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

D Zheng 1, J Worthington 2, JF Timms 2 and P Woo 1

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 electrophoretic mobility shift assay (EMSA) and transient expression experiments. Here we have combined surface plasmon resonance technology with mass spectrometry analysis and have 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 of HNRNPA2B1. Knocking down the HNRNPA1 expression by small interfering RNA 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 and B cells) and the epithelial, endothelial and muscle cells. Its function includes promotion of inflammation by induction of chemokines and adhesion molecules, but it also produces the IL-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.

IL-6 gene transcription can be induced by other pro-inflammatory cytokines, such as IL-1 and tumour necrosis factor-α, in addition to other stimuli, such as bacterial lipopolysaccharide. Functional cis-regulatory elements described here to date are transcription factor-binding sites for NF-κB (nuclear factor-κB),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 single-nucleotide polymorphism at −174 bp upstream of the IL-6 TSS (rs1800795) is the most extensively studied single-nucleotide polymorphism and showed association with diseases, including systemic onset juvenile arthritis,7,8 systemic lupus erythematosus9 and cardiovascular disease.10,11

Previously, we have reported that the IL-6 gene transcription could be regulated beyond the 1 kb 5′-flanking region and had 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 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 (MS) 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 to −5152 bp of the 5′-flanking region of IL-6

To identify proteins bound to the IL-6 5′-flanking region at −5307 to −5152 bp (referred to as IL6-155 in the following text), we utilised SPR technology and MS. 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 sensor chip. Approximately 1500 RU (arbitrary resonance units) 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 Figure 1A. On the DNA-immobilised surface, RU increased with time during injection, whereas 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 (Figure 1b). As 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 MS analysis.

Analysis of eluted material by SDS-polyacrylamide gel electrophoresis
and silver staining showed several protein bands between 35 and 45 kDa, which were not seen in the bovine serum albumin (BSA) buffer alone (Figure 1c). Sufficient material was collected from repeat injections to reveal visible bands by colloidal Coomassie Blue staining. These were excised, the proteins were digested with trypsin and extracted peptides were analysed by liquid chromatography/tandem MS. The majority of identified proteins were again identified. The presence of these bound isoforms B1 of HNRNPA2B1. In a repeat experiment, both of these four most intense bands were isoform A1-B of HNRNPA1 and specific peptide sequences. The highest scoring proteins in the

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, electrophoretic mobility shift (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 (Figure 2a). These results confirmed the involvement of HNRNPA1 in complex with the IL6-155 sequence in vivo. The binding of
HNRNPA2B1 to the DNA sequence was not confirmed by this
method.

To test whether 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 (Figure 2b). On the
other hand, when immunoprecipitated with anti-HNRNPA2B1, a
faint band was observed for the region 5368 to 5152, but
was not confirmed by quantitative PCR.

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 small interfering RNA (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 a 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 h of transfection, but both their mRNA
levels decreased by an average of 90% (Figure 3a and b). Despite
the presence of a level of ~50% HNRNPA1 protein, significant
expression by HNRNPA1 was also demonstrated in HeLa cells. The positive regulation of IL-6 mRNA expression in transfected cells had significantly lower IL-6 mRNA expression, and so is 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. As HeLa cell is a papilloma-transformed epithelial cell line in vivo, and is easy to transfect in tissue culture, we have continued to use the approach experimentally. More importantly, HeLa cells have always been shown to produce IL-6 in response to biologic stimuli that are similarly effective in peripheral blood mononuclear cell cultures, and similar to healthy peripheral blood mononuclear cells. HeLa cells 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 and −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, the reduction of HNRNPA1 protein levels by 50% in cells by siRNA significantly inhibited IL-6 promoter activity and mRNA expression. The relatively small effect

abolished protein binding to IL6-155, with two probes −5220 to −5181 and −5191 to −5152 exhibiting partial competition (Figure 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 (Figure 4b). Interestingly, a motif 5′-TACAGA-3′, located in the middle of the region (−5325 to −5280 bp), is similar to the sequence found to be responsible for HNRNPA1 binding to the IL-10 promoter (5′-TACACA-3′; Figure 4c). 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. Figure 4d shows that mut1 had little protein–DNA binding, whereas mut2 could still form a protein–DNA complex similar to 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 MS 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 MS 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. 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 is 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. As 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 approach experimentally. More importantly, HeLa cells have always been shown to produce IL-6 in response to biologic stimuli that are similarly effective in peripheral blood mononuclear cell cultures, and similar to healthy peripheral blood mononuclear cells. HeLa cells do not have high levels of constitutive expression.

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. 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

**Figure 2.** EMSA supershift assay and ChIP assay. (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 a percentage of input DNA under the corresponding bands.

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. 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

adjusted protein binding to IL6-155, with two probes −5220 to −5181 and −5191 to −5152 exhibiting partial competition (Figure 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 (Figure 4b). Interestingly, a motif 5′-TACAGA-3′, located in the middle of the region (−5325 to −5280 bp), is similar to the sequence found to be responsible for HNRNPA1 binding to the IL-10 promoter (5′-TACACA-3′; Figure 4c). 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. Figure 4d shows that mut1 had little protein–DNA binding, whereas mut2 could still form a protein–DNA complex similar to 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 MS 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 MS 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. 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 is 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. As 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 approach experimentally. More importantly, HeLa cells have always been shown to produce IL-6 in response to biologic stimuli that are similarly effective in peripheral blood mononuclear cell cultures, and similar to healthy peripheral blood mononuclear cells. HeLa cells do not have high levels of constitutive expression.

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. 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
HNRNPA1 interacts with a 5'-flanking distal element

D Zheng et al

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 h 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 five experiments and were normalised to those of TBP bands before, compared with those measured in siNeg#2-transfected cells (1.0). The mean and s.d. 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 nanograms of IL-6 promoter luciferase constructs, bearing IL-6 5'-flanking sequence up to −5307 or −5202 bp (pGL3-IL6-5307 or pGL3-IL6-5202), were transfected into HeLa cells using Lipofectamine LTX. Six hours later, cells were subjected to siRNA transfection as described. Cell extracts were prepared 48 h 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 empty vector. All experiments were performed at least three times. The error bars represent the s.d. of the replicate experiments and *P<0.05 was regarded as significant.

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 HNRNP and has primary roles in the 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.22,26 The fact that only one of two RNA recognition motifs is sufficient to bind telomeric DNA raises the potential for HNRNPA1 to interact with double-stranded DNA. Transcriptional regulatory roles of HNRNPs (including HNRNPA1) via binding to double-stranded DNA 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 KRAS14 and IL-10.15,16 How HNRNPA1 binds to double-stranded DNA to modulate gene expression is still unclear. The binding motifs identified from different promoters are not conserved. HNRNPA1 has been shown to bind a 5'-ATT-3' sequence with high affinity in the thymidine kinase gene. A 5'-AGGGT-3' sequence was found to be important for HNRNPA1 binding to the APOE promoter. A parallel G-quadruplex 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 5'-TACACA-3' is critical for binding. Donev et al.30 showed that HNRNPA1 bound to DNA within a 36-bp sequence and this sequence was found widely dispersed throughout the genome. These observations
imply that HNRNPA1 may have 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 IL-6 transcription; the ChIP assay suggested that a weak, but discernible interaction, knockdown of HNRNPA2B1 led to increased reporter activity, whereas 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.31,32 has implied that HNRNPA2B1 is a common transcriptional co-activator, functioning in response to mitochondrial respiratory stress, 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, as autoantibodies to the HNRNP family have been detected in systemic rheumatic diseases, such as systemic lupus erythematosus,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 ischaemic 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 Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ 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 dithiothreitol and protease and phosphatase.
inhibitors. Protein concentration was quantified using Bradford protein assay (Bio-Rad, Hercules, CA, USA).

Surface plasmon resonance

The SPR experiment was carried out using a BLAcore T100 with Sensor chip SA (GE Healthcare, Little Chalfont, UK). The DNA fragment containing sequences from −5368 to −5152bp upstream of IL-6 TSS (IL6-155) was amplified using a forward primer 5′-biotin-TGGCTCAGACATAGACCTACGCT-3′ and a reverse primer 5′-TATTGTTCCAAGGGTGCTG-3′, and was purified on Qiagen PCR columns (Qiagen, Hilden, Germany). The SA chip surface was activated by injection of 1mM NaCl in 50 mM NaOH for 1 min three times. Biotin-labelled PCR product at 2ng ml⁻¹ in 0.5mL NaCl was injected onto the chip surface at a flow rate of 5μl min⁻¹ for 30 min. Approximately 1500RU was achieved per flow cell. Empirically in the BLAcore technology, 1ng of a globular protein or 0.78 ng of a DNA molecule bound at the surface gives a response of 1000RU.37

For protein–DNA interaction analyses, 20 to 200 ng ml⁻¹ nuclear proteins were first incubated with 10 ng ml⁻¹ 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, 5 mM MgCl₂, 1 mM EDTA, 40 ng ml⁻¹ BSA and 0.05% P20 surfactant, and then applied to the DNA-immobilised chip at a rate of 1 μl min⁻¹. The sensorgrams were recorded automatically and were fitted 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 twofold injection with 0.05% SDS to regenerate the chip. For protein recovery, all four flow cells on the chip were immobilised with IL-6-155 DNA and the bound proteins recovered to collection tubes by using the ‘Injection and Recovery’ function of the BLAcore T100. Multiple cycles and repeats were applied to obtain sufficient protein for MS 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).

MS 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 dithiothreitol in 5 mM ammonium bicarbonate pH 8.0 for 45 min at 50°C and were alkylated with 50 mM iodoacetamide in ammonium bicarbonate for 1 h at room temperature in the dark. Gel pieces were washed in 50% acetonitrile vacuo-dried, resuspended in 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 amionium bicarbonate was added and gel pieces were incubated at 37°C for 15 min. Tryptic peptides were extracted three times with 50% (v/v) acetonitrile containing 5% (v/v) formic acid from each gel piece, pooled and vacuum centrifuged to dryness. Peptides were resuspended in 5 μl of 0.1% (v/v) formic acid and stored at −20°C before MS analysis.

Analysis of tryptic peptides from digested bands was performed by nanoflow reversed-phase liquid chromatography–tandem 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, Amsterdam, The Netherlands) and washed for 3 min with 95% solvent A (water + 0.1% FA) at a flow rate of 25 nl min⁻¹ 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 MS was operated in the data-dependent mode to automatically switch between MS and tandem 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 six most intense ions were selected for collision-induced dissociation. Target ions that had been selected for tandem MS were dynamically excluded for 60 s. 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 ± 0.01 ppm and the tandem 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 significant threshold of 1.3 and protein identifications had to have at least two unique peptides matched per protein.

EMSA assay and supershift

EMSA assay was performed using the non-radioactive LightShift Chemiluminescent EMA kit (Pierce, Thermo Scientific). The biotinylated IL6-155 probe was the same as that used for the SPR experiments. Short 39bp probes, used previously37 or with mutations, were made by annealing primer pairs and were labelled at the 3′-end using terminal deoxynucleotidyl transferase and biotin-11-dUTP (Fermentas, Thermo Scientific). EMSA assays were performed using 10 μM biotinylated probe incubated with 2 μg of nuclear proteins in 1 × binding buffer (8% Ficoll, 20 mM HEPES, 50 mM KCl, 1 mM EDTA, 0.5 mM dithiothreitol, 40 ng ml⁻¹ of poly[dI-dC] and 40 ng ml⁻¹ 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 for 15 min before 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% 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 biotinylated DNA. The nylon membranes were then visualised by exposing to X-ray film. Antibodies to HNRNPA1 (4B10), HNRNPA2B1 (D3P83) and normal mouse IgG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Pure HNRNPA1 protein was obtained from Origene (Rockville, MD, USA) and 200 ng used in EMSA reactions as a positive control.

Chromatin immunoprecipitation assay

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 three times with cold phosphate-buffered saline, collected and resuspended in kit Lysis buffer supplemented with protease inhibitors. Chromatin was sheared by sonication using a Bioruptor sonicator (Diagenode, Waldbronn, France). The lysate was sheared at 100 g for 5 min, 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 × 10⁵ 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′-TATGGCTTCAAAGGGTGAGAG-3′ and 5′-TATTGCTCAAGGGTGAGAG-3′, encompassing −5368 to −5152 bp. The PCR conditions were as follows: 95°C for 5 min, 30 cycles of 95°C for 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′-AATGGTGTCT-GAAATCCATGC-3′ and 5′-GGTGGGCTTGAGGTATGAGA-3′. The level of enrichment of the target DNA sequence was determined by real-time PCR using the 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) in serum-free medium, according to the manufacturer’s instructions. 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'-GAAGUGGUUAUAAGUGUAAU-3' and 5'-AUCACUUUAUAACACAUUCU-3', and those of HNRNPA2B1 siRNA were 5'-GCACCAUUUCUACACUGUGTT-3' and 5'-ACGUGGAUAGAAGGUGGUGG-3'.

For co-transfection experiments, 100 ng of IL-6 promoter-luciferase constructs, bearing IL-6 promoter sequence up to 5'-GAAUGGUUAUAAAGUGAUtt-3' were transfected into HeLa cells in a 96-well plate, using Lipofectamine LTX (Life Technologies). Six hours later, the media was replaced and cells were 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.

Western blotting

Protein from whole-cell lysates was resolved on 10% NuPAGE Bis-Tris gels (Life Technologies) and transferred to polyvinylidene difluoride membrane (GE Healthcare). The membrane was blocked with 5% BSA (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 horseradish peroxidase-conjugated antibody at room temperature for 1 h, washed again and proteins of interest visualised using an enhanced chemiluminescence detection system (Thermo Scientific). Membranes were probed with antibody against TBP (TATA box-binding protein) to control for loading. The corresponding bands were quantified by an image processing software, Image J (NIH, http://rsb.info.nih.gov/ij/), on underexposed images and were normalised to those of TBP bands before, compared with those measured in siNeg2-transfected cells. Antibodies against HNRNPA1, HNRNPA2B1 and RPLP0 were purchased from Santa Cruz Biotechnology.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Life Technologies) and Solaris qPCR Gene Expression Master Mix (Thermo Scientific) for IL6, HNRNPA2B1 and RPLP0. The HNRNPA1 transcripts were detected using Quantitect reverse transcription kit (Qiagen), and quantitative PCR was performed using Taqman gene expression assay (Life Technologies) and Solaris qPCR Gene Expression Master Mix (Thermo Scientific) for IL6, HNRNPA2B1 and RPLP0. The HNRNPA1 transcripts were detected using Quantitect SYBR green PCR kit with a pair of primer, 5'-GCT TGGGTGGAGAAGCCATA-3' and 5'-GCACCAUUUCUACACUGGUG-3'. All quantitative PCR reactions were performed on a Mastercycler ep realplex PCR system (Eppendorf, Hamberg, Germany) with cycling conditions as follows: 15 min of denaturation at 95°C and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Relative levels of mRNA expression were calculated according to the ΔΔCT method and were normalised by comparison with the RPLP0 mRNA expression.

Statistical analysis

All experiments were repeated at least three times. Data are presented as the mean ± s.d. 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by the Arthritis Research UK (no. 17287). The work was undertaken at UCLH/UCL that 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.
27 Tay N, Chan SH, Ren EC. Identification and cloning of a novel heterogeneous nuclear ribonucleoprotein C-like protein that functions as a transcriptional activator of the hepatitis B virus enhancer II. J Virol 1992; 66: 6841–6848.

28 Michelotti EF, Michelotti GA, Aronsohn AI, Levens D. Heterogeneous nuclear ribonucleoprotein K is a transcription factor. Mol Cell Biol 1996; 16: 2350–2360.

29 Lau JS, Baumeister P, Kim E, Roy B, Hsieh TY, Lai M et al. Heterogeneous nuclear ribonucleoproteins as regulators of gene expression through interactions with the human thymidine kinase promoter. J Cell Biochem 2000; 79: 395–406.

30 Donev RM, Doneva TA, Bowen WR, Sheer D. HnRNP-A1 binds directly to double-stranded DNA in vitro within a 36 bp sequence. Mol Cell Biochem 2002; 233: 181–185.

31 Guha M, Pan H, Fang JK, Avadhani NG. Heterogeneous nuclear ribonucleoprotein A2 is a common transcriptional coactivator in the nuclear transcription response to mitochondrial respiratory stress. Mol Biol Cell 2009; 20: 4107–4119.

32 Guha M, Tang W, Sondheimer N, Avadhani NG. Role of calcineurin, hnRNPA2 and Akt in mitochondrial respiratory stress-mediated transcription activation of nuclear gene targets. Biochim Biophys Acta 2010; 1797: 1055–1065.

33 Siapka S, Patrinou-Georgoula M, Vlachoyiannopoulos PG, Guialis A. Multiple specificities of autoantibodies against hnRNP A/B proteins in systemic rheumatic diseases and hnRNP L as an associated novel autoantigen. Autoimmunity 2007; 40: 223–233.

34 Caporali R, Bugatti S, Bruschi E, Cavagna L, Montecucco C. Autoantibodies to heterogeneous nuclear ribonucleoproteins. Autoimmunity 2005; 38: 25–32.

35 Murakami M, Nishimoto N. The value of blocking IL-6 outside of rheumatoid arthritis: current perspective. Curr Opin Rheumatol 2011; 23: 273–277.

36 Thiele BJ, Doller A, Kahne T, Pregla R, Hetzer R, Regitz-Zagrosek V. RNA-binding proteins heterogeneous nuclear ribonucleoprotein A1, E1, and K are involved in post-transcriptional control of collagen I and III synthesis. Circ Res 2004; 95: 1058–1066.

37 Buckle M, Williams RM, Negroni M, Buc H. Real time measurements of elongation by a reverse transcriptase using surface plasmon resonance. Proc Natl Acad Sci USA 1996; 93: 889–894.

38 Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29: e45.

Supplementary Information accompanies this paper on Genes and Immunity website (http://www.nature.com/gene)