The immunoglobulin heavy chain enhancer, or $\mu$ enhancer, is required for B cell development. Only the appropriate combination of transcription factors results in B cell-specific enhancer activation. HMGA1 (formerly HMG-I(Y)) is a co-activator of the ETS transcription factors required for $\mu$ enhancer activity. HMGA1 associates with the ETS factor PU.1, resulting in changes in PU.1 structure, and enhanced transcriptional synergy with Ets-1 on the $\mu$ enhancer in non-lymphoid cells. New data show HMGA1 directly interacts with Ets-1 in addition to PU.1. In vitro HMGA1/Ets-1 interaction facilitates Ets-1/ $\mu$ enhancer binding in the absence of an HMGA1/Ets-1-DNA complex. To address whether HMGA1 is present in the transcriptionally active $\mu$ nucleoprotein complex, we completed DNA pull-down assays to detect protein tethering in the context of protein/DNA interaction. Results show that HMGA1 is not tightly associated with $\mu$ enhancer DNA through PU.1 or Ets-1, despite strong associations between these proteins in solution. However, chromatin immunoprecipitation assays show HMGA1 associates with the endogenous enhancer in B cells. Furthermore, antisense HMGA1 substantially decreases $\mu$ enhancer activity in B cells. Taken together, these data suggest that HMGA1 functions as a transcriptional $\mu$ enhancer co-activator in B cells through indirect association with DNA.

B lymphocyte development is critically dependent on the immunoglobulin heavy chain intronic enhancer, or $\mu$ enhancer. The $\mu$ enhancer is required for V- to (D)J rearrangement of the immunoglobulin heavy chain (1, 2) and subsequent tissue-specific transcription of the properly rearranged locus (3, 4). $\mu$ enhancer transcriptional activity has been extensively analyzed in the context of a three-element minimal enhancer, containing the $\mu$A, $\mu$E3, and $\mu$B sites necessary for activity in B cells (5). Functional analysis of the minimal enhancer has demonstrated that transcriptional activation requires combinatorial activity of transcription factors binding the three elements in a precise stereochemistry (6, 7).

Two proteins that are required for synergistically activating the $\mu$ enhancer, PU.1 and Ets-1, are members of the ets family of transcription factors. PU.1 and Ets-1 activate transcription by binding to the $\mu$B or $\mu$A sites, respectively. The third minimal enhancer activator is the basic helix-loop-helix leucine zipper protein TFE3 that binds to the $\mu$E3 site of the enhancer. Arguably, the transcription factor CBF may instead occupy an overlapping site and activate the enhancer in lieu of TFE3 (8). Of these critical $\mu$ enhancer activators, only PU.1 is restricted to the hematopoietic lineage (9, 10). The importance of PU.1 in B cell development is highlighted in PU.1 null mice, which lack B cells (11, 12). In the absence of Ets-1 (13, 14) or TFE3 (15), B cell development is apparently normal. However, B cell activation and plasma cell development are perturbed in these mice (13-15), indicating that each of these proteins is key for terminal B lineage differentiation after exit from the bone marrow.

It has been previously demonstrated that the transcriptional co-activator HMGA1 (formerly designated HMG-I(Y)) forms a protein-protein interaction with PU.1 in solution (16, 17). HMGA1a (formerly HMG-I) and HMGA1b (formerly HMG-Y) are splice variants of a single gene. Both proteins contain peptide sequences designated A-T hooks, which are involved in DNA binding and have multiple surfaces capable of protein-protein interactions (18). In many cases HMGA1 co-activates transcription by aiding assembly of a large nucleoprotein complex. Perhaps the best-characterized transcriptional nucleoprotein complex, or enhanceosome, is dependent on sequence-specific HMGA1 binding to the inducible interferon $\beta$ (IFN-$\beta$)1 enhancer (19, 20). HMGA1 binding alters IFN-$\beta$ DNA structure, decreasing the activation energy required for the transcription factors NF-$\kappa$B and ATF/c-jun to bind (19). HMGA1 apparently serves a similar function in many other promoters and enhancers. For example, HMGA1 is necessary for increased binding of c-rel (21) and the AP-1 complex (22) to the interleukin-2 promoter. HMGA1 also facilitates formation of a transcriptional complex at the interleukin-2 receptor promoter/enhancer through both DNA and protein-protein interactions (23, 24). In contrast, HMGA1 does not directly bind $\mu$ enhancer DNA (16); therefore, the mechanism of action at the aforementioned promoters/enhancers cannot be applicable to demonstrated co-activation of the $\mu$ enhancer by HMGA1 (16).

Recent work (16) has begun to uncover the novel mechanisms by which HMGA1 co-activates the $\mu$ enhancer. HMGA1 first interacts with the critical $\mu$ enhancer regulator PU.1 in

* This work was supported by NIH AI54611, The Evans Medical Foundation, an Arthritis Foundation grant (to B. N.), and NIH T32-CA64070 (to K. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work is dedicated to the memory of Glenn Harris, a longtime mentor and friend.

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solution (16, 17). This interaction results in increased PU.1/µ enhancer binding likely through an HMGAI-induced change in PU.1 structure. This increased binding manifests itself in the ability of HMGAI to potentiate PU.1/Ets-1 functional synergy on the µ enhancer. Overall, the data suggest that HMGAI activates the µ enhancer through an indirect mechanism. Because these assays were completed in non-lymphoid cells, we questioned whether HMGAI is a co-activator of the µ enhancer in B cells. The current analysis begins to address the role HMGAI plays in enhancing the enhancer in B cells.

To determine whether HMGAI playing a role in assembling the µ enhancerosome versus activating the enhancer through other mechanisms, we have extended our initial analysis to detail how HMGAI interacts with other minimal µ enhancer activators, TFE3 and Ets-1. HMGAI increases TFE3/µE3 binding (25) reminiscent of the effect HMGAI has on PU.1. New data demonstrate that HMGAI also forms protein-protein interactions with Ets-1 and facilitates Ets-1/µ enhancer binding. Although recombinant HMGAI does not associate with µ enhancer DNA through a PU.1/Ets-1 tether in vitro, chromatin immunoprecipitation assays show HMGAI associates with the enhancer in B cells. Furthermore, antisense HMGAI substantially decreases enhancer activity in B cells. Our model for HMGAI functioning through indirect association with the enhancer may explain how HMGAI functions on a wide variety of promoters and enhancers independent of direct HMGAI/DNA binding.

MATERIALS AND METHODS

Cell Lines and Transfections—The mature plasmacytoma Ag8.653 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 0.05% penicillin/streptomycin. 2017 pro-B or 38B9 pre-B cells were grown in RPMI supplemented with 10% heat-inactivated fetal calf serum, 0.05% penicillin/streptomycin, and 10⁻⁵ M β-mercaptoethanol. BALB mature B cells were grown similarly, except using 5% serum. For enhancer activity assays 5–8 × 10⁶ cells were transfected with 4 µg CAT or luciferase reporter plasmid plus 4 µg antisense HMGAI (21) or control plasmid using Superfect (Qiagen). Cells were harvested 48 h after transfection, and equivalent amounts of whole cell extracts were assayed for the presence of the CAT reporter by enzyme-linked immunosorbent assay (Roche Applied Science). Luciferase activity was measured using luciferase assay reagents from Promega. Note that in functional assays, different activities of HMGAIa and HMGAIb cannot be distinguished because they both associate with HMGAI depleted Ag8.653. Resulting data is therefore interpreted based on effects of HMGAIa.

DNA and Oligonucleotides—The control Moloney murine leukemia virus (Mo-MLV) enhancer reporter and the minimal µ enhancer reporter construct (µ10CAT), have been previously described (5, 26). The dominant negative Ets-1 protein Ets-1Δ2286 is a 5’ deletion of Ets-1 to amino acid 2286 cloned into the EVRF mammalian expression vector as previously described (27). Ets-1Δ2286 binds µ enhancer DNA in an electrophoretic mobility shift assay (EMSA) but does not transactivate DNA, and equivalent amounts of whole cell extracts were assayed for the ability of HMGAI to potentiate PU.1/Ets-1 functional synergy. EMSAs were performed as previously described (7, 16). For EMSA analysis, a Pst-BamHI fragment (base pairs 376–433 in the numbering of Ephrussi et al. (30) or a PvuII-BamHI fragment (base pairs 383–456) of the µ enhancer was analyzed. Biotinylated DNA Pull-downs—Approximately 7 × 10⁶ streptavidin conjugated Dynabeads (Dynal) were washed with PBS-BSA (PBS, pH 7.4, 0.1% BSA) for each sample. Biotinylated µ enhancer DNA (500–1000 ng) was incubated with the streptavidin beads for 4 h at 4 °C with rotation. Dynabead-DNA complexes were extensively washed with PBS-BSA to remove unbound DNA. Beads were resuspended in pull-down buffer (PBS, pH 7.4, 0.1% BSA, 1 µg/ml polycl[di(CdC)di(CdC)] before addition of 10 µg BSA as a nonspecific protein competitor. We then added recombinant His-PU.1 (200–300 ng) alone, recombinant His-ets-1 (200–300 ng) alone, or recombinant His-HMGAIa (200–300 ng) alone or in pair-wise or three-way combinations. Samples were incubated overnight. Dynabead-DNA complexes were spun down and washed three times with ice cold PBS-BSA. Samples were transferred to fresh microfuge tubes prior to final wash to avoid eluting plastic bound proteins. Dynabead-DNA-protein complexes were eluted in SDS-reducing sample buffer by heating at 95 °C. Quadruplicate samples were pooled and equal volumes loaded onto either 10% (for analysis of PU.1 and Ets-1) or 16% (for analysis of HMGAI) polyacrylamide gels for SDS-PAGE. Samples were transferred to polyvinylidene difluoride membranes and subjected to Western blot analysis as described above.

Chromatin Immunoprecipitations—for each immunoprecipitation 1 × 10⁸ 2017 or 38B9 cells were fixed by adding formaldehyde to a final concentration of 1% (1% formaldehyde growth media for 10 min). The reaction was quenched with glycine at a final concentration of 0.125 M. Cells were washed once in ice-cold PBS containing protease inhibitor mini-mixture (Roche) and 1 µmol phenylmethylsulfonyl fluoride. Nuclei were pelleted at low speed, lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8) for 10 min on ice and diluted to 1 ml in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8, 16.7 mM NaCl, 1 µmol phenylmethylsulfonyl fluoride, protease inhibitor mini-mixture). Chromatin was sonicated to ~500-bp fragments and centrifuged at 13,000 rpm for 10 min at 4 °C to remove debris. A portion (5%) of each sample was set aside to measure input DNA, and the remainder was diluted to 2 ml in dilution buffer and split into 4 × 10⁶ aliquots. Nonimmune background was prepared with 0.5 µl of salmon sperm DNA/protein A agarose beads (Upstate) and 1 µl of normal mouse IgG (Upstate) for 1 h at 4 °C with rotation. Supernatants were incubated with either 5 µl of a-actinylated histone H3 (Upstate), α-HMGAIa (MR19), or normal mouse IgG overnight at 4 °C with rotation before immune complexes were collected with 30 µl salmon sperm DNA/protein A-agarose beads in 50 µl wash buffer (1% sodium deoxycholate, 1 mM EDTA, 10 µM Tris, pH 8.0) then twice in TE (10 mM Tris 1 mM EDTA) (pH 8.0). Pellets were resuspended in 40 µl of immobilized chromatin immunoprecipitation (ChIP) elution buffer (1% SDS, 0.5 M NaHCO₃) and rotated at room temperature for 15 min. Samples were centrifuged and eluates removed. Elution was repeated one time and eluates were combined. Input DNA (5% of total sample previously set aside) was diluted to 300 µl in elution buffer, and
cross-links were reversed in all samples with the addition of NaCl to a concentration of 0.3 M and 20 μg of RNase A for 5–6 h at 65 °C. Proteins were removed from samples with 10 mM EDTA, 53 mM Tris-HCl, pH 6.5, and 50 μg proteinase K overnight at 37 °C. Samples were extracted once with phenol, once with 1:1 phenol/chloroform, and once with chloroform and precipitated in EtOH with 30 μl of 5 M NaCl and 20 μg glycogen at −80 °C for one hour. DNA was quantified using PicoGreen quantification reagent (Molecular Probes). 500 pg of each sample was then analyzed with quantitative real-time PCR.

Real-Time PCR and Analysis—Duplicates or triplicates of each sample were analyzed in a quantitative PCR reaction using the ABI Prism 7000 sequence detector and QPCR Mastermix Plus SYBR Green kit (VWR Scientific Products). Data was analyzed with a threshold set in the linear range of amplification, which for most experiments was 0.05. The cycle number that any particular sample crossed that threshold (CT) was then used to determine fold difference (enrichment). Fold difference was calculated as 2^(-ΔΔCt(input−ChIP)) (31). Melt curves of each amplified sample indicated formation of a single product in all cases.

**RESULTS**

**HMGA1 Associates with ETS Proteins in Solution—**HMGA1 has been shown to interact with two μ enhancer activators in vitro, PU.1 and TFE3 (16, 17, 25). Because activation of the μ enhancer also requires Ets-1 binding (5), we questioned whether HMGA1 interacts with this third protein of the minimal μ enhancerosome. Using a GST pull-down assay, we tested whether a GST-tagged HMGA1α forms a stable protein–protein interaction with Ets-1 in solution. Ets-1 interacts with HMGA1α as shown in Fig. 1A (lanes 3 and 5, representing independent duplicate samples). The precipitated Ets-1 co-migrated with recombinant Ets-1 loaded directly onto the gel as a control (lane 9). In the presence of the GST tag alone, Ets-1 did not precipitate (lanes 4 and 6), indicating that Ets-1 interacts with HMGA1α rather than the GST tag. The data clearly demonstrate a stable protein–protein interaction between HMGA1α and Ets-1 in vitro.

Although recombinant HMGA1α interacts with recombinant Ets-1 or PU.1 in a GST pull-down assay, this interaction must occur in the context of a complex mix of nuclear proteins to be physiologically relevant. Fig. 1B demonstrates that GST-tagged HMGA1α forms a stable interaction with PU.1 (lane 3) in the context of 38B9 pre-B cell nuclear extracts. Recombinant PU.1 (lane 1) loaded directly onto the gel co-migrated with the HMGA1α-associated PU.1, suggesting identity of the immunoreactive bands. The GST tag alone does not interact with the PU.1 naturally present in 38B9 nuclear extracts (lane 2). PU.1 identity was further confirmed by co-migration of the HMGA1α-associated protein with PU.1 directly immunoprecipitated from 38B9 nuclear extracts using an α-PU.1 antibody (lane 4). In contrast, α-GST did not immunoprecipitate PU.1 from 38B9 nuclear extracts (lane 5).

GST pull-down analyses of HMGA1α-interacting proteins in Ag8 plasmacytoma nuclear extracts demonstrated that a likely Ets-1 proteolytic fragment bound HMGA1α but not GST. The putative nuclear Ets-1 fragment associated with HMGA1α co-migrated with an HMGA1α-associated recombinant Ets-1 fragment (data not shown). Full-length Ets-1 could not be recovered from the HMGA1α-associated protein pool, likely due to the relatively high protease level in Ag8 cells in combination with the relative lability of Ets-1 to proteases. Taken together these data demonstrate PU.1 and Ets-1 form protein–protein interactions with HMGA1 in both simple and more physiological contexts.

**HMGA1 Increases Ets-1/μ Enhancer Binding—**HMGA1α facilitates PU.1/μ binding through protein–protein interaction (16). Our demonstration that HMGA1α interacts with Ets-1 in solution raises the possibility that HMGA1α augments Ets-1/μ enhancer binding in a similar fashion. Ets-1 specifically binds to the μA site of the μ enhancer, and mutation of this site destroys Ets-1/μDNA interaction and Ets-1-mediated enhancer activation (5). EMSA analysis with full-length Ets-1 is difficult to interpret as the inhibitory domains cause weak binding to the μA site under EMSA conditions (32, 33). To simplify analysis of how the demonstrated Ets-1/HMGA1α interaction affects Ets-1 function, we used a truncated form of Ets-1, ETS(Ets-1), containing only the ETS DNA binding domain. At relatively high amounts of protein (64 ng), ETS(Ets-1) complexed with μ enhancer DNA (Fig. 2A, lane 2). At relatively low levels of ETS(Ets-1) (6 ng), no detectable complex was formed with the μ enhancer (lane 3). To determine whether HMGA1α enhances ETS(Ets-1)/μ enhancer complex formation, we added HMGA1α protein to the EMSA reaction. HMGA1α increased ETS(Ets-1)/μ enhancer binding at low levels of Ets-1 (compare lanes 4–6 to lane 3). Very low levels of HMGA1α had no effect on complex formation (lanes 7 and 8). In the presence of equivalent amounts of BSA (instead of HMGA1α), the ETS(Ets-1)/μ enhancer complex was undetectable using 6 ng ETS(Ets-1) (data not shown). To further demonstrate the specificity of the enhanced binding complex, we repeated the analysis using a μA mutated probe. Lanes 11–15 demonstrate that the μA binding site was necessary for ETS(Ets-1)/DNA complex formation in the presence of HMGA1α. Similar results were obtained for full-length Ets-1 protein despite the generally weak μ enhancer binding activity as previously published (data not shown and Refs. 26 and 29). Overall, the data suggest that μ enhancer co-activation by HMGA1 stems from its ability to

![Figure 1. ETS proteins associate with HMGA1.](image-url)

**FIG. 1.** ETS proteins associate with HMGA1. A, recombinant GST- or GST-HMGA1α-associated Ets-1 was separated on a 12% polyacrylamide gel and probed with a rabbit α-Ets-1 antibody. Lanes 1 and 2, GST-HMGA1α or GST tag alone, respectively; lanes 3 and 5, GST-HMGA1α incubated with full-length Ets-1; lanes 4 and 6, GST tag alone incubated with Ets-1; lane 7, glutathione beads incubated with Ets-1; lane 8, beads alone; lane 9, recombinant Ets-1 directly loaded onto gel. B, 38B9 pre-B cell nuclear extract proteins associated with GST (lane 2) or GST-HMGA1α (lane 3) were separated on a 10% acrylamide gel and probed with α-Pu.1 antibodies on Western blots. Lane 1 shows recombinant Pu.1 loaded directly onto the gel as a positive control. Alternatively, nuclear extracts were subjected to immunoprecipitation with rabbit α-Pu.1 (lane 4) or α-GST (lane 5) antibodies and associated proteins were probed with goat α-Pu.1 antibody to demonstrate mobility of bona fide B cell Pu.1. Results are representative of multiple pull-down assays independently completed on multiple protein preparations.
HMGA1 Is Not Tightly Associated with an in Vitro μ Enhancer Nucleoprotein Complex—Because the ETS(μ)/HMGA1 DNA complex induced by the addition of HMGA1 co-migrated with the nucleoprotein complex formed by high amounts of ETS(μ), the data suggest that HMGA1a is absent from the ETS(μ)-DNA complex. Similarly, the EMSA mobility of the HMGA1a-induced PU.1/μ enhancer complexes are not altered when compared with samples using high protein levels that bind the enhancer in the absence of HMGA1a (16, 25).

However, for HMGA1a to supershift a protein-DNA complex, HMGA1a would have to remain in the EMSA complex for the duration of the gel run (>2 h). The absence of a trimolecular complex in EMSA is therefore not a reliable indication of the presence or absence of HMGA1a in the ETS protein-DNA complex. To more rigorously challenge the apparent absence of HMGA1a in the PU.1-ETS-1-μ enhancer complex in a more physiological setting, we used biotinylated DNA pull-downs. In this analysis, μ enhancer DNA will precipitate associated proteins forming either protein-DNA or protein-protein interactions. Because the final washes in this protocol are completed within 5–10 min, loosely bound proteins are much more likely to be retained in the nucleoprotein complex as compared with an EMSA complex. To test the validity of this strategy, we precipitated PU.1 and Ets-1 with either wild type or μ DNA. The μ− mutation is a 3 base pair mutation that efficiently destroys both PU.1-μB binding and PU.1-mediated functional synergy with Ets-1 (5). Because PU.1 and Ets-1 interact in a yeast 2-hybrid assay (34), we predicted Ets-1 could tether PU.1 to a μ− enhancer if our pull-down assay was a valid measure of protein-protein interactions on DNA. Biotinylated wild type or μ− enhancer DNA was conjugated to streptavidin beads and subsequently incubated with PU.1 and Ets-1, alone or in combination. Bound proteins were detected by Western blot. 

Fig. 3A shows PU.1 is precipitated by wild type biotinylated DNA alone or in the presence of Ets-1 as expected (lanes 1 and 3). μ− DNA does not bind PU.1 (lane 7) consistent with previous results (5). However, in the presence of Ets-1, μ− DNA precipitates PU.1 likely through protein-protein interactions between PU.1 and Ets-1 (lane 5). By independently verifying PU.1/Ets-1 interaction using biotinylated DNA pull-downs, we conclude that this method detects DNA-bound protein complexes and protein-protein tethers that have been independently validated.

To determine whether HMGA1a is stably associated with the ETS protein-DNA nucleoprotein complex, we used the pull-down protocol to precipitate proteins stably associated with the μ nucleoprotein complex. Recombinant proteins alone or in combination were incubated with biotinylated wild type μ enhancer DNA as indicated at the top of Fig. 3B. Wild type μ enhancer DNA clearly precipitates recombinant PU.1 (top panel, arrowhead) or recombinant His-tagged Ets-1 (bottom panel, arrowhead) alone or in combination with other proteins. Interestingly, PU.1/DNA interaction was increased in the presence of HMGA1a (top panel, compare lanes 6 and 7 to lanes 3 and 5), consistent with the published EMSA analysis demonstrating the same phenomenon (16). In some analyses, Ets-1/DNA interaction was likewise increased in the presence of HMGA1a, but variability of this finding made interpretation difficult. Western analysis for His-tagged HMGA1a (middle panel) demonstrated HMGA1a was not precipitated with wild type μ enhancer DNA alone (middle panel, lane 1), or in the presence of PU.1 (lane 5), Ets-1 (lane 4), or PU.1 plus Ets-1 (lane 3). This result shows PU.1 and/or Ets-1 cannot stably tether HMGA1a to the μ enhancer through protein-protein interactions and further confirm HMGA1 does not bind μ enhancer DNA directly.

One trivial explanation for the apparent lack of an ets protein/HMGA1a tether in the biotinylated pull-down assay is that the assay conditions preclude HMGA1 binding. To test this possibility, we took advantage of the fact that HMGA1 interacts with both DNA and proteins through an A-T hook motif, suggesting that the basic requirements for HMGA1 interacting with both species are likely to be similar. To test the compatibility of the pull-down assay conditions with general

**Fig. 2.** HMGA1a facilitates ETS/DNA binding. A, the wild type μ enhancer was incubated with high (+ + + = 64 ng, lane 2) or low (+ = 6 ng, lanes 3–8 and 10–15) amounts of ETS(μ). HMGA1a was added to the EMSA reaction in lanes 4–8 (64, 32, 16, 8, or 4 ng, respectively). Lanes 11–15 are identical to lanes 4–8 except the probe contained a 3-base pair mutation at the μA site (5). B, the wild type μ enhancer was incubated with low amounts (15 ng) of the ETS DNA binding domain of PU.1 (ETS(PU.1)) in the presence of BSA (lanes 1–3, 32, 16, or 8 ng, respectively) or HMGA1 (lanes 4–6, 32, 16, or 8 ng, respectively).

increase DNA binding by Ets-1 (Fig. 2A), TFE3 (25), and PU.1 (16).

Taken together, our GST pull-down and EMSA assays suggest HMGA1 directly interacts with the ETS domain of Ets-1 to facilitate DNA binding. To determine whether HMGA1 function in this context is more generally applicable to other ETS domains, we extended our binding analysis to the DNA binding ETS domain of PU.1, ETS(PU.1). In the presence of BSA, low levels of ETS(PU.1) do not form a complex with μ enhancer DNA (Fig. 2B, lanes 1–3). In contrast, HMGA1a facilitates ETS(PU.1)/μ enhancer interaction (lanes 4–5). This complex co-migrates with the ETS(PU.1)/μ enhancer complex formed using relatively high levels of ETS(PU.1) (data not shown). These data demonstrate that HMGA1a facilitates nucleoprotein complex formation between the μ enhancer and the DNA binding domains of both PU.1 and Ets-1. Interestingly, the protein-DNA complex migrates identically in the presence or absence of HMGA1a in all cases (for example compare Fig. 2A, lane 2 with lanes 4–6).

**HMGA1 Is Not Tightly Associated with an in Vitro μ Enhancer Nucleoprotein Complex**—Because the ETS(μ)/HMGA1 DNA complex induced by the addition of HMGA1a co-migrated with the nucleoprotein complex formed by high amounts of ETS(μ), the data suggest that HMGA1a is absent from the ETS(μ)-DNA complex. Similarly, the EMSA mobility of the HMGA1a-induced PU.1/μ enhancer complexes are not altered when compared with samples using high protein levels that bind the enhancer in the absence of HMGA1a (16, 25).
HMGA1 A-T hook function, we took advantage of the demonstration that HMGA1 binds to the IFN-β enhancer via direct protein/DNA contact in both EMSA and in vitro footprint assays (20). We biotinylated an HMGA1a-binding fragment of the IFN-β enhancer and demonstrated that this fragment could precipitate HMGA1a under conditions identical to conditions in Fig. 3B. Although HMGA1a is not precipitated in the presence of ρ-μ bound PU.1 and Ets-1, the protein can be precipitated by IFN-β DNA (Fig. 3C, lane 1) and co-migrates with recombinant HMGA1a loaded directly onto the gel (lane 2). PU.1 was not precipitated by IFN-β DNA and was included as a negative control (data not shown). Based upon these analyses, we conclude that, although HMGA1a facilitates the binding of PU.1 and Ets-1 to the ρ enhancer, HMGA1a may not remain stably in the DNA-bound transcriptionally active minimal enhancer (μAμE3-μB) complex.

HMGA1 Is Associated with μ Enhancer in B Cells—Although protein-protein interactions measured in EMSA and DNA pull-down assays may accurately reflect similar interactions in the cell, these assays cannot recapitulate the more complicated protein-DNA complex formed at the cellular μ enhancer. The lack of detectable interactions between ETS domain proteins and the μ enhancer in the pull-down assay suggests that a PU.1/Ets-1 combination cannot tether HMGA1 to DNA. However, additional transcription factors such as TFE3, CBF, IRF-1, and E47 activate the enhancer by various measures (8, 29, 35, 36) and could participate in retaining HMGA1 in the cellular μ enhancosome. To test whether HMGA1 is associated with the μ enhancer in B cells, we performed ChIPs using α-HMGA1 antibody to precipitate chromatin complexes from 38B9 pre-B or 2017 pro-T cells. The left set of bars in Fig. 4A show α-HMGA1 antibody precipitated ~2-fold the amount of μ enhancer in 38B9 cells (striped bars) as compared with 2017 pro-T cells (open bars), consistent with the interpretation that HMGA1 preferentially associates with an active μ enhancer.

α-HMGA1 precipitated very low levels of β-globin, a gene known to be inactive in both B and T cells (middle set of bars and Ref. 37). Control IgG precipitated equivalent amounts of μ DNA in 2017 pro-T and 38B9 pre-B cells (0.84 and 0.88 of normalized input amounts, respectively; rightmost bars). Qualitatively similar, but quantitatively more dramatic results demonstrated HMGA1 associates with the μ enhancer in BAL-17 cells, a line similar to primary splenic B cells (Fig. 4B). μ enhancer DNA associated with the α-HMGA1 antibody 3.3-fold more efficiently in BAL-17 cells as compared with 2017 pro-T cells (leftmost bars; 6.7 and 2.0-fold increase, respectively). α-HMGA1 precipitated negligible amounts of the inactive β-globin locus in both cell types (middle set of bars; values of 0.37 and 0.53 for 2017 and BAL-17 cells, respectively). Association of the enhancer with the control IgG was identical in the two cells types (rightmost bars; 2.7-fold) and approximated the inefficiency of the α-HMGA1 antibody precipitating μ in 2017 cells. Overall, the data demonstrate HMGA1 associates specifically with the μ enhancer in a tissue-restricted pattern.

To confirm that HMGA1 is associated with only active μ loci, we asked whether the 38B9 μ enhancer is packaged into hyperacetylated histones. Previous analyses have demonstrated that active μ loci are packaged by hyperacetylated histones.

![Biotinylated DNA pull-downs precipitate DNA-associated proteins](image-url)

Fig. 3. Biotinylated DNA pull-downs precipitate DNA-associated proteins. A, biotinylated wild type (lanes 1–4) or μB− (lanes 5–8) μ enhancer DNA was bound to streptavidin beads and then incubated with BSA (500 ng, lanes 4 and 8), PU.1 (200 ng, lanes 3 and 7), Ets-1 (200 ng, lanes 2 and 6), or PU.1 plus Ets-1 (lanes 1 and 5). Nucleoprotein complexes were separated on 8% reducing SDS-PAGE gels. Shown is Western detection of DNA-associated proteins using an α-PU.1 antibody. Blot is representative of four independent experiments. B, biotinylated DNA pull-downs to detect tethering of HMGA1a to the μ enhancer through protein-protein interactions. 1–2 μg of biotinylated μDNA was incubated with recombinant PU.1, Ets-1, or HMGA1a alone or in combination and the associated proteins analyzed on Western blots. Precipitated samples containing proteins listed at the top of the figure were separated on either 10% (top and bottom panels) or 16% (middle panel) polyacrylamide gels and detected by Western blot with α-PU.1 (top panel) or α-His antibody (middle and bottom panels). α-His reacts with the histidine tags of the recombinant proteins. Recombinant protein was loaded directly as a positive control (lane 9, top and middle panels). C, biotinylated PRDII/NRDI DNA precipitates HMGA1a. 2 μg of annealed biotinylated PRDII/NRDI DNA was incubated with recombinant HMGA1 and the associated proteins separated on a 15% gel, then analyzed by Western blot using an α-His antibody to detect the HMGA1 tag. Recombinant HMGA1 was directly loaded and was used as a positive control (lane 2). Blots are representative of three independent experiments using two separate protein preparations.
whereas inactive loci are associated with hypoacetylated histones (37). We completed ChIP assays using an antibody specific for acetylated histone H3 in 38B9 pre-B and 2017 pro-T cells, which have active or inactive μ loci, respectively. Fig. 4C (leftmost bars) demonstrates that, as expected, acetylated H3 associated with the μ enhancer in 38B9 pre-B cells 3.6-fold more than in 2017 pro-T cells. This level of increase is consistent with analyses on primary bone marrow B cells (38). The β-globin locus associates with hypoacetylated histone H3 in both cell types (middle bars), and the nonspecific IgG control did not precipitate μDNA (rightmost bars). The data confirm HMGA1 associates only with an active μ enhancer in B cells and not with an inactive T cell enhancer, strongly supporting the contention that HMGA1 co-activates the enhancer in vivo. 

**HMGA1 Determines μ Enhancer Activity in B Cells**—The ChIP analyses (Fig. 4), in combination with the demonstration that HMGA1 synergizes with an Ets-1/PU.1 combination to activate the μ enhancer in non-B cells (16), are consistent with the interpretation that HMGA1 plays a role in tissue-specific μ enhancer activation in B cells. Transient transfection assays in multiple B cell lines reproducibly demonstrated HMGA1 overexpression increased μ enhancer activity only 50–90% (data not shown), to a level difficult to interpret using this method. This low level increase is consistent with our previous demonstration that HMGA1/ETS protein stoichiometry is critical for activating the enhancer (16). We speculate that B cells contain the appropriate concentrations of factors for optimal μ enhancer activation, such that overexpression of any given factor will not hyperactivate the endogenous enhancer substantially.

To more definitively test whether HMGA1 functions in combination with endogenous B cell proteins to activate the μ enhancer, we completed transient transfection assays with an antisense HMGA1 construct, which decreases HMGA1 protein levels to ~5% of normal lymphoid cell levels (21). For this analysis, antisense HMGA1 and the μ enhancer reporter (μ70)2 CAT were transiently co-transfected into the plasmacytoma line Ag8. Alternatively, we transfected the antisense parent vector RcCMV in combination with (μ70)2 CAT. As shown in Fig. 5A, endogenous B cell proteins, likely PU.1 and Ets-1, drive CAT reporter protein expression in the presence of the parent RcCMV vector by activating the μ enhancer (left bar). Co-transfection of antisense HMGA1 decreases μ enhancer activity ~71% (right bar) as compared with activity in the presence of RcCMV. This data demonstrates that HMGA1 expression is necessary for robust activation of a relatively accessible μ enhancer by endogenous B cell proteins.

One possible explanation for decreased μ enhancer activity in the presence of antisense HMGA1 is that antisense HMGA1 decreases transcription nonspecifically. To test the specificity of the antisense construct, we measured the effect of antisense HMGA1 on a CAT expression vector driven by the Mo-MLV enhancer. This vector contains binding sites for several transcription factors including Ets-2, Fl-1, GA-binding protein, and core binding factor, and is active in B cells (26, 39). Co-transfection of either the parent RcCMV vector or the antisense HMGA1 resulted in approximately equivalent activation of the Mo-MLV enhancer (Fig. 5B). This data suggests that the antisense HMGA1 is not interfering with transcription in general, but is specifically inhibiting μ enhancer activity.

To get a sense of the significance of a ~70% decrease in μ enhancer activity, we compared the effect of antisense HMGA1 to the effect of an Ets-1 truncation mutation on μ enhancer activation in B cells. For this analysis we used N-terminally truncated Ets-1 Δ286, which is incapable of activating the enhancer in combination with PU.1 in non-lymphoid cells, yet retains wild type DNA binding properties (27). We transfected Ag8 plasmacytoma cells with the (μ70)2 CAT reporter in the presence or absence of Ets-1 Δ286 (Fig. 5C). Ets-1 Δ286 decreased enhancer activity 88.2% (left bar) as compared with co-transfection of an empty expression vector (right bar). In contrast, Ets-1 Δ286 had no significant effect on activity of an unrelated SV40 promoter-driven luciferase gene lacking an Ets-1 binding site in Ag8 cells (Fig. 5D). These data indicate that Ets-1 Δ286 neutralizes μ enhancer activation mediated by
endogenous B cell proteins likely through competitive binding, and hence is a dominant negative \( \mu \) enhancer regulatory protein. Furthermore, antisense HMGA1 is approximately as effective as Ets-1 \( \Delta S86 \) in decreasing \( \mu \) enhancer activity. Taken together, the functional data is consistent with ChIP data in suggesting that endogenous HMGA1 is important for \( \mu \) enhancer activity in B cells in the presence of normal transcription factor levels.

**DISCUSSION**

Although the identities of DNA binding proteins and transcriptional co-activators regulating the \( \mu \) enhancer have been the subject of intense investigation for over a decade, the detailed mechanism of enhancer activation remains unclear. The data strongly suggest HMGA1 plays an important role in activating the \( \mu \) enhancer in B cells through modulating function of the *ets* family transcription factors Ets-1 and PU.1. Three findings bolster this conclusion. First, multiple biochemical assays show functional importance of an HMGA1/ETS protein interaction in *vitro* with respect to DNA binding activity. Second, we have directly demonstrated that HMGA1 specifically associates with the \( \mu \) enhancer in B cells but not in pro-T cells. Third, decreasing HMGA1 levels substantially decreases \( \mu \) enhancer activity in B cells but does not affect transcription in general. We hypothesize that in B cells, which express relatively low levels of PU.1 (7, 40), PU.1 cannot efficiently bind and activate the \( \mu \) enhancer in the absence of HMGA1 or an analogous co-activating factor. Extension of this hypothesis to include Ets-1 is likely upon more rigorous characterization of interactions described in our work.

A second level of interpretation addresses the biochemical mechanism of HMGA1 activity on the \( \mu \) enhancer. HMGA1 interacts with the \( \mu \) enhancer indirectly, likely through weak protein-protein interactions. Although ChIP assays show HMGA1 associating with the \( \mu \) enhancer in the context of cellular chromatin, multiple assays, including EMSA, DNase I footprinting (16), methylation interference, and DNA pull-downs (this study) show no specific \( \mu \) enhancer/HMGA1 binding under a variety of experimental conditions. We interpret this apparent conundrum as follows. Although the sole interpretation of the ChIP data is that HMGA1 is preferentially associated with the \( \mu \) enhancer through either protein-protein or protein-DNA interactions in B cells, there are multiple alternative explanations for our inability to detect HMGA1/ETS protein association in the context of ETS protein/DNA binding in *vitro*. First, HMGA1 may associate with the \( \mu \) enhancer through an *ets* protein-independent mechanism, because multi-ple transcription factors occupy the enhancer in *in vivo* footprint analyses (30). Preliminary pull-down assays using a TFE3/\( \mu \) enhancer combination show TFE3 does not tether HMGA1 to the enhancer. One possibility is that CBF, a transcription factor that can activate the \( \mu \) enhancer via a site overlapping the TFE3 site (8), tethers HMGA1 to the enhancer. This possibility is currently under investigation. Because HMGA1 functions on an enhancer fragment containing only the two *ets* sites and the TFE3/CFB site (16), it is unlikely that interactions outside this core enhancer are necessary to explain the data. A second possibility explaining the apparent contradictory data is that the combination of three proteins (PU.1, Ets-1, and TFE3) forms an HMGA1-associating platform. We deem this possibility unlikely due to the small size of HMGA1 which could limit its availability for interaction with multiple proteins as further discussed below. A third scenario is that protein post-translational modification absent from the recombinant proteins used in the various binding assays is required.

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*Fig. 5. Expression of \( \mu \) enhancer CAT reporter requires HMGA1 in B cells.* A, Ag8 plasmacytoma cells were co-transfected with 4 \( \mu g \) of a (\( \mu 70\)) reporter CAT construct plus either 4 \( \mu g \) of empty RCMM vector (left bar) or 4 \( \mu g \) of antisense HMGA1 in RCMM (right bar). B, Ag8 plasmacytoma cells were co-transfected with 4 \( \mu g \) of a Mo-MLV viral enhancer reporter construct and either 4 \( \mu g \) of RCMM (left bar), or 4 \( \mu g \) of antisense HMGA1 alone (right bar). C, Ag8 plasmacytoma cells were transfected with 4 \( \mu g \) of a (\( \mu 70\)) reporter CAT construct plus either 4 \( \mu g \) truncated Ets-1 \( \Delta S86 \) (left bar) or 4 \( \mu g \) pcDNA vector (right bar). D, Ag8 plasmacytoma cells were transfected with 4 \( \mu g \) of an SV40 promoter driven luciferase construct lacking an Ets-1 binding site plus either Ets-1 \( \Delta S86 \) (left bar) or empty pcDNA vector (right bar). All cells were harvested 40–48 h post-transfection, and reporter activity was measured by CAT ELISA or a luciferase activity assay. Results shown are representative of at least three assays with each sample performed in triplicate or quadruplicate. Error bars represent range of the data points.

\footnote{B. S. Nikolajczyk, unpublished observation.}
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for HMGA1 association with the \( \mu \) enhancer. HMGA1 can be modified by phosphorylation, ubiquitination, or acetylation in vitro. Biological significance of these modifications has been demonstrated in multiple analyses (18, 41–43). Such modifications could increase affinity of an ETS protein-HMGA1 complex in cells, countering the apparent absence of a stable association in vitro. Alternatively, phosphorylation sites within PU.1 have a proposed role in regulating multiple immunologically important enhancers (44, 45). A role for HMGA1 has not been tested in these contexts. One additional possibility is that formaldehyde cross-linking in the ChIP assays locks HMGA1 onto the DNA, to avoid potential dissociation during the course of the assay. However, we maintain that HMGA1 does not bind \( \mu \) enhancer DNA directly, as described above, despite extensive evidence that HMGA1 associates with bona fide target elements in the IFN-\( \beta \) enhancer (Ref. 20 and this study, Fig. 3C).

Our preliminary analysis of in vitro acetylated or phosphorylated recombiant HMGA1 show these modifications do not lead to \( \mu \) enhancer binding activity (data not shown), further discounting mechanisms based on direct HMGA1/DNA interaction. Further analyses will refine our current interpretation, that HMGA1 associates with DNA-bound ets proteins to indirectly associate with \( \mu \) enhancer DNA in B cells and thereby regulate \( \mu \) enhancer activity.

We previously demonstrated that the stoichiometry between ETS proteins and HMGA1 determined levels of \( \mu \) enhancer activity (16). Ectopic HMGA1 expression modestly increases enhancer activity by endogenous proteins in B cells (50–90% increase, data not shown). Because optimal stoichiometry between limited concentrations of \( \mu \) enhancer activators and HMGA1 is required for transcriptional co-activation (16), the inability to artificially optimize this stoichiometry in a B cell is not surprising. The new antisense HMGA1 results strongly suggest the HMGA1/ETS protein stoichiometry is naturally optimal for co-activation in B cells. Now that we know HMGA1 can increase protein binding at three sites in the minimal enhancer, we envision several possible models of the in vitro stoichiometry between HMGA1 and \( \mu \) enhancer activators.

First, one HMGA1 molecule may interact with all three members of the minimal \( \mu \)-enhancing complex simultaneously. A single HMGA1 molecule can interact with multiple proteins because HMGA1 has several demonstrated protein-protein binding surfaces (20, 46). However, we consider this possibility unlikely as the ultimate positioning of PU.1, Ets-1, and TFE3 on the \( \mu \) enhancer shows PU.1 and Ets-1 on one face of the DNA helix while TFE3 binds 120° away around the circumference of the helix (6). HMGA1a and b, at 10 and 11 kDa, are likely too small to interact with 3 proteins to simultaneously escort them to the DNA in the stated configuration. Second, HMGA1 may be associated with and affect each activator of the minimal \( \mu \) enhancer individually. One piece of data perhaps inconsistencies with this possibility is our demonstration that antisense HMGA1 has no effect on the Mo-MLV enhancer, a transcriptional element that requires an Ets-1/2 site for activity. If a bimolecular interaction between Ets-1 (or the transcriptional element that requires an Ets-1/2 site for activity) and multiple transcription factors are consistent with a role for HMGA1 in coordinating transcription factor binding to the \( \mu \) enhancer much like a protein chaperone. It remains to be seen whether, in the context of chromatin, the association of HMGA1 with transcription factors facilitates chromatin remodeling, in part, through alteration of ETS protein and concomitant recruitment/release of chromatin structural complexes. HMGA1 itself may determine chromatin structure on transcriptional target sequences (23). However, the demonstrated lack of direct HMGA1/\( \mu \) enhancer interaction precludes a direct role for HMGA1 in determining \( \mu \) chromatin structure. One partner of HMGA1, PU.1, is a demonstrated chromatin accessibility factor for the \( \mu \) locus, although its ability to increase accessibility of a closed chromatin structure is weak (49). Knowing that HMGA1 is associated with the \( \mu \) enhancer in B cells but not T cells is important for determining how these various molecules cooperate in the context of chromatin, either re-enforcing or neutralizing activities attributed to single proteins. Taken together, our findings that HMGA1 functions on the \( \mu \) enhancer in B cells spurs our interest in understanding precisely how this transcriptional co-activator regulates an important tissue-specific protein in vitro.

Acknowledgments—We would like to thank M. Atchison for critically reviewing the manuscript. Expert technical support was provided by Kate Morwood. We further appreciate assistance from Dipanjan Chowdhury and Amada Keyes in setting up the ChIP assay.

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HMGA1 Co-activates Transcription in B Cells through Indirect Association with DNA

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J. Biol. Chem. 2003, 278:42106-42114.
doi: 10.1074/jbc.M308586200 originally published online August 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M308586200

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