High-mobility-group A1 (HMGA1) proteins down-regulate the expression of the recombination activating gene 2 (RAG2)

Sabrina BATTISTA*, Monica FEDELE*, Josefina Martinez HOYOS*, Francesca PENTIMALLI*, Giovanna Maria PIERANTONI*, Rosa VISONE*, Ivana De MARTINO*, Carlo Maria CROCE† and Alfredo FUSCO*‡[1]

*Dipartimento di Biologia e Patologia Cellulare e Molecolare e/o Istituto di Endocrinologia ed Oncologia Sperimentale del CNR, Facoltà di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli 'Federico II', via Pansini, 5, 80131 Naples, Italy, †Kimmel Cancer Center, Thomas Jefferson University, 233 S 10th Street, Philadelphia, PA 19107, U.S.A., and ‡NOGEC (Naples Oncogenomic Center)–CEINGE, Biotecnologie Avanzate, via Comunale Margherita 482, 80145, Naples, Italy

HMGA1 (high-mobility-group A1) proteins are architectural transcription factors that are found overexpressed in embryogenesis and malignant tumours. We have shown previously that they have a role in lymphopoiesis, since the loss of HMGA1 expression leads to an impairment of T-cell development and to an increase in B-cell population. Since RAGs (recombination activating genes) are key regulators of lymphoid differentiation, in the present study we investigate whether RAG2 expression is dependent on HMGA1 activity. We show that RAG2 gene expression is up-regulated in Hmga1−/− ES (embryonic stem) cells and EBs (embryoid bodies) as well as in yolk sacs and fibroblasts from Hmga1−/− mice, suggesting that HMGA1 proteins control RAG2 gene expression both *in vitro* and *in vivo*. We show that the effect of HMGA1 on RAG2 expression is direct, identify the responsible region in the RAG2 promoter and demonstrate binding to the promoter *in vivo* using chromatin immunoprecipitation. Since RAG2 is necessary for lymphoid cell development, our results suggest a novel mechanism by which HMGA1 might regulate lymphoid differentiation.

Key words: electrophoretic mobility-shift assay (EMSA), embryonic stem cell, high-mobility-group A1 (HMGA1), lymphopoiesis, MEF, recombination activating gene 2 (RAG2).

INTRODUCTION

HMGA1 (high-mobility-group A1) proteins are 'architectural transcription factors' capable of binding the minor groove of AT-rich DNA sequences and inducing the bending of DNA intermediates. HMGA1 proteins have been found overexpressed in many kinds of human malignancies and rearranged in benign tumours [1]. They seem to play a major physiological role during development and cell differentiation [1]. We showed previously that HMGA1 proteins play a pivotal role in lymphocyte differentiation [2]. In particular, we suggested that the loss of Hmga1 gene expression might force the B-cell/T-cell common lymphoid precursor to differentiate to B-lymphocytes rather than to T-lymphocytes, probably by regulating the expression levels of cytokines involved in B- and T-cell proliferation/differentiation. In fact, the loss of HMGA1 induces a decrease in interleukin-2 expression and an increase in interleukin-6 expression both *in vitro* and *in vivo* [2] (M. Fedele, V. Fidanza, S. Battista, F. Pentimalli, A. J. P. Klein-Szanto, R. Visone, I. De Martino, A. Curcio, C. Morisco, L. Del Vecchio, G. Baldassarre, C. Arra, G. Viglietto, C. Indolfi, C. M. Croce and A. Fusco, unpublished work). More strikingly, the lack of HMGA1 in homozygous knockout mice leads to the development of different B-cell neoplasias (M. Fedele et al., unpublished work), probably due to the alteration in B-cells/T-cells balance.

To investigate further the molecular mechanisms involved in HMGA1 regulation of lymphopoiesis, we analysed the expression of RAGs (recombination activating genes). RAG1 and RAG2 are key performers of the V(D)J recombination, through which the specific antigen receptors in lymphocytes are generated [3,4]. In particular, they initiate the process of recombination, introducing double-strand breaks in target sequences of the Ig and T-cell receptor genes. In the absence of either RAG1 or RAG2 gene product, the development of mature lymphocytes is completely abrogated, leading to immunodeficiency, both in mouse and humans [5–7]. On the other hand, to limit recombinase activity, their expression is tightly regulated both at the transcriptional and post-transcriptional levels [8,9]. At the transcriptional level, the alteration of chromatin structure in the 5′-region of RAG1 and RAG2 genes has been shown to be responsible for their tissue- and stage-specific regulation [10,11]. The RAG2 promoter is differently regulated in B- and T-cells [12]. Moreover, in the RAG2 promoter region, a 300 bp 5′-upstream region from the major transcription initiation site is conserved between mice and humans [12], indicating that this region is important for the promoter activity. Human RAG2 promoter has been shown to be activated both in lymphoid and non-lymphoid lineages [13]. A core promoter of mouse RAG2 confers lymphoid specificity and may be regulated by distinct transcription factors in B- (Pax-5) and T- (GATA-3 or c-Myb) cells [8,12,14]. It has been shown that the LEF-1−β-catenin complex regulates the RAG2 promoter activation, together with c-Myb and Pax-5 in immature B-cells [9]. In the present study, we report that lack of HMGA1 proteins, which are known to regulate lympho-specific genes, is associated with increased RAG2 expression in mouse ES (embryonic stem) cells. RAG2 up-regulation is also found in yolk sacs and MEFs (mouse embryonic fibroblasts) from Hmga1-null embryos. Conversely, introduction of an Hmga1-expressing construct into Hmga1−/− ES cells restores RAG2 gene expression at levels comparable with wild-type ES cells. Functional assays demonstrate that HMGA1 proteins are capable of repressing the RAG2 promoter in 293T cells and that the HMGA1 repressive activity is noticeably

Abbreviations used: c/EBP-β, CAAT/enhancer-binding protein β; CMV, cytomegalovirus; dpc, days post-coitum; EB, embryoid body; EMSA, electrophoretic mobility-shift assay; ES cell, embryonic stem cell; FBS, fetal bovine serum; HA, haemagglutinin; HMGA1, high-mobility-group A1; MEF, mouse embryonic fibroblast; MTG, monothioglycerol; RAG, recombination activating gene; RT, reverse transcriptase.

[1] To whom correspondence should be addressed, at Dipartimento di Biologia e Patologia Cellulare e Molecolare (email afusco@napoli.com).

increased by co-expression of c/EBP-β (CAAT/enhancer-binding protein β). Finally, we show that the repressive effect of HMGA1 on RAG2 promoter is due to a direct specific interaction of the architectural factor with the RAG2 promoter *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell cultures

Wild-type, Hmga1+/− and Hmga1−/− AB2.1 ES cells have been described in [2]. Hmga1−/−R and Hmga1−/−CMV (where CMV stands for cytomegalovirus) clones were generated by electroporating Hmga1−/− ES cells with 20 μg of pc-*Hmga1*/Hygro construct or the empty vector respectively [2]. Transgene expression was detected by Northern blotting and RT (reverse transcriptase)–PCR. ES cells were cultured on a layer of mytomicin D-inactivated fibroblasts. Before RNA extraction, fibroblasts were removed by three passages in 0.1 % gelatin-treated plates and the maintenance of the undifferentiated state was ensured by the addition of leukaemia inhibiting factor (10^3 units/ml; Chemicon, Temecula, CA, U.S.A.). MEFs were obtained from 12.5-day-old embryos. Cells were cultured at 37 °C (5 % CO_2) in Dulbecco's modified Eagle's medium containing 10 % (v/v) FBS (fetal bovine serum) supplemented with penicillin and streptomycin. The human embryonic kidney 293T cell line [14] was cultured in Dulbecco's modified Eagle's medium + 10 % FBS.

Differentiation of ES cells

Differentiation of ES cells in a methylcellulose-based medium has been described in [2]. Briefly, 48 h before differentiation, 2×10^5 ES cells were plated on gelatin-coated plates in Iscove's modified Dulbecco's medium supplemented with 15 % FBS, sodium pyruvate (1 mM), L-glutamine (2 mM) and non-essential amino acids (0.1 mM; Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.), MTG (monothioglycerol; 100 μM; Sigma), leukaemia inhibiting factor (10 ng/ml; Chemicon), penicillin G and streptomycin. Materials for differentiation were purchased from Stem Cell Technologies (Vancouver, BC, U.S.A.) unless otherwise specified. To obtain EBs (embryoid bodies), 2×10^3 ES cells were plated on low-adherence 35 mm Petri dishes as a single cell suspension in 'primary differentiation medium', constituted by 0.9 % methylcellulose in Iscove's modified Dulbecco's medium, 15 % FBS, L-glutamine (2 mM), MTG (150 μM) and murine stem cell factor (40 ng/μl). Feeding medium (0.5 % primary differentiation medium, 15 % FBS, 150 μM MTG and 160 ng/ml murine stem cell factor) was added after 7 days in culture and subsequently every 3–4 days.

Generation of Hmga1+/− and Hmga1−/− mice

Hmga1+/− and Hmga1−/− mice have been described in [2]. Briefly, Hmga1+/− ES cell clones were microinjected into 3.5 dpc (days post-coitum) C57BL/6J blastocysts and reimplanted into foster mothers (the Animal Facility in Thomas Jefferson University). Chimaeric mice were crossed with wild-type and some of them gave germline transmission. Single knockout mice were then intercrossed to obtain double knockout mice. Pregnant mothers were killed at 14.5 dpc and the embryo genotype was evaluated [2].

RT–PCR analyses of embryos, MEFs and ES cell cultures

Tissues from mice were rapidly dissected, frozen on solid CO_2 and stored at − 80 °C. Total RNA from embryos and cell cultures was extracted with TRI Reagent solution (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's instructions and treated with DNase I (GenHunter Corporation, Nashville, TN, U.S.A.). RNA (1 μg) was reverse-transcribed using a mixture of poly-dT and random exonucleotides as primers and MuLV RT (PerkinElmer, Boston, MA, U.S.A.). PCR amplifications were performed as described in [15] in a GeneAmp PCR System 9600. Primers for RAG2, Hmga1 and Gapdh have been described in [2,16]. Non-reverse-transcribed RNA was amplified (results not shown) to rule out the possibility of DNA amplification. The PCR products were separated on 2 % (w/v) agarose gel and, if necessary, blotted and hybridized with specific probes.

Plasmids

The pc-Hmga1/Hygro and pCEFL/HA-HMGA1 constructs (where HA stands for haemagglutinin) have been described in [2,17]. For the RAG2prom-luc construct, the region − 279/+ 21 of the mouse RAG2 gene [12] was amplified using the following primers: forward primer, 5′-ACGCGTAAGCTTAAGACAGTC-ATT-3′, containing an MluI restriction site, and reverse primer, 5′-CTCGAGCTGAAGGCTGCAGGGTAG-3′, containing an XhoI restriction site. The resulting fragment was subcloned into the pGL3 vector (Promega, Madison, WI, U.S.A.). C/EBP-β expression vector has been described in [17].

Production of recombinant proteins

Production of the recombinant HMGA1b–His protein has been described in [18]. Recombinant HMGA1b(1–53) is constituted by the first 53 amino acids spanning the first two AT-hook domains, whereas HMGA1b(54–96) contains the spacer region between the second and third AT-hook, the third AT-hook domain and the C-terminal region of the protein. The recombinant HMGA1 proteins were generated by cloning the full-length or truncated Hmga1b cDNAs in the pET2c (Novagen, Madison, WI, U.S.A.). BL21/DE3 cells transformed with each vector were grown in Luria–Bertani medium, induced with isopropyl β-D-thiogalactoside, sonicated and purified by using the His-Trap purification kit (Amersham Biosciences) according to the manufacturer's instructions. The proteins were dialysed and analysed by SDS/12.5 % PAGE.

EMSA (electrophoretic mobility-shift assay)

DNA-binding assays with the recombinant proteins were performed as described previously [19]. Briefly, 5 (14 nM) to 50 ng of wild-type recombinant protein or 5 ng of truncated proteins were incubated with radiolabelled double-strand oligonucleotides, corresponding to the region spanning bases 14–53 of the murine RAG2 promoter region (RAG2pr) (NCBI accession no. AF159439). The full-length protein was also assayed with truncated or mutated oligonucleotides (Figure 2B) representing different AT-rich segments of the RAG2pr region. For EMSA on mouse spleens, 8 μg of protein extracts from wild-type, Hmga1+/− and −/− adult spleens were incubated in a solution made up of 20 mM Hepes (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM PMSF, 1 μg of poly(dC-dG), 2 μg of BSA and 10 % (v/v) glycerol to a final volume of 20 μl for 10 min at room temperature (25 °C). The samples were incubated for 10 min after the addition of 2.5 fmol of a ^{32}P-end-labelled oligonucleotide (specific activity, 8000–20000 c.p.m./fmol). In some experiments, a 100-fold molar excess of unlabelled oligonucleotide was added as a specific competitor. For antibody competition analyses, extracts were preincubated with 0.5 μg of

anti-HMGA1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or an unrelated antibody (anti-Pit-1; Santa Cruz Biotechnology) on ice for at least 30 min. The Sp1 oligonucleotide was purchased from Santa Cruz Biotechnology. The DNA–protein complexes were resolved on 6 % (w/v) non-denaturing acrylamide gels and visualized by exposure to autoradiographic films.

Chromatin immunoprecipitation

Briefly, approx. 3×10^7 wild-type, Hmga1+/− and Hmga1−/− ES cells were grown on 75 cm^2 dishes. Chromatin immunoprecipitation was performed as described in [2,20] using antibodies binding to the N-terminal region of the HMGA1 proteins [21] and not reactive to other members of the HMGA family. Input DNA (500 ng) and immunoprecipitated DNAs were analysed by PCR for the presence of RAG2 promoter sequences or the prolipase promoter region, as negative control. PCRs were performed with AmpliTaq gold DNA polymerase (PerkinElmer). The primers used to amplify the sequence of RAG2 promoter were: forward, 5′-AAGCTTAAGACAGTCATT-3′; and reverse, 5′-CTGAAGGCTGCAGGGTAG-3′. Primers for prolipase promoter were: forward, 5′-ACCAAAGTGTCAAGGGCAAC-3′; and reverse, 5′-ATTCCCTAAACCCAGCATCC-3′. PCR products were resolved on a 2 % agarose gel, stained with ethidium bromide and scanned using a Typhoon 9200 scanner.

Transient transfections

Before transient transfections in ES cells, feeder fibroblasts were removed as described above. A total of 4×10^5 wild-type or double knockout ES cells or 293T cells were plated on 6-well plates and transfected after 48 h with 1 μg of reporter plasmid (either RAG2prom-luc or pGL3), by FuGene6 (Roche, Indianapolis, IN, U.S.A.). Where indicated, 3 μg of HA–Hmga1 and/or 3 μg of c/EBP-β were co-transfected. Cells were harvested 48 h post-transfection and lysates were analysed for luciferase activity. Transfection efficiency was normalized using the β-galactosidase activity, and fold activation was calculated by dividing by pGL3 luciferase activity. All the assays were performed in triplicate and repeated in three independent experiments.

RESULTS

Loss of HMGA1 is correlated with an increased RAG2 expression in ES cells

We previously generated Hmga1−/− mouse ES cells and showed that their ability to differentiate in lymphohaematopoietic lineages is greatly compromised [2]. In particular, we showed that the T-cell population is decreased, whereas the B-cell population is increased in Hmga1−/− ES cells, yolk sacs and fetal livers compared with wild-type. To investigate further the role that HMGA1 proteins play in B-cell/T-cell differentiation, we analysed the expression of RAG2, a lymphoid-specific gene, by RT–PCR analyses. As shown in Figure 1(A), we detected an 8-fold increase in RAG2 expression in Hmga1−/− ES cells compared with wild-type and single knockout ES cells and a 2.5-fold increase in ES cell-derived Hmga1−/− EBs compared with wild-type (lanes 6 and 7). No band was detected when the RNA was not reverse-transcribed before amplification (results not shown).

To verify that the lack of HMGA1 was responsible for RAG2 up-regulation, we transfected the pc-Hmga1/Hygro construct in double knockout ES cells [2]. We verified the rescue of Hmga1 expression in some clones (−/−R1) by RT–PCR (Figure 1A). A decreased RAG2 expression was observed in Hmga1-transfected cells (−/−R1) compared with Hmga1 null cells and

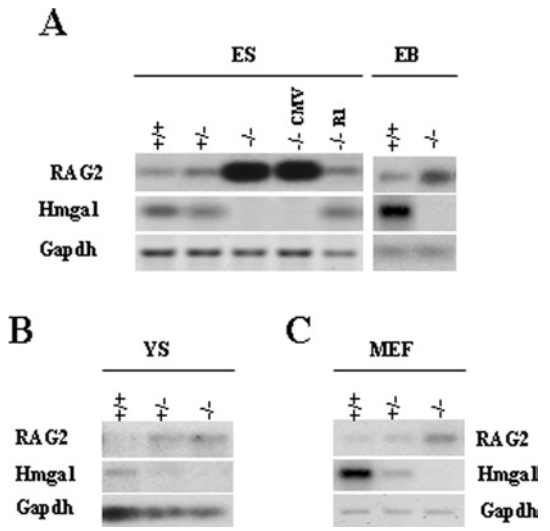

Figure 1 RAG2 expression in wild-type and Hmga1 knockout ES cells, EBs, yolk sacs and MEFs

RT–PCR analyses for RAG2 and Hmga1 expression were performed on RNA extracted from (**A**) ES cells and EBs; (**B**) yolk sacs (YS); and (**C**) MEF. Gapdh expression was evaluated as internal control. −/− CMV and −/− R1 indicate Hmga1−/− ES cells transfected with the empty vector and with the pc-Hmga1-expressing construct respectively.

Hmga1−/− cells transfected with the empty vector (−/−CMV) (compare lanes 3, 4 and 5 in Figure 1A). These results suggest that HMGA1 proteins regulate RAG2 gene expression in ES cells and in ES cell-derived EBs. Moreover, when the mammary epithelial cells MCF-7 were stably transfected with the Hmga1/Hygro construct (results not shown), the expression of RAG2 was down-regulated, suggesting that HMGA1 proteins induce RAG2 down-regulation in different cell types.

Loss of HMGA1 determines up-regulation of RAG2 gene expression *in vivo*

We next investigated RAG2 expression *in vivo*. We analysed RAG2 expression in 14.5 dpc yolk sacs from wild-type, Hmga1+/− and −/− embryos. As shown in Figure 1(B), RAG2 expression was sensibly higher in Hmga1-null and heterozygous yolk sacs, compared with wild-type. RT–PCR analyses for Hmga1 expression were performed as a control of the genotype of the tissues analysed (Figure 1B, middle panel). Moreover, RAG2 overexpression was observed also in the spleen from Hmga1−/− adult mice, compared with wild-type (M. Fedele et al., unpublished work), indicating that HMGA1 affects RAG2 expression both *in vitro* and *in vivo*. Next, we investigated whether lack of HMGA1 affects RAG2 expression in non-lymphoid cells. Again, RAG2 expression in MEFs from wild-type, Hmga1+/− and −/− embryos was inversely related to HMGA1 expression (Figure 1C).

These results indicate that RAG2 expression is inversely related to Hmga1 expression both *in vitro* and *in vivo*, suggesting a suppressive role for the HMGA1 proteins in the regulation of RAG2 transcription.

HMGA1 proteins directly bind the RAG2 promoter

To investigate whether HMGA1 proteins are directly involved in RAG2 transcriptional regulation, we evaluated the HMGA1 DNA-binding activity to the RAG2 promoter. In particular, we analysed a region spanning nt 14–53 of the murine RAG2 promoter

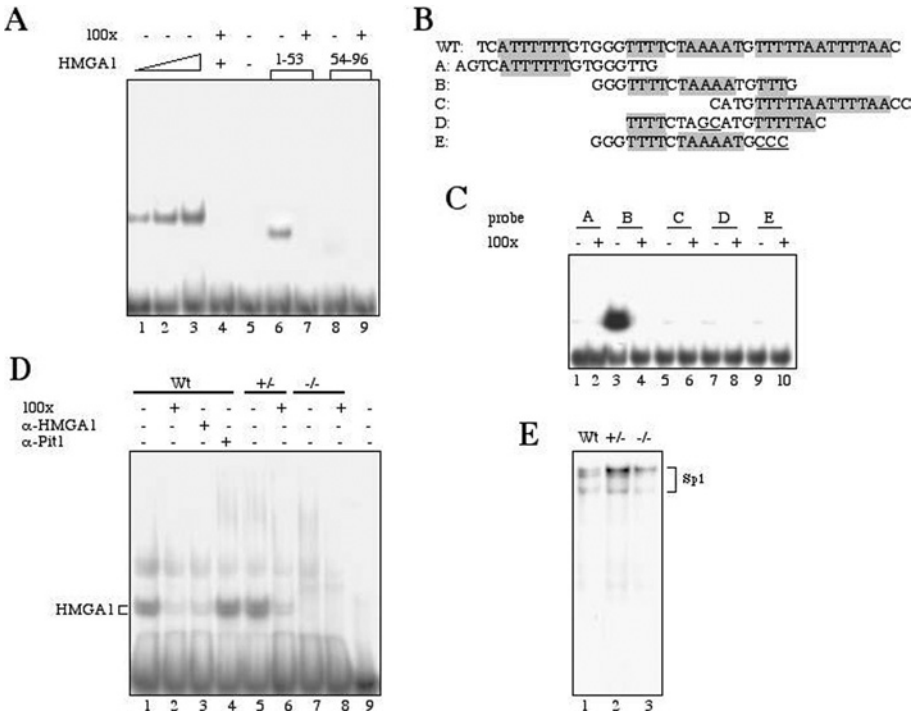

Figure 2 HMGA1 binding to RAG2 upstream regulating region

(**A**) EMSA was performed by incubating the radiolabelled wild-type RAG2 promoter oligonucleotide (RAG2pr) with 5 ng (lanes 1 and 4), 20 ng (lane 2) or 50 ng (lane 3) of the recombinant full-length HMGA1b–His protein or with 5 ng of truncated HMGA1b(1–53) (lanes 6 and 7) or HMGA1b(54–96) (lanes 8 and 9) proteins. Where indicated, a 100 times molar excess of unlabelled RAG2pr oligonucleotide was incubated as a specific competitor. (**B**) Sequences of RAG2 promoter oligonucleotides used in electrophoretic binding assays. Hypothetical HMGA1-binding sites are boxed in grey. WT, the wild-type sequence. Oligonucleotides A–C are shorter wild-type sequences, encompassing a few hypothetical HMGA1-binding regions. In oligonucleotides D and E, some nucleotides were mutated (underlined). (**C**) EMSA was performed with deleted or mutated oligonucleotides. The probes used (A–E) are the same as in (**B**). Full-length HMGA1b–His protein (5 ng) was incubated with the indicated probes (A–E) in the presence (+) or absence (–) of a 100 times molar excess of the corresponding unlabelled oligonucleotides, as specific competitor. (**D**) EMSAs were performed by incubating 8 μg of protein extracts from wild-type (wt), Hmga1+/– and –/– mouse spleens with the RAG2pr probe. Where indicated, the samples were preincubated either with anti-HMGA1 (α-HMGA1) or unrelated antibodies [anti-Pit-1 (α-Pit-1)] or with a 100 times molar excess of unlabelled RAG2pr. Two main specific complexes, corresponding to isoforms a an b of HMGA1, were observed in wt and Hmga1+/– extracts, whereas no binding activity was detected in Hmga1–/– extracts. (**E**) EMSA was performed with the same extracts as in (**D**), incubated with a probe corresponding to the Sp1 consensus sequence to normalize the amount of protein extracts.

[12] (Rag2pr) and containing four AT-rich putative HMGA1-binding sites (Figure 2B). As shown in Figure 2(A), increasing amounts (5, 20 and 50 ng) of a recombinant HMGA1 protein [18] were capable of binding the ^{32}P-end-labelled double-strand oligonucleotide in EMSA. This binding was specific, as demonstrated by competition with 100-fold molar excess of unlabelled RAG2pr oligonucleotide (lane 4) and by lack of competition when an unrelated unlabelled oligonucleotide was used as competitor (results not shown). To evaluate the regions of HMGA1 proteins involved in binding to the RAG2 promoter, we performed EMSAs by incubating 5 ng of truncated recombinant HMGA1 proteins with the RAG2pr probe. We demonstrated that the HMGA1 DNA-binding activity is due to the first two AT-hook domains, since HMGA1b(1–53), containing the first two AT-hook domains, was capable of binding RAG2pr, whereas HMGA1b(54–96), containing the spacer region between the second and third AT-hook, the third AT-hook domain and the C-terminal region of the protein was not (compare lanes 6 and 8). To map better the preferential HMGA1-binding sites on the RAG2 promoter, we assayed three shorter oligonucleotides, oligos A–C (Figure 2B), representing three different AT-rich segments of the RAG2 promoter. As shown in Figure 2(C), HMGA1 binds oligo B with high affinity, whereas the binding to oligos A and C is almost undetectable (lanes 1 and 5). Binding specificity was demonstrated by competition experiments after the addition of a 100-fold molar excess of unlabelled oligo B (lane 4). These results restrict the HMGA1-binding region to the central part of the sequence, which retains three of four putative HMGA1-binding sites (TTTT, AAAA and TTT). To identify which site is responsible for the binding, we mutated the A-stretch region (oligo D): we found that the HMGA1 protein was no longer able to bind the sequence (lane 7), demonstrating that the A-stretch is important for the binding. On the other hand, when the downstream T-stretch was mutated (oligo E), the A-stretch was not sufficient to carry on the binding (lane 9), showing that both the A-stretch and the downstream T-stretch are necessary for the binding (Figure 2C). Conversely, the upstream T-stretch does not consistently co-operate with the A-stretch for the binding, since oligo E contains both the upstream T- and A-stretches, but does not show a significant binding to HMGA1.

To verify the binding of HMGA1 to RAG2 promoter also in mouse tissues, we assayed the DNA-binding activity of total protein extracts from spleens of wild-type, heterozygous and knock-out mice to the RAG2pr probe. As shown in Figure 2(D), two specific complexes, with a mobility corresponding to isoforms a and b of HMGA1 proteins, were present in extracts from both wild-type and heterozygous spleens, whereas they were absent from extracts derived from homozygous Hmga1 knockout spleens. These complexes were specifically displaced by incubation with an antibody directed against the HMGA1 proteins (compare lanes 3 and 1), but not by an unrelated antibody (lane 4), showing that they do consist of HMGA1 proteins. Binding activity was normalized using an oligonucleotide probe for the ubiquitous Sp1 transcription factor (Figure 2E).

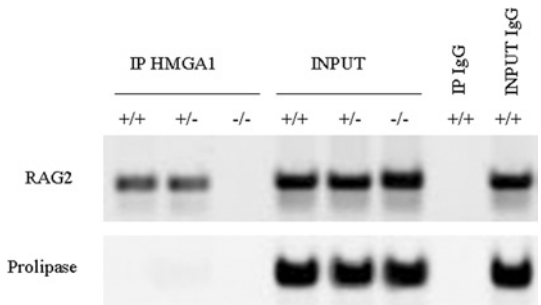

Figure 3 ***In vivo*** **binding of HMGA1 proteins to the RAG2 promoter region**

Chromosomes and nuclear proteins from Hmga1+/+, +/− and −/− ES cells were cross-linked and immunoprecipitated (IP) with anti-HMGA1 antibodies. The presence of the −279/+21 sequence of the RAG2 promoter was detected by PCR. INPUT indicates PCR products with chromosomal DNA without immunoprecipitation. As an immunoprecipitation control, IgG was used (lane 7). The lower panel shows PCR amplification of the immunoprecipitated DNA using primers for the prolipase gene promoter.

In conclusion, these results indicate that HMGA1 proteins are capable of binding directly a specific sequence in the RAG2 promoter region and that the normal spleens contain a binding activity that is lost in Hmga1−/− spleens.

To verify whether HMGA1 proteins bind the −297/+21 RAG2 promoter region *in vivo*, we performed chromatin immunoprecipitation experiments in Hmga1+/+, +/− and −/− ES cells (Figure 3). Anti-HMGA1 antibodies precipitated the −297/+21 RAG2 promoter region from Hmga1+/+ and +/− ES cells, but not from Hmga1−/− ES cells (Figure 3). The RAG2 promoter was immunoprecipitated by anti-HMGA1 antibodies, whereas no precipitation was observed with normal rabbit IgGs (lane 7); moreover, when primers for a control promoter (prolipase) were used, no band was detected (Figure 3, lower panel), suggesting that the reaction is specific for the RAG2 promoter. The results indicate that HMGA1 proteins bind the RAG2 promoter region *in vivo*.

HMGA1 proteins repress RAG2 promoter activity in functional assays

To investigate the effect of HMGA1 proteins on RAG2 promoter, we transiently transfected wild-type and double knockout ES cells with a construct (RAG2prom-luc) expressing the luciferase gene under the control of the mouse RAG2 promoter region, −279 to +21. The region spanning −279 to +123, conserved between mice and humans, has been shown to be necessary for maximal activity of RAG2 promoter [12]. As shown in Figure 4(A), Hmga1−/− ES cells showed a 2-fold increase in RAG2 promoter activity compared with wild-type.

We next transfected 293T cells with the RAG2prom-luc reporter construct. As observed previously [12], the promoter showed extremely low but reproducible activity in non-lymphoid cell lines, such as 293T. However, when the HMGA1 expression vector was transfected, a decrease in the luciferase activity was observed (Figure 4B). We showed previously that c/EBP-β co-operates with HMGA1 in activating the leptin gene promoter [17] by direct interaction with HMGA1. At least two binding motifs for c/EBP-β are present in the promoter region of the human RAG2 gene (at −146 to −138 and at −137 to −129) and mutations in the −137 to −129 region abrogate promoter activity [22]. In the mouse RAG2 promoter, a c/EBP-β consensus sequence is located at −154 to −146. To evaluate whether c/EBP-β and HMGA1 co-operate in regulating RAG2 promoter activity, we co-transfected a construct expressing c/EBP-β together with the HMGA1 expression construct: the co-transfection of c/EBP-β, together with Hmga1, induced a further decrease of luciferase activity (Figure 4B), whereas c/EBP-β by itself induced just a slight decrease.

These results indicate that HMGA1 proteins negatively regulate the RAG2 promoter in different cell types and that c/EBP-β is capable of co-operating with HMGA1 in repressing RAG2 expression.

DISCUSSION

The aim of the present study was to investigate the role of HMGA1 proteins in the regulation of RAG2 gene expression. The rationale for this study was our previous observation that disruption of the Hmga1 gene leads to alterations of lymphopoiesis [2].

The results presented here demonstrate that Hmga1−/− ES cells express higher levels of RAG2 and that increased RAG2 expression also occurs in MEFs and yolk sacs of Hmga1−/− mice. The increase in RAG2 expression is due to the lack of HMGA1 proteins, since the introduction of an Hmga1-expressing construct brings RAG2 expression to levels comparable with wild-type ES

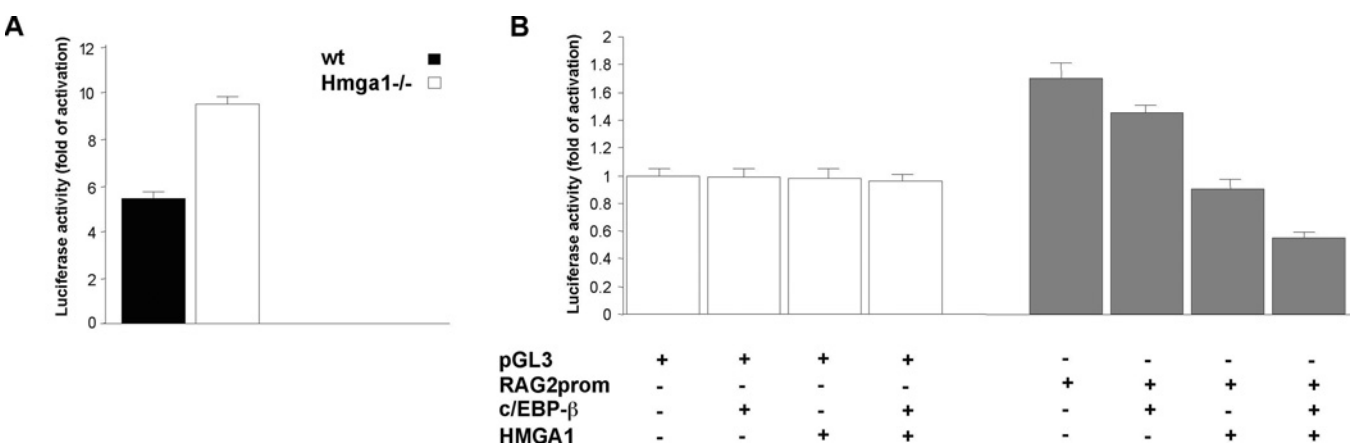

Figure 4 Functional assays of RAG2 promoter activity

Luciferase activity (fold activation) of RAG2 promoter in Hmga1+/+ and −/− ES cells (**A**) and in 293T cells (**B**). Where indicated, 3 μg of HMGA1 and/or 1 μg of c/EBP-β expression vectors were co-transfected.

cells. In support of this statement, we show that the HMGA1 proteins bind to the RAG2 promoter, *in vitro* and *in vivo*, by EMSA and chromatin immunopreciptiation assays respectively. This suggests a direct regulation of RAG2 by HMGA1. We conclude that the binding is mediated by two specific A and T adjacent stretches on the RAG2 promoter and by the first two AT-hook domains of the HMGA1 proteins. Moreover, we demonstrate that an HMGA1-expressing construct is capable of repressing the activity of a RAG2 promoter-driven luciferase gene in functional assays. We also show that the HMGA1 repressive activity is increased by the co-expression of c/EBP-β, a transcription factor already known to co-operate with HMGA1 in the regulation of other promoters [17] and possibly affecting human RAG2 promoter activity [22]. The slight decrease in gene expression, exerted by HMGA1 in transient transfections, matches with transient assays on other HMGA1-responsive promoters, where the light stimulating effect of HMGA1 is potentiated by co-operating transcription factors [23,24]. Together, these results demonstrate that HMGA1 proteins down-regulate RAG2 expression. On the other hand, the repressive activity of HMGA1 proteins on RAG2 promoter does not seem to be exerted through the down-regulation of other factors involved in RAG2 transcription such as Pax-5, c-Myb and GATA-3, since their expression is unchanged in Hmga1−/− ES cells compared with wild-type (results not shown). Since RAG2 plays a major role in B-cell/T-cell differentiation, the HMGA1-mediated down-regulation of RAG2 expression suggests a possible mechanism by which loss of HMGA1 expression may lead to an impairment of lymphoid differentiation *in vitro*.

Given the role of architectural transcription factor ascribed to HMGA1 (capable of recognizing the DNA structure rather than sequence), a probable mechanism by which HMGA1 proteins exert their action on the RAG2 promoter might be in opening the chromatin structure of RAG2 promoter region and improving its accessibility to appropriate transcriptional factors. Similar mechanisms have been already described for transcriptional regulation of the RAG1 gene [10]. Consistent with our findings, it has been recently reported that another member of the HMG-box family, LEF-1 (lymphoid enhancer-binding factor-1), binds the −41/−17 RAG2 promoter region and, together with c-Myb and Pax-5, is capable of activating the RAG2 promoter [9].

The negative regulation of RAG2 by HMGA1 might have quite important implications since an increased RAG2 expression may induce a higher V(D)J recombination and, in the absence of an appropriate cell-cycle checkpoint, lead to increased susceptibility to develop neoplasias of the lymphoid tissues. RAG proteins may also mediate the insertion of cleaved recombination signals into new DNA sites [25] and this mechanism has been proposed to be responsible for certain types of DNA translocation associated with lymphatic tumours. Interestingly, Hmga1−/− mice develop B-cell lymphomas, characterized by a high frequency of aberrant V(D)J rearrangements in the *IgH* gene (M. Fedele et al., unpublished work).

Since RAG2 activates double-strand breaks, our results suggest that HMGA1 proteins may have an indirect role in regulating processes such as double-strand breaks and recombination. We previously showed that HMGA1 is inherently involved in the down-regulation of the DNA repair protein BRCA-1 [20] and that this function is converted into a greater ability of Hmga1−/− ES cells to repair cisplatinum-induced DNA breaks [26]. On the other hand, HMGA1 can bind a fourway (Holliday) junction DNA, an intermediate structure formed by DNA during recombination processes [27,28], competing for its binding with histone H1 and HMG1 [29]. Consistently, a possible involvement of HMGA1 in *in vivo* processes such as genetic recombination, DNA repair and chromosome rearrangements have been proposed [28]. Together, these observations suggest that HMGA1 might have a specific and pleiotropic role at different steps of DNA break repair and recombination. Other than an indirect role (such as down-regulation of BRCA1 and RAG2), it might play a direct role in binding and bending of DNA, allowing the apposition of sequences to be recombined.

It has been shown that inactivating mutations in either RAG1 or RAG2 are responsible for the so-called 'Omen syndrome', in which no circulating mature B-cells are found, while a large number of poorly functional T-lymphocytes could be detected [30]. Conversely, overexpression of RAG proteins may contribute to some cases of human immunodeficiency [31] and lymphocytic leukaemia. Interestingly, Hmga1-null mice develop B-cell lymphomas (M. Fedele et al., unpublished work), whereas those overexpressing the full-length construct develop T-cell lymphomas [18]. Therefore it can be hypothesized that the impairment of the HMGA1 function might be responsible for some cases of human immunodeficiency.

In conclusion, the results presented here demonstrate that HMGA1 down-regulates the RAG2 promoter and suggest an additional mechanism for the modulation of lymphopoiesis by HMGA1 proteins.

This study was supported by Associazione Italiana per la Ricerca sul Cancro, by Programma Italia-USA sulla Terapia dei Tumori co-ordinated by Professor C. Peschle and by MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica) Terapie antineoplastiche innovative. We are grateful to D. R. Keene (Micro-Imaging Center, Shriners Hospital for Children, Portland, OR, U.S.A.) for a revision of this paper.

REFERENCES

1 Fedele, M., Battista, S., Manfioletti, G., Croce, C. M., Giancotti, V. and Fusco, A. (2001) Role of the high mobility group A proteins in human lipomas. Carcinogenesis **22**, 1583–1591
2 Battista, S., Pentimalli, F., Baldassarre, G., Fedele, M., Fidanza, V., Croce, C. M. and Fusco, A. (2003) Loss of *hmga1* gene function affects embryonic stem cell lympho-hematopoietic differentiation. FASEB J. **17**, 1496–1498
3 Weaver, D. T. (1995) V(D)J recombination and double-strand break repair. Adv. Immunol. **58**, 29–85
4 Fugmann, S. D., Lee, A. I., Shockett, P. E., Villey, I. J. and Schatz, D. G. (2000) The RAG proteins and V(D)J recombination: complexes, ends, and transposition. Annu. Rev. Immunol. **18**, 495–527
5 Shinkai, Y., Rathbun, G., Lam, K. P., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M. et al. (1992) RAG-2 deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangment. Cell (Cambridge, Mass.) **68**, 855–867
6 Schwarz, K., Gauss, G. H., Ludwig, L., Pannicke, U., Li, Z., Lindner, D., Friedrich, W., Seger, R. A., Hansen-Hagge, T. E., Desiderio, S. et al. (1996) RAG mutations in human B cell-negative SCID. Science **274**, 97–99
7 Villa, A., Santagata, S., Bozzi, F., Giliani, S., Frattini, A., Imberti, L., Gatta, L., Ochs, H., Schwarz, K., Notarangelo, L. D. et al. (1998) Partial V(D)J recombination activity leads to Omen syndrome. Cell (Cambridge, Mass.) **98**, 885–896
8 Kishi, H., Wei, X.-C., Jin, Z.-X., Fujishiro, Y., Nagata, T., Matsuda, T. and Muraguchi, A. (2000) Lineage-specific regulation of the murine RAG-2 promoter: GATA-3 in T cells and Pax-5 in B cells. Blood **95**, 3845–3852
9 Jin, Z. X., Kishi, H., Wei, X. C., Matsuda, T., Saito, S. and Muraguchi, A. (2002) Lymphoid enhancer-binding factor-1 binds and activates the recombination-activating gene-2 promoter together with c-Myb and Pax-5 in immature B cells. J. Immunol. **169**, 3783–3792
10 Kitagawa, T., Mori, K., Kishi, H., Tagoh, H., Nagata, T., Kurioka, H. and Muraguchi, A. (1996) Chromatin structure and transcriptional regulation of human RAG-1 gene. Blood **88**, 3785–3791
11 Wei, X. C., Kishi, H., Jin, Z. X., Zhao, W. P., Kondo, S., Matsuda, T., Saito, S. and Muraguchi, A. (2002) Characterization of chromatin structure and enhancer elements for murine recombination activating gene-2. J. Immunol. **169**, 873–871
12 Lauring, J. and Schlissel, M. S. (1999) Distinct factors regulate the murine RAG2 promoter in B- and T-cell lines. Mol. Cell. Biol. **19**, 2601–2612

13 Zarrin, A., Fong, I., Malkin, L., Marsden, P. and Berinstein, N. (1997) Cloning and characterization of the human recombination activating gene 1 (RAG1) and RAG2 promoter regions. J. Immunol. **159**, 4382–4394
14 Wang, Q.-F., Lauring, J. and Schlissel, M. (2000) c-Myb binds to a sequence in the proximal region of the RAG-2 promoter and is essential for promoter activity in T-lineages. Mol. Cell. Biol. **20**, 9203–9211
15 Battista, S., Fidanza, V., Fedele, M., Klein-Szanto, J. P., Outwater, E., Brunner, H., Santoro, M., Croce, C. M. and Fusco, A. (1999) The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. Cancer Res. **59**, 4793–4797
16 Potocnik, A. J., Nielsen, P. J. and Eichmann, K. (1994) *In vitro* generation of lymphoid precursors from embryonic stem cells. EMBO J. **13**, 5274–5283
17 Melillo, R. M., Pierantoni, G. M., Scala, S., Stella, A., Battista, S., De Biasio, M. C., Fedele, M., Chiappetta, G., Fidanza, V., Condorelli, G. et al. (2001) Critical role of the HMGI(Y) proteins in adipocytic cell growth and differentiation. Mol. Cell. Biol. **21**, 2485–2495
18 Baldassarre, G., Fedele, M., Battista, S., Vecchione, A., Klein-Szanto, A. J., Santoro, M., Waldmann, T. A., Azimi, N., Croce, C. M. and Fusco, A. (2001) Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway. Proc. Natl. Acad. Sci. U.S.A. **98**, 7970–7975
19 Thanos, D. and Maniatis, T. (1995) Virus induction of human IFN β gene expression requires the assembly of an enhanceosome. Cell (Cambridge, Mass.) **83**, 1091–1100
20 Baldassarre, G., Battista, S., Thakur, S., Belletti, B., Pentimalli, F., Trapasso, F., Fedele, M., Pierantoni, M. G., Croce, C. M. and Fusco, A. (2003) Negative regulation of BRCA1 gene expression by HMGA1 proteins accounts for the reduced BRCA1 protein levels in sporadic breast carcinoma. Mol. Cell. Biol. **23**, 2225–2238
21 Chiappetta, G., Berlingieri, M. T., Manfioletti, G., Santoro, M., Bandiera, A., Visconti, R., Giancotti, V. and Fusco, A. (1995) The expression of the high mobility group HMGI (Y) proteins correlates with the malignant phenotype of human thyroid neoplasms. Oncogene **10**, 1307–1314
22 Fong, I. C., Zarrin, A. A., Wu, G. E. and Berinstein, N. L. (2000) Functional analysis of the human RAG2 promoter. Mol. Immunol. **37**, 391–402
23 John, S., Reeves, R., Lin, J., Child, R., Leiden, J. M., Thompson, C. B. and Leonard, W. J. (1995) Regulation of cell-type-specific interleukin-2 receptor-chain gene expression: potential role of physical interaction between Elf-1, HMG-I(Y), and NF-B family proteins. Mol. Cell. Biol. **15**, 1786–1796
24 Yie, J., Liang, S., Merika, M. and Thanos, D. (1997) Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter. Mol. Cell. Biol. **17**, 3649–3662
25 Hiom, K., Melek, M. and Gellert, M. (1998) DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocation. Cell (Cambridge, Mass.) **94**, 463–470
26 Baldassarre, G., Belletti, B., Battista, S., Nicoloso, M. S., Pentimalli, F., Fedele, M., Croce, C. M. and Fusco, A. (2005) HMGA1 protein expression sensitizes cells to cisplatin-induced cell death. Oncogene, in the press
27 Zlatanova, J. and van Holde, K. (1998) Binding to four-way junction DNA: a common property of architectural proteins? FASEB J. **12**, 421–431
28 Hill, D. A., Pedulla, M. L. and Reeves, R. (1999) Directional binding of HMG-I(Y) on four-way junction DNA and the molecular basis for competitive binding with HMG-1 and histone H1. Nucleic Acids Res. **27**, 2135–2144
29 Varga-Weisz, P., von Holde and Zatlanova, J. (1994) Competition between linker histones and HMG1 for binding to four-way junction DNA: implications for transcription. Biochem. Biophys. Res. Commun. **203**, 1904–1911
30 Wada, T., Takei, K., Kudo, M., Shimura, S., Kasahara, Y., Koizumi, S., Kawa-Ha, K., Ishida, Y., Imashuku, S., Seki, H. et al. (2000) Characterization of immune function and analysis of RAG gene mutations in Omen syndrome and related disorders. Clin. Exp. Immunol. **119**, 148–155
31 Abe, T., Tsuge, I., Kamachi, Y., Torii, S., Utsumi, K., Akahori, Y., Ichihara, Y., Kurosawa, Y. and Matsuoka, H. (1994) Evidence for defects in V(D)J rearrangements in patients with severe combined immunodeficiency. J. Immunol. **152**, 5504–5513

Received 20 September 2004/25 January 2005; accepted 14 February 2005
Published as BJ Immediate Publication 16 February 2005, DOI 10.1042/BJ20041607