IFN-γ gene expression is controlled by the architectural transcription factor HMGA1

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Abstract

We report for the first time that IFNG gene expression requires high mobility group (HMG)A1, the architectural transcription factor mediating enhanceosome formation. This finding is supported by our direct studies of T cells isolated from the HMGA1-transgenic mice displaying an up-regulation of IFN-γ production and of HMGA1-deficient mice exhibited a decreased IFN-γ induction. In parallel transfection studies in EL4 cells, we observed elevated IFNG gene promoter activity in cells stably over-expressing HMGA1 and a reduction of such activity in cells expressing dominant-negative HMGA1. In vitro binding assays further demonstrated a specific interaction of HMGA1 to defined regions of the IFNG gene proximal promoter.

Introduction

IFN-γ is predominantly produced by CD8+ T cells, and to a lesser degree in polarised CD4+ T cells and NK cells. IFN-γ is the signature cytokine product as well as a key inducer of Tn, development. Its expression occurs in response to a variety of stimuli, such as TCR stimulation and a supportive cytokine milieu (e.g. IL-2, IL-12 and IL-18) (1–5). Different pathways are found inducing IFN-γ expression in distinctive cell types.

Mapping of the IFNG gene locus for transcription activities has defined several cis-acting regulatory regions: one associated with the TATA box—the core promoter (nt –1 to –100), containing proximal and distal regions at nt –44 to –71 and nt –78 to –98, respectively (1, 6, 7). The human and mouse promoter regions over nt –1 to –265 are 77% identical and differ by only 11 positions in the core promoter region (8, 9). The proximal element has strong homology to the IL-2Rα gene promoter (6). Further upstream, a non-core promoter has been localised, with binding sites for the transcription factors YY1, nuclear factor of activated T cells (NFAT), activated protein-1 (AP-1), Stats and nuclear factor κB (10–15). Importantly, a T-bet interaction site was identified at nt –415 to –565 on the IFNG gene promoter (7)—T-bet is a key lineage commitment factor in Tn,1 cell differentiation and a major factor promoting IFN-γ production (16, 17). The distal regulatory region has been shown to be important for lineage-specific expression of the IFNG gene rather than determining basal levels of transcription (18). The first and third introns of the IFNG gene have also been found to possess enhancer-like activity (19, 20). Two reports were published recently on the epigenetic control events of the IFNG gene (21, 22). Overall, control of the IFNG gene expression appears to be multi-factorial and involves multiple steps.

We chose to investigate if IFNG gene expression is supported by the high mobility group (HMG)A1 architectural transcription factor, based on the two facts that IFN-γ is rapidly produced by activated T lymphocytes (thereby specific transcription machinery must be in place to meet the demand

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and HMGA1 may optimise the process) and the burst in HMGA1 synthesis during T lymphocyte activation and clonal expansion (23, 24). HMGA1 is likely to participate in IFNG gene transcription, for it may serve in mediating ordered recruitment of activated transcription factors on the IFNG gene proximal promoter.

HMGA1 (old name HMG1/Y) constitutes a family of non-histone chromosomal proteins that contribute to the regulation of inducible genes by altering local chromatin structure and by facilitating the formation of multi-protein enhanceosomes on gene proximal promoters (25-28). Enhanceosomes are composed of multiple transcription factors whose assembly is mediated by protein-protein and DNA-protein interactions. Enhanceosomes have been shown to enable temporal, spatial and inducible gene expression and to optimise the rate of transcription (29, 30). Emerging evidence supports a view that HMGA proteins first interact with the target gene proximal promoters (exhibit an affinity for the minor groove of AT-rich DNA sequences) and increase accessibility of trans-acting factors to their corresponding cis-elements. An enhanceosome, once assembled through further interactions between participating factors on gene proximal promoter, facilitates the activity of RNA polymerase II and thereby gene transcription (31). Expression of >30 genes has been reported to be modulated by HMGA1, with many of these genes having defined roles in the immune system (23, 32). Indeed, the best-studied HMGA1-dependent genes are IFN-β, IL-2 and IL-2Ra (33-35).

Our evidence that HMGA1 participates in the IFNG gene transcription comes from studies of both the HMGA1-transgenic/knockout mice and reporter gene assays. In order to investigate if HMGA1 influences the IFNG gene expression in vivo, transgenic mice were generated by us, where HMGA1 levels are enhanced in peripheral T lymphocytes. Analysis of lymphocytes isolated from three founder mice demonstrated that enhanced levels of IFN-γ transcript and cytokine were produced following their activation. Examination of the T cells collected from HMGA1-deficient mice developed by the Fusco group (manuscript submitted) revealed a reduced IFN-γ transcript level upon stimulation. Subsequent reporter gene studies in EL4 cells also showed that the transcription of a heterologous IFNG reporter gene was proportional to the levels of functional HMGA1 in EL4 cells. Finally, specific high-affinity binding sites for HMGA1 have been mapped within the IFNG gene proximal promoter. Collectively, the data support a role for HMGA1 in the IFNG gene transcription. This knowledge should contribute to our understanding of IFNG and Tc1-specific gene regulation and supports the view that the burst of HMGA1 expression detected in activated lymphocytes is germane to cytokine expression in effector T cells.

Methods

Creation of HMGA1-transgenic mice and characterisation of transgene expression

The mouse HMGA1 coding region (in form of -b isoform) was sub-cloned using the BamH1 site into the transgene plasmid pW120 (36; obtained from R. Perlmutter), where expression is driven by the distal-1ck promoter. The entire 5.5-kb transgene cassette was injected into the pronuclei of single-cell stage fertilised eggs from superovulated C57BL/6 females to generate founders. Three independent transgenic founders were identified by Southern blot hybridisation as described (37). Expression of the transgene was detected by northern and western blot analyses methods as detailed previously (38), and the specific polyclonal antibodies recognising HMGA1 were used (39).

Spleen cell preparation, culture and enrichment

Dissociated spleen cells were cultured in complete T cell medium (TCM) consisting of RPMI 1640 supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 0.05 mM 2-mercaptoethanol and 10% foetal bovine serum (all from Invitrogen, Carlsbad, CA, USA).

CD4+ and CD8+ T cells were enriched from isolated spleen cells by using IMMULAN columns (Biotex Laboratories, Houston, TX, USA) coupled with anti-CD8a (Ly-2)/anti-B220 (RA3-6B2) or anti-CD4 (L3T4)/anti-B220, respectively (BD Biosciences, San Diego, CA, USA). The purity of antibody-enriched splenocytes was measured by flow cytometry.

IFN-γ and IL-5 ELISA and ribonuclease protection assays on anti-CD3 activated splenocytes

Isolated spleen cells (after adherent cells were removed) were grown in complete TCM in tissue culture plates, pre-coated with anti-CD3e (145-2C11, 2.5 µg ml⁻¹), for 4 days and culture supernatants were collected. For IFN-γ ELISA, plates were coated with 2 µg ml⁻¹ anti-IFN-γ mAb R4-6A2 capture antibodies, and the residual binding sites were blocked with 1% BSA. Culture supernatants and standards (rIFN-γ, BD Biosciences) were added and incubated overnight at 4°C. The plates were washed and 0.2 µg ml⁻¹ biotin-conjugated anti-IFN-γ mAb XMG1.2 was added. After incubation at room temperature for 1 h, the plates were further washed and to each well 100 µl of streptavidin–HRP (KPL, Gaithersburg, MD, USA, in 1:400 dilution) was added, prior to the ABTS solutions (KPL) and the absorbance was read at 405 nm with the Dynex MRX ELISA reader (Chantilly, VA, USA).

Expression of IFN-γ in the culture supernatants were calculated from the standard curve. For IL-5 ELISA, the pair of anti-IL-5 TRFK5 and TRFK4 and rIL-5 (all from BD Biosciences) were used and manufacturer’s instructions was followed (40).

Reverse transcription–PCR analyses on splenocytes prepared from HMGA1-deficient mice

Standard protocols were used for the gene-specific and semi-quantitative amplification. Individual sequence of the pairs of primers, synthesised by Proligo (Boulder, CO, USA), is as follows: IFN-γ: 5' AGCGGCTGACTGAACTCAGATTGTAG-3' / 5' GTCAACAGTTTCCGCTGTAAAGG-3'; HMGA1: 5’-CTGGCCTCAAACAGGAAAA-3’/5’-GAGATGCCCTCCTTCC-TC-3’ and glyceraldehyde-3-phosphate dehydrogenase
(GAPDH): 5′-CAGTCTTCTGAGTGCGATG-3′/5′-CTG-GTGCAGATGUCATGCGTG-3′. Densitometry was performed on PCR products resolved onto agarose gels stained with ethidium bromide, in order to determine the ratios of IFN-γ/GAPDH transcript level.

Establishment of EL4 stable transfectants for measurement of promoter activities

EL4 cells were maintained in complete TCM as described above. Five million EL4 cells were re-suspended in 0.3 ml RPMI and transfected with 19 µg of either HMGAI (-a isoform) expression construct or its parental uncloned pcDNA3.1 plasmid (Invitrogen), and 1 µg of a plasmid expressing green fluorescent protein (GFP), in an electroporator (Invitrogen) set for 250 V and indefinite resistance, using cuvettes of 4-mm gap supplied by BTX (San Diego, CA, USA). Transfected cells were selected with 0.4 g/ml G418 (Invitrogen), and they exhibit green under a fluorescent microscope with a FITC filter.

Lines of stably transfected EL4 cells with HMGAI or empty expression plasmid were transiently transfected, with 10 µg of human IFNG promoter-pGL-2 Basic Luc reporter construct, or the pGL-2 Promoter construct (Promega, Madison, WI, USA), and 10 µg of a plasmid expressing chloramphenicol acetyl transferase (CAT). Transfected cells were divided into two portions and were grown separately for 40 h, before one portion being stimulated by PMA (50 nM)/ionomycin (1 µg/ml) for 4 h. Transfected cells were collected, washed and lysed ready for Luc and CAT measurement using reagents purchased from Promega and Roche (Indianapolis, IN, USA), and results were measured by a luminometer (Lumat LB9507, Berthold Technologies, Bad Wildbad, Germany) and the Dynex ELISA reader, respectively. Each Luc reading was normalised by its corresponding CAT activity. Normalised Luc level driven to produce by the cloned IFNG promoter fragment was compared with that of the SV40 promoter conferred by the pGL-2 Promoter construct and then fold change was determined. Mean and standard errors were calculated from all experiments and plotted. Student’s t test was the statistical method used and P value was limited to 0.01.

Activities of the human IL-5 gene promoter (−450 to +35) cloned into pCAT Basic reporter vector were quantitated at CAT level and normalised by the amount of Luc expressed by the co-transfected pGL-2 Control plasmid, into the panel of EL4 cells to which the GATA-3 expression plasmid was also delivered. For control, the pCAT Control vector was replaced in the transfection.

Generation of recombinant lentiviral vectors and gene transduction

The plasmid expressing the non-DNA-binding mutant form of HMGAI was obtained from R. Reeves (34), and was amplified by PCR in order to create BamHI and Nof sites for cloning into the pHR-SIN-CSGWAnotl plasmid, by replacing the GFP with mutant HMGAI, and a His-tag was inserted in its N-terminal. DNA sequence of the cloned fragment was confirmed. 293T cells, maintained in DMEM-based medium were transfected with three plasmids, namely, pCMV.R8.91, pMDG and pHR-SIN-CSGWAnotl or pHR-SIN-CSGWAnotl-HMGAI by lipofectamine (Invitrogen) and medium supporting growth of the transfected 293T cells for 3 days contains recombinant viral particles (41). EL4 cells were infected with the medium containing the recombinant virus and centrifuged at 800 r.p.m. for 2 h at room temperature. The degree of transduction in EL4 cells with recombinant lentivectors expressing GFP was visualised by a fluorescent microscope with FITC filter. Transduced expression in EL4 cells was measured by western blot analysis.

DNase I footprinting assays

The double-stranded DNA fragment subjected to analysis was amplified and radio-labelled by PCR, from the primers, namely, hIFN-γP2F (spanning nt −358 to −386 of the hIFN-γ promoter, 5′-GAGGGCCCTAGAATTTCGTTTTTCACTTTACCTGT-3′) and hIFN-γP1R (spanning nt −40 to −79, TCTCTTGAGGAT-TACGTATTTTCACAAAGTCTTTAAGATG-3′), with Taq polymerase purchased from Promega in the presence of [α-32P]dCTP (ICN, Costa Mesa, CA, USA). The amplified DNA fragment was purified by PAGE, and 10 000 c.p.m. of probe was incubated with recombinant HMGAI (50 and 200 ng, respectively) in 50 µl of a buffer of 25 mM HEPES (pH 7.6), 50 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 10 µg BSA, 0.01% NP40 and 1 µg poly(dG–dC), on ice for 30 min and then at room temperature for 10 min. Subsequently, MgCl2 was added to 4 mM and the probe was partially digested with DNase I (Promega). The digests were purified and resolved on an 8% sequencing gel for autoradiography as described (42). Preparation of the G + A ladder was as described (42).

Preparation of nuclear extracts from EL4 cells and electrophoretic mobility shift assays

EL4 were stimulated by PMA (50 nM)/ionomycin (1 µg/ml) for 4 h, and nuclear extract of that and that of unstimulated cells was prepared by published method (42).

Probes for electrophoretic mobility shift assays (EMSA) were labelled by [γ-32P]dATP (ICN) and T4 PNK (New England Biolabs), F(forward) and R(reverse) oligonucleotides were annealed and PAGE-purified. They are hIFN-γP1F: 5′-CATCT-TAAAAACCTGTTGAAAACTGTAATCTCCAGAGAA-3′ and hIFN-γP1R: 5′-TCTCTTGAGGAT-TACGTATTTTCACAAAGTTTTTTAAGATG-3′ spanning nt −40 to −79 of hIFN-γ promoter, hIFN-γP1UF: 5′-CATCTTAAAAACCTGTTGAA-3′ and hIFN-γP1UR: 5′-TCTCAAGATTITTATTTGAGATG-3′ spanning nt −60 to −79 and hIFN-γP1DF: 5′-AACCCTGGAAAAACTCGTATACTCCAGAGAA-3′ and hIFN-γP1DR: 5′-TCTCTTGAGGAT-TACGTATTTTCACAAAGTTTTTTAAGATG-3′ spanning nt −40 to −69.

Probes of specific activity 10 000 c.p.m. were incubated with recombinant HMGAI or nuclear extracts in 10 µl of a buffer of 20 mM HEPES (pH 7.9), 40 mM KCl, 2 mM MgCl2, 1 mM DTT, 12.5% glycerol, 5 µg BSA and 400 ng poly(dG–dC), at room temperature for 25 min, before being electrophoresed through a 5% polyacrylamide (0.5× Tris borate EDTA) gel at room temperature. Antibodies recognising HMGAI (39), or its isotype control, were first incubated with nuclear extracts on ice for 30 min before mixing with the probe. An equivalent amount of nuclear extract was used in every EMSA, after the protein concentration was measured by the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA).
Results

Transgenic HMGA1 expression in lymphocytes enhances IFN-γ induction in vivo

In order to test if HMGA1 is involved in the regulation of IFNG gene, transgenic mice were created by us with the distal-lick promoter-HMGA1 (-b isoform)-coding region cassette (Fig. 1A). This promoter has been well-characterised by Perlmutter and co-workers to drive the expression of associated transgenes in T lymphocytes from the CD4+CD8+ stage. We deliberately chose this promoter as we wanted to avoid/minimise any deleterious impact of HMGA1 level during T cell development. Three transgenic mouse founders were characterised by Southern, northern and western blot analyses (Fig. 1B–D) for transgene integration/copy number and HMGA1 expression. We initially measured the distribution of T/B and CD4+/CD8+ lymphocytes within the transgenic mice and found no significant difference from that of control mice (data not shown). This indicated that the transgene was not having a general influence on lymphocyte development.

Anti-CD3-stimulated splenocytes of the transgenic HMGA1 founders produced a mean 12-fold greater level of IFN-γ protein than splenocytes from non-transgenic controls (as assessed by ELISAs, Fig. 2A), in contrast to the insignificant increase of IL-5. Quantitative mRNA measurement by RNase protection assays showed an 8-fold elevation in the quantity of IFN-γ transcripts in these cells (relative to non-transgenic controls) when re-stimulated with PMA/ionomycin (Fig. 2B). Flow cytometric analysis indicated that single cells of the transgenic mice expressed higher level of IFN-γ upon activation (data not shown). Taken together, these data show that over-expression of HMGA1 in CD4+CD8+ T lymphocytes strongly enhances the transcription of the endogenous IFNG gene in vivo, resulting in enhanced levels of IFN-γ cytokine secreted from activated cells.

Inactivation of HMGA1 gene expression limits in vivo IFN-γ stimulation in lymphocytes

Existence of the HMGA1-deficient mice expressing no HMGA1-a or -b isoforms (generated by the classical gene inactivation approach by the Fusco group; manuscript submitted) allowed us to examine the impact of HMGA1 on expression of the IFNG gene, using a different approach. As shown in Fig. 3, a lower level of amplified IFN-γ transcripts (a mean of 3.5-fold over four independent semi-quantitative measurements), thereby an impeded IFN-γ activation (upon anti-CD3 stimulation followed by PMA/ionomycin re-stimulation), was observed in splenocytes of homozygous-null (+/-) mice that did not express HMGA1 transcript, compared with that of their wild-type counterpart (+/+). This underscores the role of HMGA1 in maximising induction of the endogenous IFNG gene.

Over-expression of HMGA1 in EL4 cells allows increased activation of heterologous IFNG gene promoter

As has been pointed out in the Introduction, several cis-regulatory regions have been implicated in the regulation of IFNG gene expression. Since HMGA1 typically influences gene activation via the proximal promoter of genes (23, 24, 26–28), we tested whether this also applied to the IFNG gene. A cell model was first established in order that the aforementioned studies could be carried out. EL4 cells were stably transsected separately with a plasmid expressing HMGA1 (specifically its -a isoform) and its parental empty expression plasmid. Such over-expression was demonstrated by western blot analysis (Fig. 4A). This pair of cell lines was transiently transfected with a panel of Luc reporter constructs of the human IFNG gene promoter, namely, ‘−110’, ‘−210’ and ‘−565’ (7), and all sharing the same 3’ end at nucleotide +64, and their 5’ end span to nt −110, −210 and −565, respectively. As shown in Fig. 4B, higher PMA/ionomycin induction (from 5.5- to 7.6-fold) of IFNG promoter activities was observed in EL4 cells over-expressing HMGA1, compared with that in HMGA1-low transfected EL4 cells that ranged from 2.4- to 4.3-fold, and 1–3-fold in EL4 cells. The HMGA1-responsive region was mapped within the 110 bp proximal promoter. An increased heterologous IL-2 promoter activity (8, 9; our data not shown), but not that of IL-5 (Fig. 4C), was supported by the HMGA1high-EL4 line, suggesting the specificity of the IFN-γ induction.
of IFN-γ assays on the above pooled splenocytes, of transgenic (T, lane 1) and control (N, lane 2) mice, re-stimulated with PMA/ionomycin for 4 h and level (left panel) and IL-5 (right panel) in the culture supernatant of anti-CD3-stimulated (and non-stimulated, denoted as media) spleen cells isolated from transgenic (black bars) and control mice (striped bars) grown for 4 days. Error bars represent standard error. B, Ribonuclease protection assays on the above pooled splenocytes, of transgenic (T, lane 1) and control (N, lane 2) mice, re-stimulated with PMA/ionomycin for 4 h and level of IFN-γ transcript measured, confirming an increased IFNG expression in T cells of transgenic HMGA1 expression. Same amount of total RNA was used, as demonstrated by the equivalent levels of L32 and GAPDH.

**Fig. 2.** Enhanced IFN-γ production in transgenic HMGA1 T cells activated via primary and re-stimulation. A, Mean ELISA measurement of IFN-γ (left panel) and IL-5 (right panel) in the culture supernatant of anti-CD3-stimulated (and non-stimulated, denoted as media) spleen cells isolated from transgenic (black bars) and control mice (striped bars) grown for 4 days. Error bars represent standard error. B, Ribonuclease protection assays on the above pooled splenocytes, of transgenic (T, lane 1) and control (N, lane 2) mice, re-stimulated with PMA/ionomycin for 4 h and level of IFN-γ transcript measured, confirming an increased IFNG expression in T cells of transgenic HMGA1 expression. Same amount of total RNA was used, as demonstrated by the equivalent levels of L32 and GAPDH.

**Fig. 3.** Inactivation of HMGA1 gene leading to diminished induction of IFNG gene expression. A, Agarose gel photo (stained with ethidium bromide) of a representative reverse transcription (RT)–PCR analysis (of semi-quantitative quality) to measure the mRNA level of IFN-γ and HMGA1, respectively, of the total RNA prepared from re-stimulated splenocytes (by PMA/ionomycin after initial anti-CD3 stimulation) isolated from wild-type (+/+), and homozygous-null (−/−) HMGA1-knockout mice, in which a lower level of IFN-γ transcript was detected in that of −/− mice (with no HMGA1 expression). Amplification products were collected by 30 PCR cycles, whereas further cycling (data not shown) assured that the amplifications were semi-quantitative. Input mRNA between samples was equivalent, as controlled by the GAPDH amplification. B, A summary of four independent RT–PCR analyses, and the mean ratios of amplified IFN-γ/GAPDH transcript is displayed with standard error of mean (as error bars), in which the ratio produced by −/− mice is set as 1. Levels of amplified products were determined by densitometry.

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**Blockade of HMGA1 function leads to diminished activity of human IFNG gene promoter.**

To provide further evidence that HMGA1 is required for maximal IFNG promoter activity, a dominant-negative form of HMGA1, a non-DNA-binding mutant (34), was then expressed in EL4 cells by lentiviral gene transduction (such expression was monitored by western blot analysis, Fig. 5A). Recombinant lentivirus transduced to express GFP (gave rise to 40% fluorescent cells measured by flow cytometry, data not shown) served as a control. Transduced EL4 cells were transfected with the −565 bp IFNG gene promoter reporter and the result, illustrated in Fig. 5B, demonstrated that IFNG promoter activity was reduced to 50% of the control in resting cells and to 51% in cells stimulated with PMA/ionomycin induction. We did not observe such response in the cloned −450 bp IL-5 gene promoter, indicating that the blockade is specific to the IFNG gene promoter function.

**HMGA1 interacts with human IFNG gene proximal promoter in vitro.**

As a first step to delineate how HMGA1 up-regulates IFN-γ expression through its −110 bp core gene promoter, we directly tested for such interactions in vitro by DNase I footprinting and EMSA. Examination for AT-rich nucleotide sequences (the DNA-binding specificity for HMGA1) suggested a priori that HMGA1 might interact with the ATF-2/AP-1/ATF-1/CREB site previously characterised within the gene proximal promoter (Fig. 6A). A double-stranded DNA fragment spanning nt −40 to −386 was amplified by PCR, incubated with recombinant HMGA1 and subjected to footprinting in vitro with DNase I as the cleaving agent. Fig. 6B shows that the regions nt −58 to −65 (site A), nt −68 to −76 (site B) and nt −114 to −133 (site C) were occupied by HMGA1 in a specific fashion. Complementary data were obtained when the anti-sense strand of the same fragment was studied (data not shown).

To show that HMGA1 found in lymphocytes can form complexes with this region, a double-stranded DNA probe hIFNγP1 spanning nt −40 to −79 containing the HMGA1 interaction sites A and B was used for EMSA, and the results are shown in Fig. 6C. A specific shifted HMGA1 probe complex was identified from mixing the probe with nuclear extract of resting or stimulated EL4 cells (left panel), and the level of complex was decreased in the presence of antibodies recognising HMGA1. Similar complexes were also observed using nuclear extracts isolated from mouse primary T cells of unskewed and polarised Tn1 phenotype (data not shown).

In an attempt to distinguish HMGA1 affinity between the two binding sites, A and B, of the examined region, subsequent EMSAs were performed using two overlapping probes hIFNγP1U (upstream) and hIFNγP1D (downstream). Competition
assay between radio-labelled full-length hIFNγP1 probe and separately with 30- and 100-fold excess cold probes of Sp1, hIFNγP1U and hIFNγP1D (middle panel) revealed that only the downstream hIFNγP1D probe is efficient in abolishing the hIFNγP1–HMGA1 complex, suggesting that site A contributes to specific HMGA1 interaction. Further, two interesting observations were made from EMSA using either the upstream or downstream probes (right panel): HMGA1 was found to

Fig. 4. Stable transfectant of forced HMGA1 expression in EL4 cells has led to increased activity of human IFNG gene promoter. A, Comparison of HMGA1 level in two lines of EL4 cells stably transfected with the HMGA1 (+pHMGA1, lane 1) or an empty expression plasmid (+pcDNA, lane 2), and non-transfected cells (lane 3), by western blot analysis on equivalent amount of nuclear extracts loaded. A higher level of HMGA1 (indicated by arrow) was observed in EL4/+pHMGA1 cells. B, Response of the human IFNG gene promoter to altered HMGA1 level in EL4 cells. The pair of EL4/+pHMGA1 (shown as black bars) and EL4/+pcDNA (grey bars) transfected cell lines, together with the parental EL4 cells (white bars), were transfected separately with a member of the panel of deletion IFNG gene promoter Luc reporters (namely, −110, −210 and −565 denoting the position of each of the 5’ end) and a CAT expression plasmid, divided into two portions and one being stimulated by PMA/ionomycin (P/I). Fold change was calculated by comparing each normalised Luc reading with that of pGL-2 Promoter, among transfections of the same cell line. Mean and standard error of mean were plotted graphically. Elevated level of activities contributed by the IFNG gene promoter fragments of three different lengths were observed in EL4 cells over-expressing HMGA1, and all increases are statistically significant. C, Activity of the cloned human IL-5 gene promoter fragment (−450 to +35) appears indifferent in EL4 cells of HMGA1high (illustrated as white and striped bars) and HMGA1low (grey and black bars), all in the presence of ectopic GATA-3 expression. CAT was the readout of the IL-5 promoter activity, and this was normalised by constitutive Luc expression by pGL-2 Control plasmid, and compared with the normalised CAT activity resulted from a separate pCAT Control plasmid transfection to calculate the fold change. Those transfections stimulated by P/I as mentioned above were denoted as striped and black bars.
interact only with probe hIFNγP1D carrying site A and downstream flanking sequence, but not with probe hIFNγP1U containing site B and part of site A, although DNase I footprinting has demonstrated that both sites are targets for HMGA1 interaction. This again argues that site A is crucial for HMGA1 binding to this region. The affinity of HMGA1 to site A on the shortened fragment hIFNγP1D is also dramatically lowered compared with sites A and B on the composite probe hIFNγP1, suggesting that intact sites A and B plus flanking regions are required for optimal HMGA1 binding to this region. Whether a single HMGA1 molecule interacts with more than one of its DNA-binding sites on this promoter requires further analysis. Taken together, however, the data provide initial information on the mechanism of binding of HMGA1 to a minimal HMGA1-responsive region.

**Discussion**

It is now well established that HMGA1 promotes inducible gene expression by facilitating the formation of enhanceosomes at gene promoters. This is accomplished by binding to AT-rich sequences, induction of local DNA conformational changes and mediating DNA–protein and protein–protein interactions. Acetylation of HMGA1 controls assembly/dis-assembly of enhanceosomes and thereby expression of target genes (33). HMGA1 has been implicated in the process of Th cell differentiation by participating in regulation of cytokine genes (23, 24). However, there has not been a direct test to this hypothesis, apart from the studies conducted by Serfling’s group on IL-4 gene promoter (43). To test the possible role of HMGA1 in Th cytokine gene transcription, a transgenic mouse model was first established to over-express HMGA1 (in form of its -b isoform) in peripheral T lymphocytes, to allow detection of any alterations in cytokine production and as a result skewed the Th cell lineage commitment. The distal-λc transgene cassette was used to drive HMGA1 transgene expression, in order to minimise any impact of ectopic expression of HMGA1 in T cell development. Normal development (determined by surface expression of CD3, CD4, CD8 and B220) of lymphocytes was unaffected in three founders that were positive for transgene-directed HMGA1 expression. To identify any roles for HMGA1 in T cell-specific gene expression in mature T cells, we chose to focus first on the cytokine IFN-γ. The analysis showed that IFNG gene and cytokine expression was dramatically enhanced (relative to non-transgenic controls) in isolated splenocytes from transgenic mice upon primary TCR activation (and no co-stimulation necessary) and PMA/ionomycin re-stimulation. The analysis of some other potential target genes showed either no or reduced influences on their gene expression (data not shown), prompting us further still to focus our efforts on the IFNG gene. We further examined the splenocytes derived from mice lacking HMGA1 (both -a and -b isoforms) as a result of inactivation of the

Fig. 5. Lentiviral-mediated expression of a mutant HMGA1 in EL4 cells reduced activity of the human IFNG gene promoter. A. Recombinant lentiviral vectors were produced to transduce EL4 cells for expressing GFP (denoted as lenti-GFP) or a mutant form of HMGA1 of abolished DNA-binding capacity with a His-tag (indicated as lenti-DN-A1). The top panel shows fluorescent pictures of cells infected separately by the two viral vectors, in which bright cells were seen in lenti-GFP transduced EL4 cells. Figure in the bottom is a western blot on PCA extracts indicating endogenous HMGA1-a and -b level in lenti-GFP transduced cells (lane 1), and that transduced to express His-DN-A1 (lane 2). B. Shown in the top panel is the mean fold change of activity of the cloned human IFNG gene promoter fragments in EL4 cells transduced to express GFP (lenti-GFP) or DN-A1 (lenti-DN-A1), followed by PMA/ionomycin (P/I) stimulation. Separated cultures of transduced EL4 cells were co-transfected with the -565 IFNG promoter–Luc construct (white bars) or the pGL-2 Promoter (black bars), and the CAT expression plasmid. After being normalised by the CAT reading, the Luc level contributed by the pGL-2 Promoter to calculate the fold change. Mean fold change was shown, and the error bars represent standard error of mean. Difference in fold change of the IFNG gene promoter activities between transduced cells before and after inductions is statistically significant. The mean fold change of the human IL-5 gene promoter activity in the pair of transduced EL4 cells was also included in the bottom panel. Activity of the cloned -450 IL-5 gene promoter fragment was measured as CAT level, in the pair of EL4 cells co-transfected with the Luc and GATA-3 expression plasmids. This normalised CAT level was compared with that exhibited by the pCAT Control vector.
functional gene and found them expressing less IFN-γ upon activation. This observation further supports the capability of HMGA1 to promote IFNG gene expression in vivo. The results from the transfection experiments with IFNG reporter constructs delivered into EL4 cells supported the data obtained in transgenic/knockout mice, indicating that the IFNG gene transcription is HMGA1 mediated. Elevated level of HMGA1 (isoform -a) expression enhanced IFNG gene promoter activity, and functional blockade of endogenous HMGA1 by lentiviral-mediated transduction to express a dominant-negative mutant of abolished DNA-binding capacity (34) halved the resting and induced IFNG promoter activities (stronger inhibition is anticipated in the presence of excessive dominant-negative expression). Worth mentioning, we did not notice differential influence exerted by isoform -a and -b of HMGA1, encoded by a single functional gene, to the IFNG gene expression, despite an earlier report suggesting their different role in cellular transformation (44).

To begin to delineate the mechanism via which HMGA1 controls IFNG gene activation, HMGA1 binding sites have been mapped within the minimal HMGA1-responsive element defined by 5’-deletion analyses of the IFNG gene core

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**Fig. 6.** HMGA1 physically interacts with the human IFNG gene core promoter. A, Nucleotide sequence of the human IFNG gene promoter, in which the three binding sites for HMGA1 determined by in vitro method are marked underneath with asterisk. The transcription initiation site is indicated as +1 by white bent arrow, while the first codon ATG is denoted in bold. The two 5’-(nt – 110 and – 210, respectively) and common 3’-(nt +64) ends of the cloned IFNG gene promoter fragments into Luc reporter plasmids are shown by black bent arrows. Nucleotides shown in gray are homologous to the mouse IFNG gene promoter sequence. Binding sites of other factors are overlined and named. Black arrows represent sequence of probes for EMSA (see below). B, Three HMGA1 binding sites were mapped onto the human IFNG gene promoter flanking nt – 40 to –386, by in vitro DNase I footprinting, using recombinant HMGA1 (rHMGA1). Shown is an autoradiograph of the examined region with two levels of rHMGA1 used (50 and 200 ng, respectively, in lanes 3 and 4). The three sites footprinted by rHMGA1 are indicated in gray. Naked fragment was used in lane 2. A G + A ladder was included in lane 1 for size determination. C, Complex formation between HMGA1 present in T cells with a DNA probe of the human IFNG proximal gene promoter sequences. Shown in the left panel, such complexes (indicated by arrow) were detected by EMSA with a probe hIFNγP1 representing the region nt – 40 to – 79 that covers the IFNG gene proximal promoter overlapping with the two putative HMGA1 binding sites, and 10 ng recombinant HMGA1 (rA1, in lanes 2 and 5), or nuclear extracts prepared from resting (R; in lanes 3 and 6) or re-stimulated (S, in lanes 4 and 7) EL4 cells. Addition of HMGA1-specific antibodies (anti-A1, in lanes 6–7) ablated such complexes. Lane 1 contained free probe only. The panel in the middle shows the EMSA result of competing radio-labelled hIFNγP1 probe with increasing (30-fold used in lanes 2, 4 and 6; 100-fold used in lanes 3, 5 and 7) excessive probes of Sp1 (lanes 2–3), hIFNγP1U (lanes 4–5) and hIFNγP1D (lanes 6–7), in the presence of 10 ng of the rHMGA1 except in lane 1. The panel on the right hand side is the EMSA using hIFNγP1 (lanes 1–3), or its sub-fragments hIFNγP1U (lanes 4–5) and hIFNγP1D (lanes 6–7), in the presence of 6 ng (lanes 2, 4 and 6) and 30 ng (lanes 3, 5 and 7) of the rHMGA1. Free hIFNγP1 probe was used in lane 1.
promoter. Initial DNase I footprinting and EMSA have located two such sites within the first 110 bp from the transcription initiation site within the IFNG gene proximal promoter. Further characterisation suggests that the downstream site, lying within the proximal promoter, is essential but that the flanking sequence is required for optimal HMGA1 interaction to this region. Our ongoing work involves creating site-specific mutants, in order to better understand inter- and intramolecular interactions occurring within this HMGA1-responsive element.

In conclusion, this work establishes for the first time that HMGA1 controls IFNG gene transcription, and maps one candidate regulatory region. Thus, the hypothesis that HMGA1 was likely to participate in Tn1-specific gene expression appears to be correct. However, our work does not exclude the possibility that other putative HMGA1 binding sites exist upstream of the first 500 bp of the IFNG gene promoter. HMGA1 may also interact with other regulatory regions on the gene locus remote from the promoter, such as the IFNG intronic enhancer. This region is highly homologous to the ARRE-2 site on the intronic enhancer. This region is highly homologous to the upstream of the first 500 bp of the IFNG gene promoter, such as the IFNG intronic enhancer. Our ongoing work involves creating site-specific interactions occurring within this HMGA1-responsive element.

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Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| AP-1         | activated protein-1 |
| CAT          | chloramphenicol acetyl transferase |
| DTT          | dithiothreitol |
| EMSA         | electrophoretic mobility shift assays |
| GAPDH        | glyceraldehyde-3-phosphate dehydrogenase |
| GFP          | green fluorescent protein |
| HMG          | high mobility group |
| NFAT         | nuclear factor of activated T cells |
| PCA          | perichloric acid |
| PMA          | phorbol myristate acetate |
| TCM          | T cell medium |

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