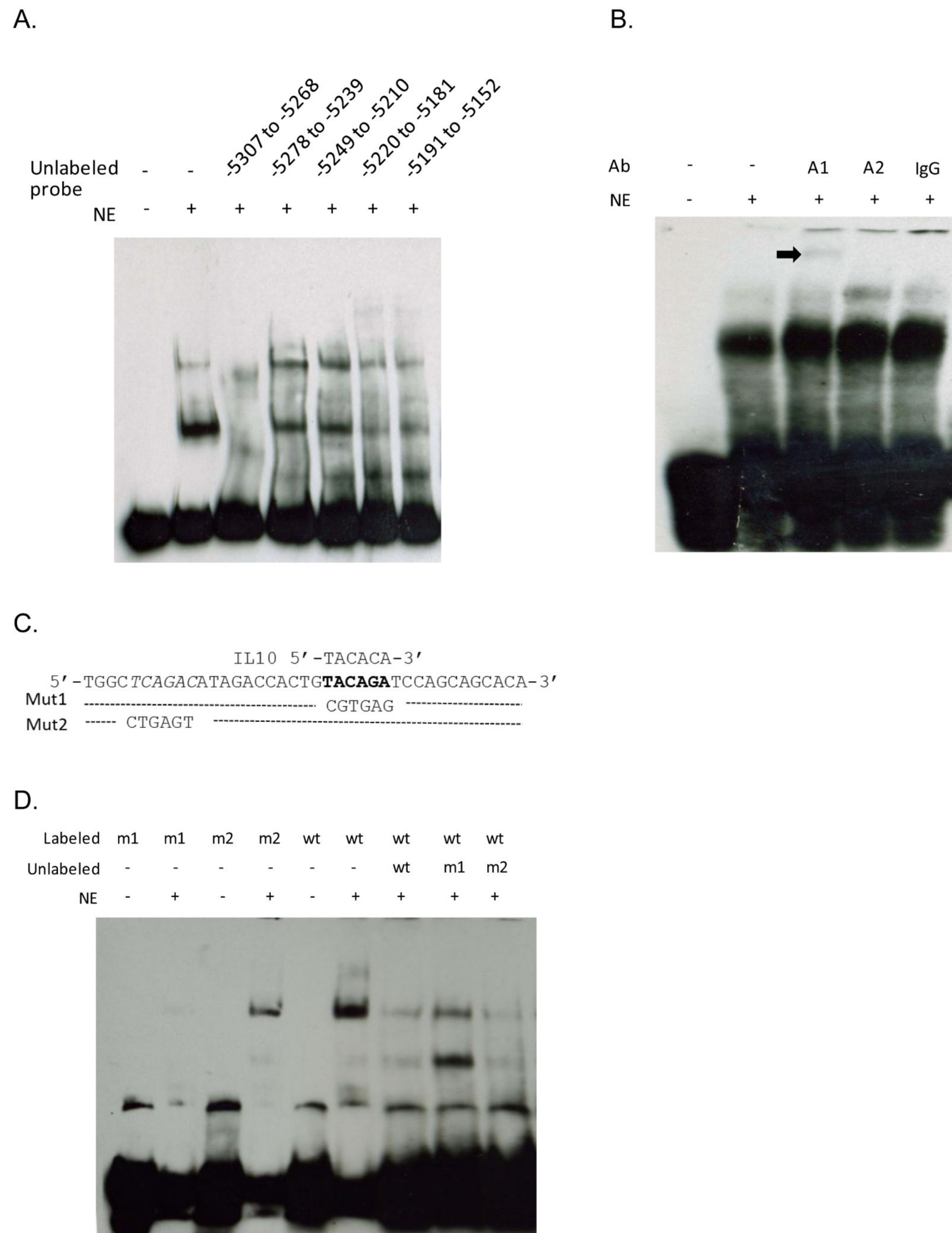

Figure 4.

Identification of HNRNPA1 binding sites in the IL-6 promoter. A) Nuclear proteins binding to biotin-labelled IL6-155 probe in EMSA. Competitive EMSA was performed using 100-fold molar excess of unlabelled short probes spanning the region. Unlabelled probes were: –5307 to –5268; –5278 to –5239; –5249 to –5210; –5220 to –5181 and –5191 to –5152. B) EMSA supershift assays of biotin-labelled probe –5307 to –5268. The super-shifted band due to anti-HNRNPA1 is indicated by a black arrow. Ab, antibody; A1, anti-HNRNPA1; A2, anti-HNRNPA2B1; IgG, mouse IgG. C) DNA sequence of the probe –5307 to –5268 and its derivatives, mut1 and mut2. The predicted binding site was highlighted in bold and the neighbouring site as italic letters. D) Nuclear proteins binding to labelled probe –5307 to

−5268 and mutants. Competitive EMSAs were performed using 100-fold molar excess of unlabeled probes: wt, wild-type probe; m1, mut1 probe; m2, mut2 probe.

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