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HMG-I(Y) proteins are mammalian architectural transcription factors. They have been demonstrated to be necessary for the transcriptional regulation of a number of genes, including the genes associated with tumorigenesis and metastasis. It was proposed in the original grant that over-expression of HMG-I(Y) proteins in breast cancer cells will fundamentally influence the expression of genes that are involved in metastatic invasion, migration, angiogenesis and colonization. In the three years of study, the results support the hypothesis. They include (I) expression of endogenous HMG-I (Y) proteins correlates tumor progression in human breast cancer cells and expression of anti-sense of HMG-I inhibits the growth of tumor cells, (II) over-expression of exogenous HMG-I (Y) proteins promotes the tumor growth in soft agar and (III) over-expression of HMG-I(Y) alters transcription of genes that are associated with tumor progression. These results indicated that HMG-I(Y) proteins indeed may play an important role in tumor metastasis.

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Table of Contents

INTRODUCTION

HMG-I and HMG-Y proteins are architectural transcription factors. They are encoded by the same gene and generated through alternative RNA splicing. They share the same primary structures that include three DNA binding domains as well as acidic C-terminal tail. The cellular functions of HMG-I (Y) proteins are still not fully understood. However, they have been implicated in the *in vivo* transcriptional regulation of a growing number of genes in either a positive or a negative fashion, such as ICAM, VCAM, E-selectin and TNF-α, among others. The HMG-I (Y) proteins have the ability to bend, unwind and supercoil DNA substrates *in vitro* as well as being able to induce changes in the rotational setting of DNA on the surface of isolated nucleosomes. In addition, to specifically interacting with DNA, the HMG-I (Y) proteins have been demonstrated to physically interact with many general and tissue specific transcription factors, including NF-κB, c-Jun, SRF, RAR, Oct2, Oct6, ATF2, and Elf-1. As a result of their ability to modulate DNA structure and to also engage in specific protein-protein and protein-DNA interactions, the HMG-I (Y) proteins are play a essential role to in transcriptional regulation by participating in the formation of stereo-specific complexes called 'enhanceosomes' on the promoter/enhancer regions of the genes they activate. The HMG-I (Y) proteins have also been demonstrated to derepress histone H1-mediated inhibition of transcription *in vitro* and to be strongly enriched in histone H1-depleted active chromatin *in vivo*. Having these properties, HMG-I (Y) proteins have been postulated to replace histone H1 from nucleosomal chromatin, thus allowing other transcription factors access their DNA binding sites so that gene specific enhancesomes can be formed and their transcriptional activity regulated, usually in an inducible fashion. Recently, it has been reported that acetylation of HMG-I (Y) by CBP disrupts the enhanceosome and turns off the gene expression of IFN-β.

The expression of HMG-I(Y) is correlated with cancer progression. This has been demonstrated in several cancer models, including human benign neoplasms: uterine leiomyomata and pulmonary chondroid hamartomas; also human carcinomas: prostate, thyroid and colorectal carcinomas. In addition, increasing levels of HMG-I (Y) are found with progression in a series of cell lines isolated from the Dunning rat model system of prostate cancer as well as from a mouse mammary epithelial cell system. Therefore, the expression of HMG-I(Y) has been proposed as a marker for tumor progression and metastatic potential.

The funded proposal is to define the role of HMG-I(Y) proteins in tumor progression and metastasis of human breast cancer. The experimental strategies are: (I) over-expression of HMG-I(Y) in non-metastatic tumor cell lines and over-expression of dominant negative HMG-I(Y) or anti-sense HMG-I(Y) in highly metastatic tumor cell lines; (II) monitor the characteristic changes of these transfectants on the expression of genes, such as adhesion molecules, matrix metalloproteases, which are involved in metastatic invasion, migration, angiogenesis and colonization. The results from the three-year study demonstrated that HMG-I(Y) proteins indeed play a critical role in tumor progression, therefore, supported original proposal.

RESULTS AND DISCUSSION

Expression of endogenous HMG-I (Y) proteins correlates with tumor progression in a human breast cancer cell line system and expression of anti-sense of HMG-I inhibits the growth of tumor cells.

Human breast cancer cell lines, Hs578Bst, Hs578T, MCF-7 and MCF-7/PKCα, have been used for determine whether the expression of HMG-I (Y) proteins correlates to tumor progression. By using Western blot analysis, **as shown in annual report II (Fig. 2, 1998),** HMG-I (Y) proteins were undetectable in the normal epithelial Hs578Bst cells and were expressed significantly lower in non-metastatic tumor MCF-7 cells than those in highly metastatic MCF-7/ PKCα and Hs578T cells. The expression levels of HMG-I (Y) correlate with tumor progression of human breast cancer cells. These results agree with published similar experiments (Liu, Cancer Res.1999 and Nacht, Cancer Res.1999).

To test whether HMG-I(Y) play a role in cell growth, a well characterized anti-sense HMG-I(Y) expression vector was tranfected into non-metastatic tumor MCF-7 cells and highly metastatic Hs578T cells. As shown in **annual report II (Fig. 1, 1998),** transient expression of anti-sense HMG-I(Y) significantly inhibits growth of MCF-7 and Hs578T tumor cells. In addition, the MCF-7 cells which transfected with anti-sense HMG-I(Y) were also examined by soft agar assay. The result demonstrated that expression of anti-sense HMG-I(Y) leads to a significant reduction of colony formation as shown in **annual report II (Table 1, 1998).**

Over-expression of exogenous HMG-I (Y) proteins promotes the growth of tumor cells.

To examine the role of over-expression of exogenous HMG-I (Y) in non-metastatic tumor cells, MCF-7 cells were transfected with cDNA of human HMG-I or HMG-Y. As shown in **annual report I (Fig. 3, 1997),** the transfectants of HMG-I or HMG-Y or both led to more colonies than those transfected with empty vector in soft agar assays. However, it has become problematic to draw definitive conclusions because low levels of exogenous HMG-I(Y) proteins were expressed and anti-HMG-I(Y) polyclonal antibodies cannot distinguish the transfected exogenous HMG-I(Y) proteins from the endogenous ones.

To overcome this problem, expression of tagged HMG-I(Y) proteins and an inducible expression system were established. Constructs encoding a 9-amino acid peptide from hemagglutinin (HA tag) fused to the N-terminal end of HMG-I and HMG-Y were generated. Monoclonal antibody 12CA5 specifically against HA-tag was used to monitor expression of exogenous HMG-I(Y) proteins. In addition, the HA-tagged HMG-I(Y) were subcloned into a tetracycline inducible vector (*tet-off* system, Clontech, CA). Expression of the HA-tagged HMG-I(Y) in MCF-7 transfectants can be manipulated by culturing them in the presence (off) or in the absence (on) of tetracycline. Several clones that stably expressed HA-HMG-I(Y) proteins have been selected and maintained in the present of tetracycline. As shown **in Figure 1** by using immunoprecipitation and Western blots, the exogenous HMG-I and HMG-Y proteins in MCF-7 cells were not expressed in the present of tetracycline with exception of clone HA-I-C7. However, these exogenous

proteins were dramatically increased after withdrawal of tetracycline in cell cultures. Increasing of exogenous HMG-I(Y) proteins in MCF-7 cells led to a significant growth in soft agar assays **(Figure 2)**. As summarized in **Table 1**, HMG-Y expressing clones HA-Y-C2, HA-Y-C10 and HA-Y-C21 have 13-39 fold more colonies in their inducing status than those in their suppressing status. Similarly, HMG-I expressing clones HA-I-C14 and HA-I-Cs have 20-39 fold more colonies. However, the HA-I-C7 clone that expressed the highest amount of exogenous HA-HMG-I protein has only about two-fold increase of colonies in the soft agar assay. This is due to the leaking expression of the exogenous protein in the present of tetracycline as shown in **Figure 1**. When comparing the HA-I-C7 with clones that transfected with empty vector M-tet, HA-I-C7 has about 19-fold more colonies.

Table 1. Over-expression of HA-HMG-I(Y) proteins leads to anchorage-independent growth of MCF-7 cells.

Clone	# of colony (on, without tet.)	# of colony (off, with tet.)	Fold
HA-Y-C2	151.75 ± 19.92	11.25 ± 3.09	13.5
HA-Y-C10	245.50 ± 21.20	6.25 ± 1.70	39.3
HA-Y-C21	143.75 ± 10.37	5.75 ± 1.70	25.0
HA-I-C7	321.75 ± 17.03	168.25 ± 12.68	2.0
HA-I-C14	118.00 ± 12.19	3.00 ± 2.16	39.3
HA-I-Cs	204.25 ± 25.32	10.25 ± 2.87	20.0
M-tet	17.00 ± 2.94	4.50 ± 2.08	3.7

Tumorigenesis and metastasis potential of MCF-7 cells that over-express exogenous HMG-I(Y) have been evaluated in BALB/c (nu/nu) nude mice. As shown in **Figure 3A**, HMG-Y expressing clone HA-Y-C21 formed a tumor nodule of 7 mm in diameter after subcutaneous injection. However, no tumor nodules were observed in the nude mice that had been injected with control M-tet **(Figure 3B)** or HA-I-C7 cells (data not shown). Due to the small size of the tumor nodules, the nude mice have not yet been sacrificed and the experiments are still in progress.

Over-expression of HMG-I(Y) alters transcription of genes that are associated with tumor progression.

It has been hypothesized in the original proposal that over-expression of HMG-I(Y) will facilitate the disregulation of expression of numerous genes, some of which will then be selected for (or against) by the immediate cellular environment during each stage of transformation and metastasis. To screen for the known genes that associated with tumor progression and regulated by HMG-I(Y), human cancer cDNA expression array from Clontech has been utilized. The array filters were hybridized with cDNA probes which were prepared from the exogenous HMG-Y expressing clone HA-Y-C21, the exogenous HMG-I expressing HA-I-C7 and the empty control vector clone M-tet, respectively. Each hybridized filter was scanned with a Phosphorimager. The resulting files were analyzed with image analysis software. Differential gene expression analysis of

the control clone M-tet versus HMG-Y expressing clone HA-Y-C21 revealed that, among 588 analyzed genes, 32 genes (0.17%) were found to be differentially expressed at least10-fold in the clone HA-Y-C21 and 54 genes (9.18%) were found to be differentially expressed about 9-4 fold. A total of 86 genes (14.63%) had significantly altered levels of expression (**Figure 4**A and **Figure 4**B). Of these genes, 73 were up-regulated and 13 were down-regulated. A similar, but less dramatic, result was observed in comparison between the exogenous HMG-I expressing clone HA-I-C7 and the empty control vector clone M-tet. The differentially expressed genes in the exogenous HMG-I(Y) expressing HA-Y-C21 and HA-I-C7 cells demonstrated a broad range of functional activity. They have been known as cell cycle/growth regulators, intermediate filament markers, apoptosis regulators, cell fate/development regulators and cell adhesion, motility as well as invasion molecules. For example, genes of intergrin-β1 and matrix metalloproteinase 13 (MMP13) were identified 10-fold and 4.7-fold increase in clone HA-Y-C21.

To verify the reliability of array data, PCR primers specific for intergrin-β1 and MMP13 were designed and semi-quantitative RT-PCRs were run for three HMG-Y expressing clones and two HMG-I expressing clones. The results (**Figure 5**A and **Figure 5**B) indicated that the differential expression pattern and the quantitative expression level of each of two genes as determined by RT-PCR were similar to those observed with cDNA arrays, confirming the reliability of the array expression profile data.

Figure Legends:

Figure 1. Expression of HA-tagged HMG-I and HMG-Y proteins in *on* or *off* condition of tetracycline inducible expression system. MCF-7 cells were transfected with HA-tagged HMG-I or HMG-Y *tet-off* constructs. The transfectants were cultured in the presence of 5 μg/ml of tetracycline (*OFF*, non-induced) or in the presence of 2 μg/ml of tetracycline (+/-, partially induced) or in the absence of tetracycline (*ON*, induced) for 48 hours. 5 x 10^6 cells were lysed and immunoprecipitated with 12CA5 specifically against HA tag. Then the HA-tagged HMG-I(Y) proteins were detected by Western blot with antibody MR18 specifically against HMG-I(Y).

Figure 2. Over-expression of exogenous HMG-I or HMG-Y in MCF-7 cells leads to more colonies than those transfected with empty control vector in soft agar. 1x 10^4 cells per petri dish (60 mm) were seeded on 0.33% agar in 10% FCS culture medium. The cells were incubated at 37 °C, and 5% CO_2 in humidified incubator for 21 days, followed by staining with p-iodonitrotetrazolium violet (INT) solution for 24 hours. A: MCF-7 cells transfected HA-tagged HMG-Y cultured in the presence of 5 μg/ml of tetracycline (*OFF*); B: MCF-7 cells transfected HA-tagged HMG-Y in the absence of tetracycline (*ON*); C: MCF-7 cells transfected HA-tagged HMG-I cultured in the presence of 5 μg/ml of tetracycline (*OFF*); and D: MCF-7 cells transfected HA-tagged HMG-I in the absence of tetracycline (*ON*).

Figure 3. Over-expression of exogenous HMG-Y in MCF-7 cells leads to formation of tumor nodule in BALB/c (nu/nu) mice. Five BALB/c (nu/nu) mice were injected subcutaneously with 5 x 10^6 MCF-7 cells in PBS; three mice were injected with MCF-7 cells transfected HA-tagged HMG-Y (HMG-Y group) and two mice with MCF-7 cells transfected empty control vector (Control group). Three months after injection, two out of three mice in HMG-Y group have a tumor nodule of about 7 mm in diameter at injection site (**A**) and none of two mice in Control group have tumor nodule (**B**).

Figure 4. Over-expression of HMG-Y alters gene expression with use of cDNA expression arrays. Atlas human cancer cDNA expression array membranes (Clontech, Palo Alto, CA) were side-by-side hybridized with [^{32}P]-labeled cDNA probes prepared from the empty control vector clone M-tet (**A**) or the HMG-Y expressing clone HA-Y-C21 (**B**). The hybridized membranes were exposed overnight to a Phosphorimager screen and the results were analysis with Phosphorimager instrument.

Figure 5. Over-expression of HMG-Y up-regulates gene expression of integrin-β1 and matrix metalloproteinase 13 (MMP13) with use of RT-PCR. The template cDNAs were prepared from the HMG-Y expressing clones, the HMG-I expressing clones and the empty control vector clone M-tet as indicated. Semi-quantitative PCRs were run with mixture of internal control primers (HPRT) and primers specific for intergrin-β1 (**A**) or MMP13 (**B**) in the presence of [^{32}P] dATP. After 25 cycles, the PCR products were separated by electrophoresis on 6% polyacrylamide gel and then scanned with Phosphorimager instrument.

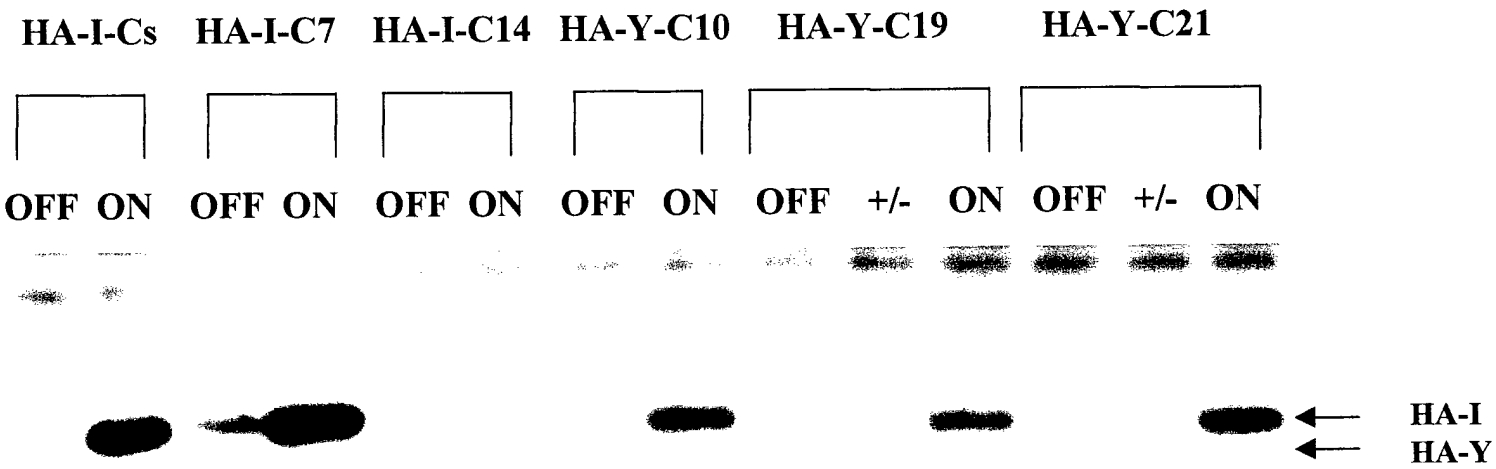
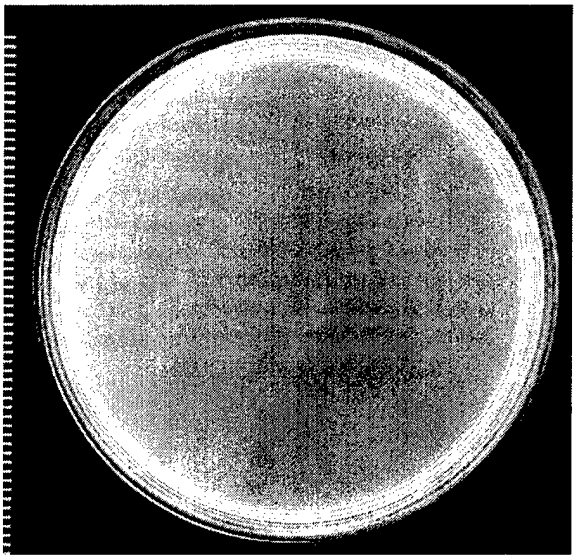

HA-I-Cs
HA-I-C7
HA-I-C14
HA-Y-C10
HA-Y-C19
HA-Y-C21
OFF ON
OFF ON
OFF ON
OFF ON
OFF +/- ON
OFF +/- ON
HA-I
HA-Y

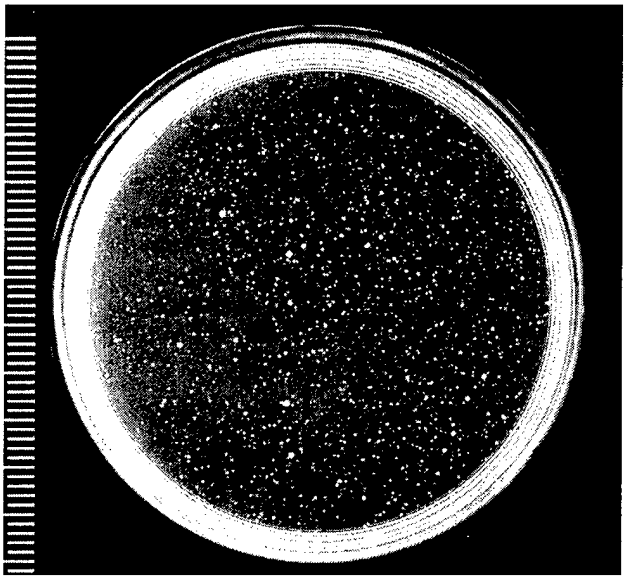
Figure 1

A



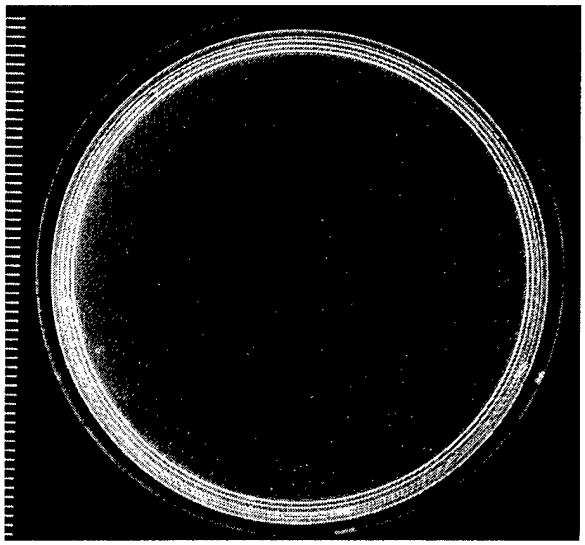
HA-Y/off

B



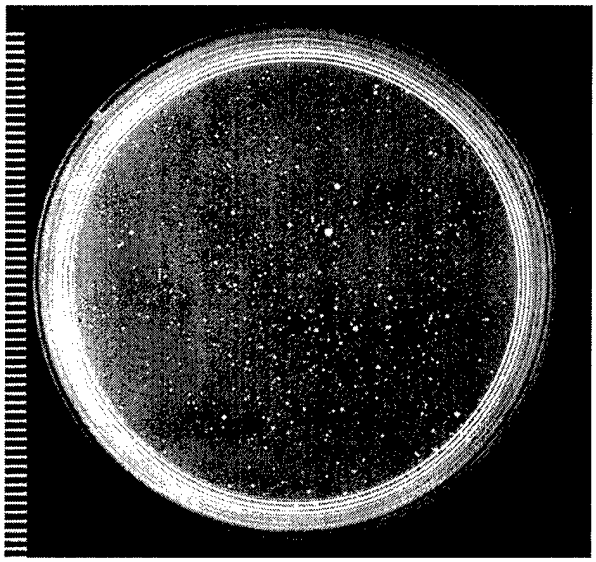
HA-Y/on

C



HA-I/off

D



HA-I/on

Figure 2

A

B

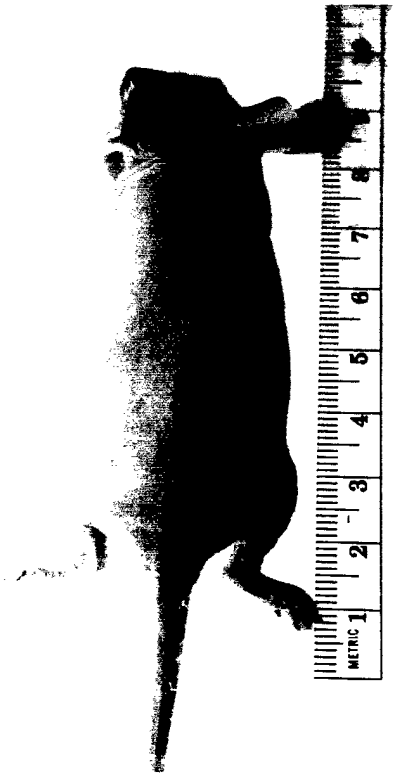


Figure 3

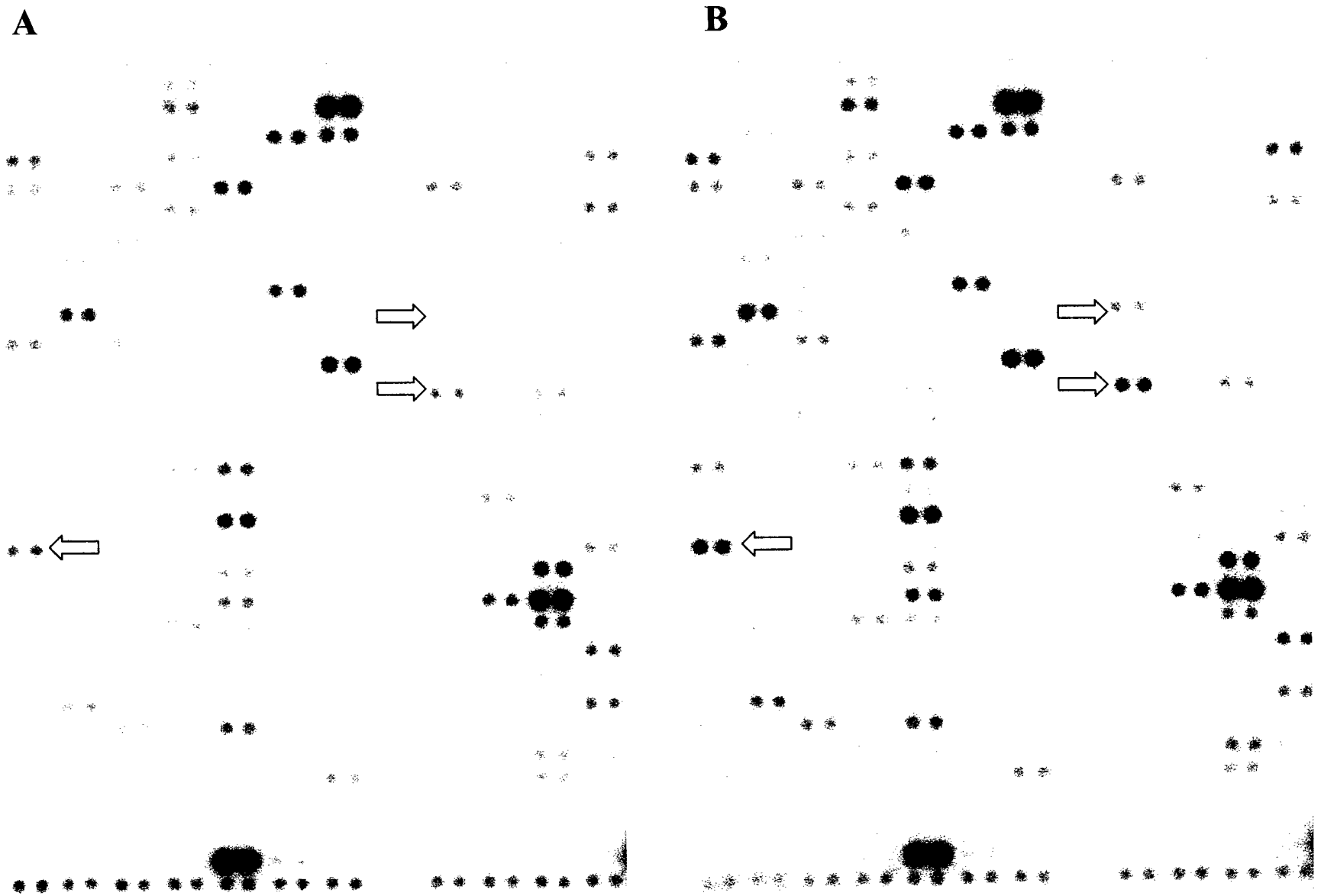


Figure 4

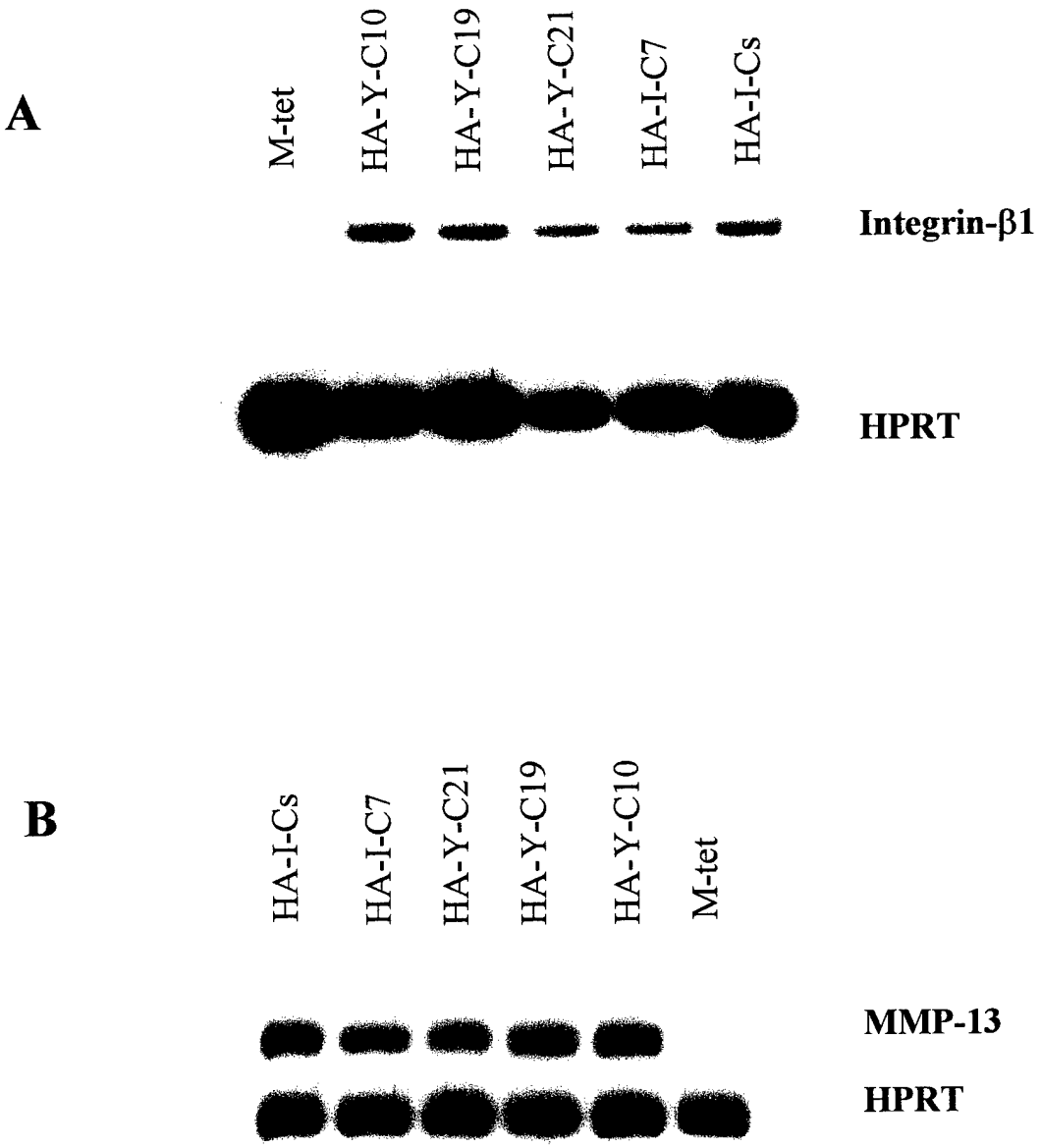

A
M-tet
HA-Y-C10
HA-Y-C19
HA-Y-C21
HA-I-C7
HA-I-Cs
Integrin-β1
HPRT
B
HA-I-Cs
HA-I-C7
HA-Y-C21
HA-Y-C19
HA-Y-C10
M-tet
MMP-13
HPRT

Figure 5

SUMMARY

During the three years of research, especially in the last year, all aspects of work has been met according to the statement of work in the original grant. The key research accomplishments were:

- Expression of endogenous HMG-I(Y) protein correlates to tumor progression in human breast cancer cells and expression of anti-sense of HMG-I inhibits the growth of tumor cells.
- Over-expression of exogenous HMG-I(Y) proteins promotes the growth of tumor cells.
- Over-expression of HMG-I(Y) alters transcription of genes that are associated with tumor progression.

Three papers published or submitted, and two manuscripts in preparation are supported either entirely, or in part, by the Army breast cancer research grant. The tiles of the three enclosed papers are:

1. Cmarik, J.L., Li, Y., Ogram, S.A., Min, HZ., Reeves, R., and N.H. Colburn (1998). Tumor promoter induces high mobility group HMG-Y protein expression in transformation-sensitive but not –resistant cells. *Oncogene* 16, 3387-3396.
2. Li, Y., and R. Reeves (1999) Phosphorylation of mammalian HMG-I(Y) by protein kinase C. *Biochem. Biophys. Res. Commun.*, submitted.
3. Himes, S.R., Reeves, R., Attema, J. Nissen, M., Li, Y., and M.F. Shannon (1999). The role of high mobility group I(Y) proteins in expression of interleukine-2 and T-cell proliferation. *J. Immunol.,* Submitted.

A Ph.D. degree in molecular and cellular biology should be awarded to Y. Li due to the work supported by, at least in part, the Army breast cancer research grant, early in the year 2000.

PHOSPHORYLATION OF THE MAMMALIAN ARCHITECTURAL TRANSCRIPTION FACTOR HMG-I(Y) BY PROTEIN KINASE C

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Abbreviations: CBP, CREB-binding protein; DAG, diacylglycerol; IFN-β, interferon-β; IL-2, interleukin-2; IL-2Rα, interleukin-2 receptor α; IL-4, interleukin-4; Igε, immunoglobulin epsilon gene; IRS, insulin receptor substrate; LT, lymphotoxin; PCA, perchloric acid; PKC, protein kinase C: PS, phosphtidylserine; HMG, high mobility group nonhistone chromatin proteins; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

ABSTRACT:

HMG-I(Y) proteins are architectural transcription factors that play an essential role in regulating the induction or repression of expression of a number of mammalian genes *in vivo*. They are believed to function during inducible gene expression by forming stereo-specific 'enhanceosome' complexes on promoter/enhancer regions that involve both specific protein-DNA and protein-protein interactions. Here we demonstrate that, contrary to a previous report, the mammalian HMG-I(Y) proteins are *in vitro* substrates for phosphorylation by the enzyme protein kinase C (PKC). We also demonstrate that *in vivo* the HMG-I(Y) proteins can be phosphorylated within 30 min of treatment of cells with the PKC activator TPA. These results suggest that the PKC signaling pathway can influence *in vivo* gene transcriptional regulation by rapidly phosphorylating the key mammalian transcription factor HMG-I(Y).

INTRODUCTION

Protein modifications play an essential role in the regulation of most biological processes in eukaryotic cells. For example, the end point of many signal transduction pathways is the phosphorylation or acetylation of transcription factors, modifications that are crucial for cells to interpret and respond to a variety of external stimuli. The architectural transcription factors HMG-I, HMG-Y and HMG-IC belong to the HMG-I(Y) family of "high mobility group" (HMG) non-histone chromosomal proteins (1). HMG-I and HMG-Y proteins are encoded by the same gene and generated through alternative RNA splicing whereas HMG-IC is coded for by a separate, but related, gene at a different locus. All of these proteins (simply referred to as HMG-I(Y), for convenience) share a similar primary modular organization in that they each contain three independent DNA binding domains, called 'A·T-hooks" because of the ability of this motif to specifically bind to the minor groove of AT-rich sequences of DNA, as well having a distinctive acidic C-terminal tail. The HMG-I(Y) proteins are subject to variety of *in vivo* secondary biochemical modifications including phosphorylation and acetylation (2).

The cellular functions of HMG-I(Y) proteins are still not fully understood. However, they have been implicated in the *in vivo* transcriptional regulation of a growing number of genes including LT, IFN-β, IL-2, IL-2Rα and IL-4, among others. In addition, to specifically interacting with A·T-rich DNA, the HMG-I(Y) proteins have been demonstrated to physically interact with many general and tissue specific transcription factors, including NF-κB, c-Jun, SRF, RAR, Oct2, Oct6, ATF2, and Elf-1

(3-11). The HMG-I(Y) proteins also have the ability to bend, unwind and supercoil DNA substrates *in vitro* (12, 23), as well as being able to induce changes in the rotational setting of DNA on the surface of isolated nucleosomes (13). As a result of their ability to recognize and modulate DNA structure, as well as engage in specific protein-protein, the HMG-I(Y) proteins are thought to play an essential role in gene transcriptional regulation by participating in the formation of stereo-specific complexes called 'enhanceosomes' on their cognate promoter/enhancer regions (4, 11, 14). The HMG-I(Y) proteins have also been demonstrated to derepress histone H1-mediated inhibition of transcription *in vitro* and to be strongly enriched in histone H1-depleted active chromatin *in vivo* (15). Consequently, HMG-I(Y) proteins have been postulated to replace histone H1 from nucleosomal chromatin during the process of gene induction thus allowing other transcription factors to gain access to their DNA binding sites so that gene-specific enhancesomes can be formed and transcriptional activity regulated. Recently, it has been reported that acetylation of HMG-I(Y) by the CBP (CREB binding protein) histone acetyltransferase disrupts an enhanceosome complex and turns off expression of IFN-β gene (16). Besides being modified by acetylation, human HMG-I(Y) proteins can also be extensively phosphorylated by cdc2 kinase *in vivo* in a cell-cycle dependent manner (17) and by MAP kinase (18) as well as by casein cinase II *in vitro* (19). The known consequence of phosphorylation of HMG-I(Y) *in vitro* is to reduce the affinity of binding of the protein to the naked DNA. Phosphorylation of HMG-I(Y) has also been proposed to be associated with controlling the proliferation of myeloid progenitor cells (20) and other cell types (21). In the case of the myeloid progenitor cells, this effect is thought to be mediated through the insulin receptor motif (I4R motif) in the promoter/enhancer

region of the IL-4Rα gene after IL-4 stimulation of cells. In response to IL-4 stimulation of the cell proliferation, the I4R motif recruits insulin receptor substrate (IRS)-1 and IRS-2 proteins and results in phosphorylation of HMG-I(Y) proteins in a pathway that is independent of activation of STAT proteins (20).

Recently, it has been reported that insect homologs of the mammalian HMG-I(Y) proteins can be phosphorylated *in vitro* by the enzyme protein kinase C (PKC) (18). A question remains, however, as to whether the mammalian HMG-I(Y) proteins are likewise substrates for PKC phosphorylation either *in vitro* or *in vivo*. Earlier work by Palvimo et. Al. (22), for example, indicated that the mammalian HMG-I(Y) proteins are not *in vitro* substrates for PKC phosphorylation. Nevertheless, inspection of the amino acid sequences of the human HMG-I(Y) proteins indicate that they contain multiple sites that correspond to known PKC phosphorylation consensus motifs (see below), suggesting that these proteins may, indeed, be substrates for PKC enzymatic modification. We, therefore, re-examined the possibility that the human HMG-I(Y) proteins might be substrates for phosphorylation by PKC, both *in vitro and in vivo*. In this report, we shown, for the first time, that mammalian HMG-I(Y) proteins are not only efficient *in vitro* substrates for phosphorylation by PKC but are also rapidly phosphorylated *in vivo* within 30 minutes of treatment of cells with the PKC activating agent12-O-tetradecanoyl-phorbol-13-acetate (TPA). These observations reopen the possibility that PKC, one of the essential signal pathways determining cell fate, regulates the function of mammalian HMG-I(Y) proteins *in vivo*.

MATERIALS AND METHODS

In vitro phosphorylation by PKCα. Each phosphorylation reaction contained 0.5 ug protein, 2U of PKC enzyme (Calbiochem, Inc. CA, Cat# 539494) and a buffer consisting of 20mM HEPES (pH7.4), 1 mM DTT, 10mM $MgCl_2$, 200ug/ml phosphtidylserine (PS), 10ug/ml diacylglycerol (DAG), 1 mM $CaCl_2$ and 0.15mM [γ-^{32}P]-ATP (0.05Ci/ml). The protein substrates were either histone H1, HMG-I, or HMG-Y, or a truncated form of the HMG-I protein containing only amino acid residues 50-91 (26). The enzymatic enreactions were carried out at 30°C for 10 minutes and terminated with 1.5% phosphoric acid. The terminated reactions then were run on 12% SDS/PAGE and exposed to an X-ray film.

Cell Culture and *in vivo* Labeling. Human breast cancer Hs578T cells were obtained from the American Type Culture Collection (ATCC) and were grown according to the suppliers instructions. The growing cells were labeled with inorganic [^{32}P] phosphate (Dupont) for the final 15 minutes of either 0, 30 or 60 minutes treatment with 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment at a final concentration of 5 ng/ml. Following ^{32}P-labeling, cells were washed with phosphate buffered saline (PBS) and the proteins were extracted by 5% perchloric acid (PCA) on ice. The PCA soluble proteins were precipitated with 25% final concentration of trichloroacetic acid to obtain a fraction containing histone H1 and the HMG proteins (23).

RESULTS and DISCUSSION

HMG-I(Y) proteins contain multiple PKC consensus sites and are substrates for PKC phosphorylation *in vitro*.

PKC plays a critical role in cell proliferation, differentiation, neoplastic transformation and apoptosis (for reviews, see: 24, 25). The influence of PKC in these processes is believed to be mediated through phosphorylation of a large number of substrates including membrane receptor molecules such as the insulin receptor and the EGF receptor, other kinase enzymes, such as $p21^{ras}$, raf-1, MAP kinase and PKA, as well as nuclear transcription factors such as Jun, Fos, myc, CREB and p53. Substrates for PKC contain 'consensus' peptide motifs that are specifically recognized and phosphorylated by the enzyme, including: S/TXK/R, K/RXXS/T, K/RXXS/TXK/R, K/RXS/T or K/RXS/RXK/R (X can be any amino acid) (24). As shown in **Fig.1A**, HMG-I and HMG-Y proteins contain either eleven or ten potential PKC phosphorylation sites, respectively. To determine whether the HMG-I(Y) proteins are the substrates of PKC *in vitro*, we preformed ^{32}P-labeling of purified recombinant HMG-I(Y) proteins with PKC enzyme isolated from rat brain (Calbiochem, Inc. CA). As shown in **Fig.1B**, PKC phosphorylates both HMG-I and HMG-Y proteins as efficiently as does it does histone H1 *in vitro*. Furthermore, a truncated form of the HMG-I protein containing only amino acid residues 50 to 91 (26) is also phosphorylated by PKC indicating that residues Thr53, Ser64 and Thr72 are the potential PKCα phosphorylation sites in this peptide fragment.

These results are seemingly at odds with an early report by Palvimo, et al (22) who failed to observe phosphorylation of the HMG-I(Y) proteins by the PKC enzyme *in vitro*. The reason for this apparent conflict are unknown but may be due to a difference

in the isoforms of the PKC enzyme utilized in the different experiments. It is now known that there are at least 12 different PKC isoform enzymes that can be sub-classified into three groups: conventional PKCs (cPKC) enzymes, novel PKCs (nPKC) enzymes and atypical PKCs (aPKC) enzymes (24, 25). The cPKC group of enzymes contains the α, β_I, β_{II}, γ isoform proteins which require phosphtidylserine (PS), Ca^{2+} and diacylglycerol (DAG or phorbol esters) for activation of their full catalytic potential; the nPKC group of enzymes contains the δ, ε, η, θ, μ isoform proteins which require both PS and DAG for their full activation; and the aPKC group of enzymes that contains the ζ, ι, λ isoforms which only require only the presence of PS for full activation. In the earlier work by Palvimo et al (22), the authors used rat brain PKC (without indicating the specific isoforms of the enzyme) with only Ca^{2+} and PS being present in the reaction mixtures. Under these this condition, the authors were able to phosphorylate the histone H1, HMG-14, and HMG-17 proteins but not the HMG-I(Y) proteins. In the present work, we phosphorylated histone H1 and HMG-I(Y) proteins by using fully activated PKCs isolated from rat brain in the present of Ca^{2+}, PS and DAG. Taken together, the results from these two different laboratories strongly suggest that HMG-I(Y) proteins can be phosphorylated by the cPKC group of enzymes and/or by the nPKC group but probably not by the aPKC group of enzymes. In contrast, histone H1 protein can be phosphorylated by all three groups of PKC enzymes *in vitro*.

Phosphorylation of HMG-I(Y) proteins can be rapidly induced *in vivo* by TPA.

The major cellular effect of short-term treatment of mammalian cells with tumor promoting phorbol esters, such as TPA, is to activate PKC enzyme activity *in vivo* (24).

This effect is due to the structure of TPA which is similar to that of the PKC activator molecule DAG; therefore, TPA can substitute for DAG and directly activate PKC both *in vitro* and *in vivo* (27, 28, 29). To test whether HMG-I(Y) proteins can be phosphorylated in response to TPA stimulation, we preformed *in vivo* ^{32}P-labeling of human breast cancer Hs578T cells following different periods of exposure of the cells to the phorbol ester. As shown in Fig.2, unstimulated Hs578T cells contain a low basal level of endogenously phosphorylated HMG-I(Y) proteins (lane 1). However, following treatment of the cells with TPA for 30 minutes (with radiolabeling occurring during the last 15 minute of exposure), the level of phosphorylated HMG-I(Y) increases more than 10 fold and remains at this continuously high level for up to one hour of TPA treatment (lane 3). Interestedly, in the same cells the level of phosphorylated histone H1 increases only slightly after 30 minutes of TPA treatment and returns to the basal level of modification after 1 hr of phorbol ester exposure (compare lane 1 and lane 3).

HMG-I(Y) gene has been described as a 'delayed early response gene' whose transcriptional expression is induced with 1-2 hours following exposure of cells to exogenous growth factors (30). It has also been well documented that TPA induces HMG-I(Y) mRNA expression in human lymphoma HUT-78 T cells (31), human erythroleukema K562 cells (22), human breast cancer Hs578T cell (33) and JB6 murine P+ cells (34) within 2 hours of exposure to the phorbol ester and that this induction depends on continued protein synthesis. In the present work, we extend these earlier observations by demonstrating that, in addition to its effect on the induction of HMG-I(Y) mRNA expression, treatment of cells with TPA also rapidly induces increased levels of HMG-I(Y) protein phosphorylation. Since this induced phosphorylation occurs with

30 minutes of exposure of cells to TPA, the results strongly imply that phosphorylation of HMG-I(Y) proteins is an immediate downstream event resulting from PKC enzyme activation by phorbol esters or other factors.

Although there is evidence suggesting that phosphorylation of HMG-I(Y) is linked to both the induction of cellular proliferation and to the transcriptional activation of certain genes, the precise molecular and cellular effects of phosphorylation of the HMG-I(Y) proteins *in vivo* are not known. It is know from both *in vitro* and *in vivo* studies, however, that phosphorylation of the HMG-I(Y) proteins by the enzyme cdc2 kinase reduces the affinity of binding of these proteins for DNA substrates, thus implicating such protein phosphorylations in the alterations in chromatin and chromosome structure that occur during the cell cycle {reviewed in 21, 35). A considerable body of evidence also indicates that, acting as architectural transcription factors, the HMG-I(Y) proteins can function *in vivo* to either activate or repress the expression of constellations of genes in response to different external stimuli. In some cases where HMG-I(Y) is involved in transcriptional repression, for example of the promoters of the Igε and IL-4 genes (36, reviewed in 37}, stimulation of phosphorylation of the HMG-I(Y) by exposure of lymphoid cells to the cytokine IL-4 leads to induced transcription activation of the genes (36, 37). This induced switch from inhibition to activation of gene expression by modulation of the phosphorylation state of the HMG-I(Y) proteins has been suggested to result from a decrease in the DNA binding affinity of the of phosphorylated HMG-I(Y) proteins. Nevertheless, it should be noted that during the process of gene activation, the HMG-I(Y) proteins not only bind to DNA but also interact with other transcription factors to form promoter-specific complexes that involve

precise protein-protein interactions. It is therefore reasonable to propose that external stimulus-induced phosphorylations of the mammalian HMG-I(Y) proteins by kinases such as PKC, MAPK or casein kinase II may affect these specific protein-protein interactions and thereby directly influence the transcriptional activity of genes *in vivo*. These possibilities are currently being investigated.

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FIGURE LEGENDS

Figure 1: Phosphorylation of recombinant HMG-I protein by PKC *in vitro*.

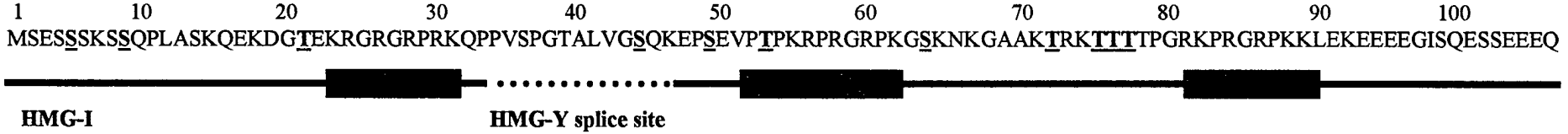
A: **Diagram of amino acid sequence of HMG-I (Y) protein. The DNA binding domains of HMG-I(Y) are shown as shaded blocks and the amino acids potentially phosphorylated by PKC are shown in bold, underlined letters. The amino acids missing from the HMG-Y isoform protein as a result of alternative mRNA splicing are indicated.**

B: Autoradiogram of *in vitro* ^{32}P-ATP-labeled proteins enzymatically phosphorylated by rat brain PKC and separated on a 12% SDS /PAGE gel. The protein samples are: histone H1 (Lanel and Lane 3); recombinant HMG-I (Lane 2); recombinant HMG-Y (Lane 4); truncated recombinant HMG-I(Y) peptide containing a.a. residues 50-90 (Lane 5); recombinant GST-50-75 a.a. peptide (Lane 6); and, GST-peptide only (Lane 7).

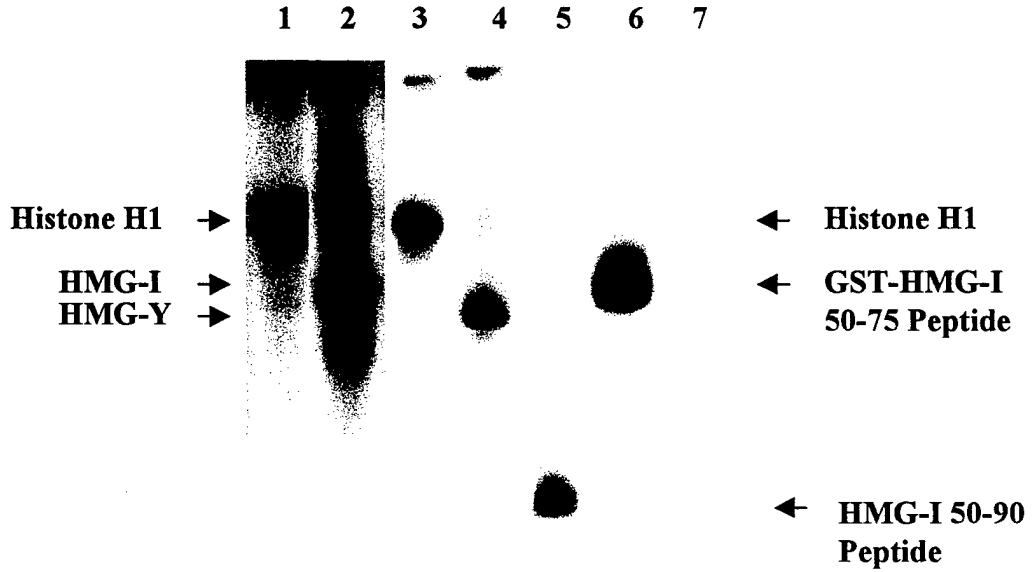
Figure 2: Rapid mitogenic induction of HMG-I(Y) phosphorylation in human breast Hs578T cells following stimulation with the PKC activating phorbol ester TPA.

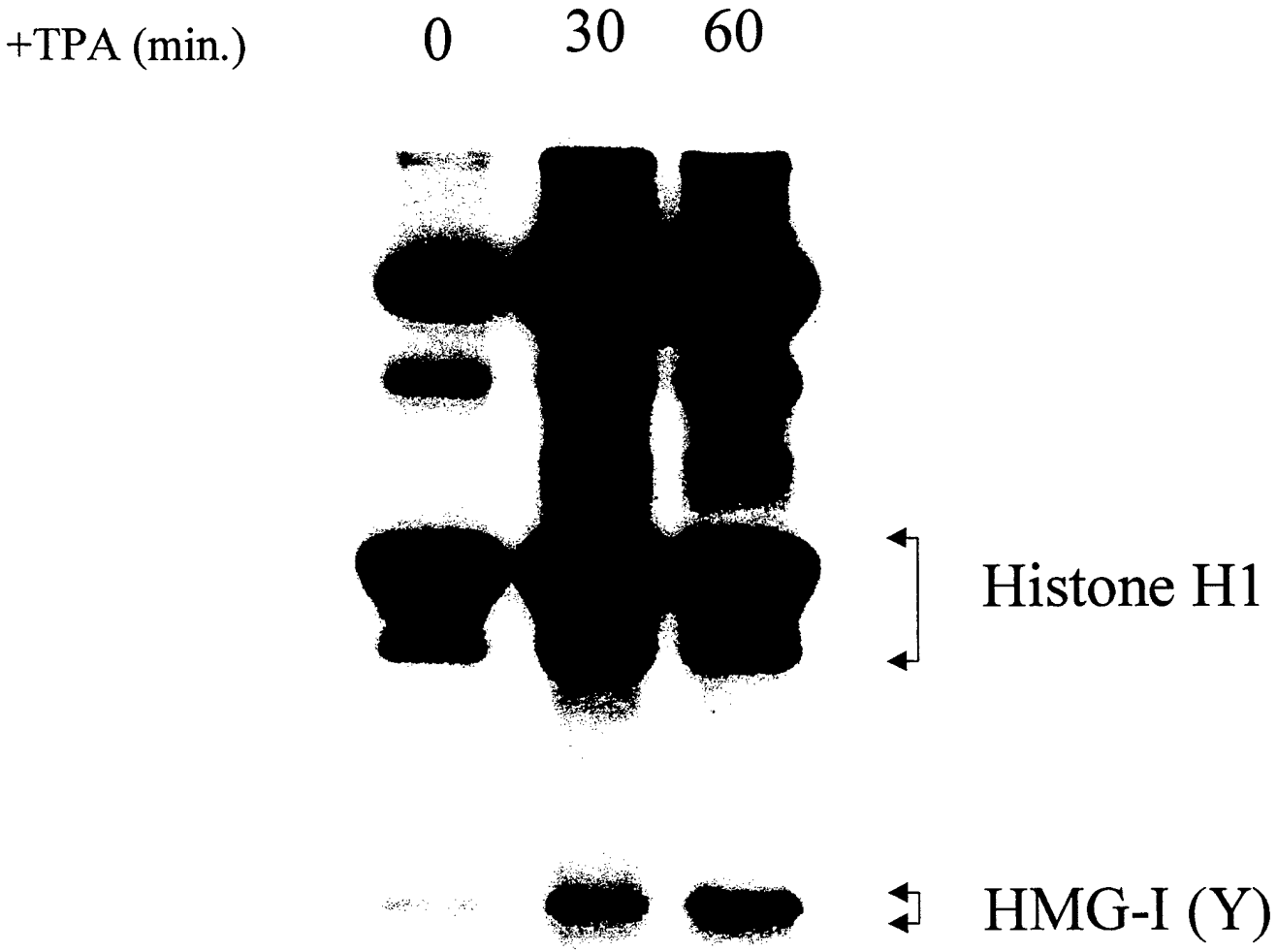
Autoradiogram of *in vivo* ^{32}P-orthophosphate -labeled total, 5% PCA extracted, proteins isolated from Hs578T and separated on a 12% SDS /PAGE gel. Lane 1, control cells without TPA stimulation labeled for 15 min with ^{32}P-orthophosphate; Lanes 2 and 3, cells treated with TPA (5 ng/ml) for either 30 or 60 min, respectively, and labeled with labeled for the last 15 min with ^{32}P-orthophosphate.

A


1 10 20 30 40 50 60 70 80 90 100
MSESSSKSSQPLASKQEKDGTEKRGRGRPRKQPPVSPGTALVGSQKEPSEVPTPKRPRGRPKGSKNKGAAKTRKTTTTPGRKPRGRPKKLEKEEEEGISQESSEEEQ
HMG-I
HMG-Y splice site

B


1 2 3 4 5 6 7
Histone H1
HMG-I
HMG-Y
Histone H1
GST-HMG-I
50-75 Peptide
HMG-I 50-90
Peptide


+TPA (min.)
0
30
60
Histone H1
HMG-I (Y)

RUNNING TITLE: HMGI(Y) and IL-2 gene transcription

The Role of High Mobility Group I(Y) Proteins in Expression of Interleukin-2 and T-cell proliferation[1]

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Keywords: T lymphocytes, gene regulation, transcription factors, cytokines, human.

ABSTRACT

The HMGI(Y) family of proteins plays an important architectural role in chromatin and have been implicated in the control of inducible gene expression. We have previously shown that expression of HMGI antisense RNA in Jurkat T cells inhibits the activity of the interleukin-2 (IL-2) promoter. Here we have investigated the role of HMGI(Y) in controlling IL-2 promoter-reporter constructs as well as the endogenous IL-2 gene in both Jurkat T cells and human peripheral blood lymphocytes. We found that the IL-2 promoter has numerous binding sites for HMGI(Y) which overlap or are adjacent to the known transcription factor binding sites. HMGI(Y) modulates binding to the IL-2 promoter of at least three transcription factor families, AP-1, NFAT and NF-kB. By using a mutant HMGI that cannot bind to DNA but can still interact with the transcription factors, we found that DNA binding by HMGI was not essential for the promotion of transcription factor binding. The non-DNA binding mutant, however, acts as a dominant negative protein in tranfection assays suggesting that the formation of functional HMGI(Y) containing complexes requires DNA binding as well as protein:protein interactions. The alteration of HMGI(Y) levels affects IL-2 promoter activity not only in Jurkat T cells but also in peripheral blood lymphocytes. Importantly, we also show here that expression of the endogenous IL-2 gene as well as proliferation of peripheral blood lymphocytes are affected by changes in HMGI(Y) levels. These results demonstrate a major role for HMGI(Y) in IL-2 expression and hence T cell proliferation.

Introduction

Activation of T helper cells requires the specific binding of the T cell receptor (TCR) to an antigen-major histocompatibility class II complex on the surface of antigen presenting cells (APCs) as well as costimulation through the interaction of cell surface molecule pairs such as CD28 on T cells and B7 on APCs (1,2). Signal transduction through the TCR and CD28 induces the activation and nuclear expression of a number of transcription factors, including NF-κB/Rel, NFAT and AP-1 family proteins (reviewed in 3,4). This cascade of events results in the coordinate expression of a variety of genes including the cytokine interleukin 2 (IL-2), which functions as a major growth factor for T cells, and expression of IL-2 receptor α (IL-2Rα), which allows the formation of a high affinity receptor for IL-2. The resulting autocrine loop can act as a strong growth stimulus allowing a specific clonal expansion of the T cell.

The requirements for activation of IL-2 gene expression in response to T cell activation have been studied in detail. Initially two major TCR responsive regions were identified in the IL-2 promoter (reviewed in 3,5). These were termed antigen response elements (ARRE), i.e., ARRE-1 (or NFIL-2A) and ARRE-2 (or NFIL-2E). The ARRE-2 region was the first to be identified as a composite binding site for NFAT and AP-1 proteins, whereas ARRE-1 was thought to function via the binding of AP-1 and Oct transcription factors (6-10). Recently, the ARRE-1 region has been found to bind a complex of NFAT and AP-1 as well as Oct (11). Several other regions of the promoter have also recently been identified as binding sites for NFAT alone or as complexes with AP-1 (11). The mechanism of costimulation through the CD28 receptor involves

enhanced nuclear expression of NF-κB and c-Rel transcription factors (12-14) as well as phosphorylation of c-Jun by JNK kinase, resulting in increased transactivation competence for AP-1 (15,16). The region of the IL-2 gene that responds to CD28 (the CD28RR) is composed of a variant NF-κB site (referred to as the CD28RE), which has a high affinity for c-Rel containing complexes, as well as an adjacent AP-1 site. These elements cooperate to lead to increased IL-2 promoter activity (17-20).

The highly inducible nature of the IL-2 promoter seems to be the result of coordinate binding of many transcription factors to their recognition sequences on the promoter leading to the assembly of a functional unit (3,4,11,14,21-24). Such a unit has been termed an enhanceosome on the interferon beta (IFNβ) promoter (25). The family of nuclear proteins known as HMGI(Y) are known to play a major role in the assembly of the IFNβ enhanceosome (26-28). We have previously shown that HMGI(Y) is essential for the selective binding of c-Rel to the IL-2 CD28RE and that modulating HMGI(Y) levels affects not only the activity of the CD28RR but of the entire promoter (14).

HMG-I(Y) proteins are small nonhistone nuclear proteins that bind the narrow minor groove of A:T sequence rich B form DNA and have numerous modes of action by which they modify gene transcription (reviewed in 29). The HMG-I(Y) family of proteins consist of three members, HMG-I and HMG-Y which are produced by alternative splicing of mRNA from the same gene locus, and HMGI-C, which is coded for by a related gene at a separate locus (29). These proteins have been classed as architectural transcription factors because they do not act as transactivators in their own right but modify the function of other proteins (30). HMG-I(Y) proteins can

interact directly with several families of transcription factors including Rel/NF-κB, bZip, Ets, homeodomain and Pou domain proteins and lead to an alteration in their DNA binding to sites that either overlap or are adjacent to A:T rich HMG-I(Y) binding sites (29). The binding of HMG-I(Y) also induces structural changes in DNA substrates (31,32) that, in turn, often leads to alterations in the assembly of transcription factors into higher order functional complexes (29). In addition, HMG-I(Y) appears to play a critical role in chromatin architecture (33) and has been shown to interact specifically with isolated nucleosome core particles (34), to alter the rotational setting of DNA on the surface of nucleosomes (35) and to antagonize H1-mediated transcriptional repression (36,37).

Here we show that HMGI(Y) can bind to numerous sites across the IL-2 proximal promoter region and modulate the binding of the major transcription factor families that are thought to control IL-2 gene transcription. Functional studies in both Jurkat T cells and primary T cells show that HMGI(Y) plays a major role in the regulation of the IL-2 gene and hence T cell proliferation.

Materials and Methods

Cell culture and transfection.

The basic medium for Jurkat cell culture was RPMI medium containing 10% fetal calf serum (FCS), supplemented with L-glutamine and penicillin-gentamycin antibiotics (RPMIJ). The basic medium for PBL culture was as above but contained 20% FCS, 100μM β-mercaptoethanol and 5% conditioned media (RPMIL). Mononuclear cells were isolated from peripheral blood using lymphoprep (Nycomed Pharma AS) and cultured for 4 days in RPMIL and PHA (5μg/ml) (Boehringer Mannheim). Cells were then stimulated with 5ng/ml of IL-12 (Pharmingen) for 4 hours to promote transition to G_1 and panned to remove macrophages. Non-adherent PBLs were pelleted and resuspended in RPMIL at $1x10^7$ cells in 400μl media. PBLs were assayed for CD3, CD4 and CD8 expression using the Cyto-Stat assay kit from Coulter. 94% of the cells were CD3 positive, 74% were CD4 and 20% CD8 positive. Cells were transfected by electroporation using a BioRad Gene Pulser II at 290V with a capacitance of 975μfarad. Jurkat cells were resuspended at $5x10^6$ cells in 400μl media and electroporated at 270V, 975μfarad capacitance. The efficiency of transfection was determined using the pCMVGFP expression plasmid and flow cytometry (Epics XL-MCL; Coulter) and ranged from 2 to 4% GFP positive in PBLs and 15 to 25% GFP positive in Jurkat cells. Transfected cells were incubated in RPMIL with 10% conditioned media (PBLs) or RPMIJ media (Jurkats) for 24 hours. Cells were then sorted for high fluorescence using a Facstar Plus cell sorter (Becton Dickinson) and the top 1 to 2% for PBLs and 10 to 15% for Jurkats collected and used in IL-2 ELISA and proliferation assays.

Reporter Assay, ELISA and proliferation assay.

pRcCMV and pcDNA3.1/Zeo were obtained from Invitrogen and pRcCMVIGMH, pIL-2luc and pHIVluc have been previously described (14). Standard site-specific mutagenesis procedures, the details of which will be reported elsewhere (Li et al.; unpublished data), were used to create a non-DNA binding mutant form of the HMGI protein designated HMGI(mII,mIII) starting with the wild-type human HMGI cDNA, clone 7C (38). Briefly, HMGI(mII,mIII) had four proline to alanine substitutions introduced at amino acid residues # 57, 61, 83 and 87 located in its second and third DNA-binding domains, the primary regions of the protein that interact with the minor groove of A:T-rich substrates (39). As a consequence, the recombinant HMGI(mII,mIII) protein lacks the ability to specifically bind with high affinity to A:T-rich DNA sequences *in vitro* but retains its ability for specific protein-protein interactions with other transcription factors (Li et al.; unpublished data). For expression in transfected mammalian cells, the mutant HMGI(mII,mIII) cDNA was subcloned into the pcDNA3.1/Zeo plasmid vector (Invitrogen) and used as described above. The HMGY cDNA was also subcloned into pcDNA3.1 to generate pcDNAHMGY for expression of the protein in cells. Jurkat cells used in reporter assay were cotransfected with 5μg of pIL-2luc or pHIVluc together with pRcCMVIGMH, pcDNAHMGI(mII,mIII) or pcDNAHMGY, in combination with the amount of parent plasmid needed to normalize DNA at 10μg. At 24 hours posttransfection, cells were pelleted and resuspended in phenol red free RPMIJ media at $1x10^6$ cells/ml. 100μl of cells/well were plated in 96 well culture plates and stimulated with 50ng PMA and 1μM Ca^{2+}Ionophore (P/I) (A23187; Boehringer Mannheim,) or with the above and a

1/10,000 dilution of ascites fluid containing activating antibody to the CD28 receptor (α-CD28) (Bristol Myer Squibb) for 9 hours. Cells were then harvested and assayed for luciferase activity using a 96 well plate Luminometer (TopCount; Packard) as previously described (14). PBLs were cotransfected with 5μg of pIL-2luc and either 10μg of pRcCMVIGMH, pcDNAHMGI(mII,mIII) or pcDNAHMGY and the appropriate parent plasmid. At 24 hours posttransfection, cells were stimulated with PHA (5μg/ml) for 16 hours. Cells were then harvested and cell extracts assayed for luciferase activity as previously described (40).

ELISA and proliferation assays were performed with PBL or Jurkat cells transfected with 10μg of the appropriate expression plasmid together with 5μg of pCMVGFP for cell sorting. $1x10^4$ sorted cells in 100μl were plated in 96 well tissue culture plates and stimulated with either PHA for 24 hours (PBL) or P/I+α-CD28 for 8 hours (Jurkat) and supernatants assayed for IL-2 using a Quantikine ELISA kit (R&D Systems). Proliferation was assayed in similarly treated cells using the chemiluminescent bromodeoxyuridine incorporation kit from Boehringer Mannheim.

Production and Purification of Recombinant Proteins

Recombinant hexahistidine-tagged full-length Fos and Jun proteins, as well as truncated Fos116-211 and Jun224-334 proteins, were prepared and purified by affinity chromatography using a Ni-NTA-agarose column (Qiagen) as described (41). Fos and Jun proteins were co-renatured *in vitro* into the heterodimer transcription factor AP-1 by step-wise dialysis from 6M urea with the final buffer containing 25 mM sodium phosphate pH 7.6, 5% glycerol and 5 mM DTT (42). Full-length recombinant human HMGI protein (i.e., the unspliced member of the HMGI(Y) protein family;29) was

produced using the expression vector pET7C carrying the wild-type human HMGI cDNA38) as described (43,44). NFATp and c-Rel were prepared as described (45; Shang et al., in press). The purity of each recombinant preparation was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (46.) Protein concentrations were determined spectrophotometrically employing either a BioRad (Richmond, CA) protein assay kit or using the extinction coefficient ε_{220} = 74,000 l/mol·cm for wild type HMGI protein (47).

Preparation of Nuclear Extracts.

Nuclear extracts were prepared from Jurkat T cells stimulated with PMA (20ng/ml), Ca^{2+} ionophore (1μm) and CD28 antibody (1:10,000) for 1h or 6h. Nuclear extract preperation was as previously described (14).

***In vitro* DNase I Footprinting**

A cloned 410 bp Xho-I/HindIII restriction fragment encompassing nucleotides (nts) -360 to +50 of the human IL-2 gene proximal promoter (48) was the starting fragment for use in *in vitro* protein footprinting. Promoter subfragments were isolated by either selective restriction enzyme digestion or PCR amplification techniques (46). A fragment from –180 to –60 was used for some of the footprinting experiments. Standard gel electrophoretic procedures (46) were used to isolate all DNA fragments followed by purification on a QIAGEN column as described by the manufacturer (QIAGEN Inc., Chatsworth, CA). Restriction enzyme fragments were 5'-end radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The ends of PCR

fragments were selectively radiolabeled by incorporating, during the final few cycles of the amplification reaction, either one or the other of the two PCR primers that had been 5' radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Footprinting of both the HMGI and AP-1 recombinant proteins on promoter DNA fragments radiolabeled on one 5' end employing the nuclease DNase-I followed published protocols (35,44). For each protein and DNA substrate, optimal conditions for footprinting were empirically determined. Single-stranded DNA cleavage products were then separated by electrophoresis on a 6 % sequencing gel with Maxam-Gilbert "G-lane" chemical cleavage products of control DNA fragments serving as reference standards (46). Band intensities were quantitatively analyzed using a PhosphorImager machine and ImageQuant software (Molecular Dynamics Corp., Sunnyvale, CA).

Electrophoretic mobility shift assays

Oligonucleotides for gel shift analysis had the following sequences:

IL-2 CD28RR, 5'TGGGGGTTTAAAGAAATTCCAGAGAGTCATCAG3';

CD28APm, 5'TCCAGAAATTCCAAAGAGagATCAGAGAT3';

CD28REm, 5'TCCAGccATTCCAAAGAGTCATCAGAGAT3'

IL-2NF-IL2A, 5'AAGTCTTTGAAAATATGTGTAATATGTAAAACATTTTGA3';

GM170, 5'GATCCTGTAGGAAACAGGGGCTTGAGTCACTCCAG3';

TRE, 5'GTGGAATTCGGGGAAGTGACTCAGCGCTCGGACG3'

Double stranded oligonucleotides were end labeled using T4 polynucleotide kinase (40). Affinity purified recombinant NFATp and truncated Fos and Jun proteins were used in binding reactions with the NF-IL2A or GM170 radiolabelled oligonucleotides (0.1ng) in a buffer containing 20mM Hepes, 5mM KCl, 20mM NaCl, 2mm $MgCl_2$,

2mM DTT and 5μg purified bovine serum albumin (New England Biolabs). Protein:DNA complexes was resolved on a non-denaturing 5% polyacrylamide gel containing 0.5xTBE buffer (0.5mM Tris, 42mM Boric acid, 1mM EDTA [pH 8.3]). Binding of recombinant proteins to the CD28RR was carried out in 10mM Tris pH7.5, 10mM $MgCl_2$, 5mM EDTA pH7.5, 10mM DTT, 0.2% NP-40, 1% glycerol, 0.4% sucrose, 0.5 mg/ml BSA and 100ng poly dG:dC in 20μl reactions. 0.2ng of radiolabelled probe was generally used with the amounts of recombinant proteins indicated for individual experiments. Reactions were separated on 5% 0.5x TBE polyacrylamide gels. EMSAs for nuclear extract were carried out as previously described except that 5% glycerol was used instead of Ficoll and 0.5mM PMSF was also added. Competitor double stranded oligonucleotides or antibodies was added to the binding reactions and incubated for 10 min prior to the addition of the radiolabelled probe.

Western Blot Analysis

For western blot, Jurkat cells were transfected, sorted and stimulated as above, except $1x10^6$ cells were recovered from the GFP positive population. A similar analysis using PBLs was not feasible due to the low transfection efficiency of these cells and the large numbers of cells required for the assay. Nuclei were isolated from cells and HMG I(Y) proteins were extracted with 5% perchloric acid and precipitated with trichloroacetic acid (44). Proteins were separated on 18% denaturing polyacrylamide gels and HMGI(Y) detected by western blot using a 1:250 dilution of a rabbit polyclonal anti-HMGI(Y) antibody (50). The ECL chemiluminescent detection system

(Amersham) was used and the resultant bands detected by x-ray film and scanned by densitometry (BioRad).

RESULTS

HMG-I(Y) binds to multiple elements within the IL-2 promoter

We have previously shown that transcription from a transfected IL-2 promoter reporter construct was inhibited by coexpression of antisense RNA for HMG-I(Y) proteins (14). A comparison of the sensitivity of the promoters for a number of T cell-expressed genes (IL-2, IL-3, GM-CSF and the HIVLTR) to HMG-I(Y) depletion found that IL-2 was the most significantly affected by changes in HMG-I(Y) levels (data not shown). The IL-2 proximal promoter contains approximately 65% A:T base pairs and has many potential binding sites for HMG-I(Y) with A:T sequences present within or adjacent to several known transcription factor binding sites. In order to identify the binding sites for HMGI(Y) on the IL-2 promoter, DNase-I footprinting was performed on a fragment of the IL-2 promoter from nt # –360 to +50 (Fig. 1). DNase I footprinting was carried out on both DNA strands by labelling each end of the DNA fragment independently. As shown in Fig. 1A and B, footprints for HMG-I(Y) were observed at a number of locations across the proximal promoter (indicated by vertical bars and filled arrowheads). Fig 1A represents footprints obtained with the DNA labelled at –360 and in Fig1B the DNA was labelled at +50. Fig. 1C indicates the location of these HMG-I(Y) binding sites in relation to the sequence and the known transcription factor binding sites on the promoter. Since different binding sites within the promoter have different affinities for HMG-I(Y) (data not shown), only those sites that are consistently seen in independent replicate experiments are indicated. The HMG-I(Y) footprints are within or adjacent to the –45 NFAT/TATA-2 site (11), the NFIL-2A region, the NFIL-2B region, the CD28 response region (CD28RR) and the –285 NFAT site in ARRE-2. All of these elements have been shown to be important in IL-2 promoter activity (reviewed in 3,5). Interestingly, at the concentrations used in this assay, HMG-I(Y) was also observed to specifically footprint at the –30 TATA-1

sequence (Fig. 1), a region of the IL-2 promoter that is known to be protected *in vivo* by proteins in both unstimulated and stimulated T cells (21). As a control for these experiments, a Fos/Jun (AP-1) heterodimer was also footprinted to the promoter DNA, both alone and in combination with the HMG-I(Y) protein (Fig. 1A and B). AP-1 was used at high concentrations in Fig 1A and lower concentrations in Fig 1B. **(Ray can you add specific levels here??).** As expected, at the high concentration of input protein used in Fig 1A, the Fos/Jun complex altered the footprinting pattern across the entire promoter but specific footprints were seen across known AP-1 sites in the promoter region (indicated by vertical dashed lines), including the low affinity NFIL-2A (ARRE-1) site located between nt # –80 and –90. These specific footprints were confirmed in Fig 1B at lower concentrations of AP-1. However, when both HMG-I(Y) and Fos/Jun were combined together in the reaction mixture, binding of Fos/Jun to the NFIL-2A site and the CD28RR was considerably reduced and this is most clearly seen at the higher concentrations of protein used in Fig 1A (see below).

HMG-I(Y) modulates AP-1 and NFAT binding to the IL-2 promoter.

HMG-I(Y) has previously been shown to promote or inhibit transcription factor binding to their regulatory elements overlapping or adjacent to the HMG-I(Y) binding sites (reviewed in: 29). We have previously shown that HMGI(Y) can modulate the binding of c-Rel to the CD28RR on the IL-2 promoter. We wished to determine if HMGI(Y) affected other transcription factors that are known to bind to the IL-2 promoter. Several of the regions on the IL-2 promoter to which HMG-I(Y) binds have been described as NFAT and AP-1 binding sites (reviewed in: 3,4). We chose to examine the effect of HMG-I(Y) on NFAT and AP-1 binding to two of these sites. The NFIL-2A (ARRE-1) region has previously been reported to cooperatively bind NFAT and AP-1 to form a higher order NFAT complex on the NFIL-2A region (11). The second site we examined, the CD28RR, has also been reported to bind NFATp (11,

Shang et al in press) and AP-1 but the binding sites are in a distinct configuration and cooperative binding is not observed (data not shown).

At low protein concentrations, recombinant truncated Fos/Jun alone does not stably bind to the low affinity AP-1 site in the NF-IL2A site in EMSA assays (Fig 2A, lane 1). The addition of recombinant HMG-I(Y), however, resulted in the appearance of an AP-1 band (Fig 2A, lanes 2-5). The intensity of the AP-1 band initially increased (up to 2ng HMG-I(Y)) and then decreased with the addition of increasing amounts of HMG-I(Y) (Fig 2A). These results demonstrating that high concentrations of HMG-I(Y) inhibit Fos/Jun binding are consistent with the previously described footprinting results obtained with varying concentrations of these proteins (Fig. 1A). Recombinant NFATp protein was added to determine the effect of HMG-I(Y) proteins on formation of the NFAT/AP-1 complex. Under the binding conditions used here NFATp did not bind alone to the probe and the formation of the higher order NFAT complex did not occur (Fig 2A, lane 6). Addition of HMG-I(Y) protein resulted in a dose-dependent increase in formation of the NFAT complex as well as the previously observed increase in AP-1 binding (Fig 2A, lanes 7-10). At higher levels of HMG-I(Y) the intensity of the NFAT complex did not significantly decline as was seen for AP-1 alone (Fig 2A, lanes 7-10). The amount of AP-1 and NFATp used in these experiments was sufficient for strong binding to the consensus sites contained in an oligonucleotide from the GM-CSF enhancer (GM170) that can bind NFATp or AP-1 alone and also form a higher order complex (Fig 2A, lanes 11-15). This oligonucleotide showed no binding of HMG-I(Y) and neither the binding of AP-1 or NFATp alone nor the formation of the NFAT higher-order complex were affected by addition of HMG-I(Y) at any concentration (Fig 2A, lanes 11-15).

Recombinant full length c-Fos/c-Jun (AP-1) bound to the CD28RR in a dose dependent manner, without the addition of HMGI(Y) (Fig 2B, lanes 1-3). An increase in AP-1 binding was observed with the addition of HMGI(Y) (Fig2B, compare lanes 3,6 and 9) but relatively high levels of HMGI(Y) were required to observe this effect

(20ng compared to 2ng for the promotion of AP-1 binding to the ARRE-1 region). We have previously shown that HMGI(Y) promotes the binding of c-Rel to an adjacent site (CD28RE) in the CD28RR (14). NFAT proteins can also bind to the CD28RR and we have previously shown that HMGI(Y) can promote truncated recombinant NFATp binding to the CD28RR (Shang et al in press). We therefore examined whether HMGI(Y) could promote the formation of an NFATp/AP-1 complex on this site. At the concentrations used to promote AP-1 binding here (20ng), the binding of NFATp was in fact inhibited (Fig 2B, lanes 10-13). Similar results were observed whether full length AP-1 (Fig 2C) or truncated AP-1 (data not shown) was used in the binding reactions.

These results show that HMGI(Y) can modulate the binding of transcription factors that are required for IL-2 promoter function to at least two major control regions of the IL-2 proximal promoter. It appears, however, that the ratio of HMGI(Y) to AP-1 or NFATp may be important in determining the functional outcome of the interaction.

HMGI(Y) modulates nuclear AP-1 binding to the CD28RR.

The CD28RR consists of an AP-1 binding site as well as a c-Rel binding site (CD28RE). We have previously shown that the c-Rel containing complexes from activated T cell nuclear extracts require HMGI(Y) to bind to the CD28RE (14). In order to determine if nuclear AP-1 was also dependent on HMGI(Y), we examined binding of AP-1 from nuclear extracts of P/I/CD28 activated Jurkat T cells. Because the Rel and AP-1 complexes migrate at the same position on EMSA gels using the intact CD28RR (17), it was necessary to generate EMSA probes with mutations in either the CD28RE (RE mutant) or the AP-1 site (AP mutant). Binding of nuclear extracts to these probes showed that an inducible complex could bind to the RE mutant in extracts made both 1 and 6h following stimulation with P/I/CD28 (Fig 3A). These

complexes migrated at the same position as the complexes binding to the wildtype probe or the Rel-containing complexes binding to the AP mutant (Fig 3A). The identity of the complexes binding to the RE mutant was confirmed by competition experiments where the RE mutant, the wt CD28RR and a consensus AP-1 site (TRE) were able to compete for complex formation but the AP mutant was not (Fig 3B). Thus these complexes appear to contain AP-1-like proteins. In order to determine if the formation of the AP-1-like complex was dependent on HMGI(Y), an anti-HMGI(Y) antibody was added to the binding reactions. The addition of the HMGI(Y) antibody to the binding reactions reduced AP-1 binding to the RE mutant as it did the c-Rel complexes binding to the AP mutant (Fig 3C). We have previously shown that HMGI(Y) antibody removes the inducible complexes containing c-Rel from the CD28RR but does not affect NF-kB complexes binding to a distinct NF-kB site (14). Ap-1 binding to a TRE was not significantly reduced by the addition of the HMGI(Y) antibody (Fig 3C).

To confirm that HMGI(Y) could promote AP-1 binding to the RE mutant, recombinant HMGI was titrated into binding reactions with a fixed amount of AP-1 using either the wildtype CD28RR or the RE mutant. As described above Ap-1 binding to the CD28RR was increased only at high concentrations of HMGI (Fig 3D lanes 7 and 8). On the other hand, a dose dependent increase in AP-1 binding was observed with increasing levels of HMGI(Y) starting at x ng HMGI on the RE mutant (Fig 3D). We also observed that HMGI(Y) no longer bound to the RE mutant (Fig 3D), probably because the RE mutant not only affects the CD28RE but also the A/T stretch within this site to which HMGI(Y) most likely binds. These results show, firstly that AP-1 binding in nuclear extracts is influenced by HMGI(Y) and secondly implies that the

ability of HMGI(Y) to promote AP-1 binding is not dependent on DNA binding by HMGI(Y).

HMGI(Y) binding to DNA is not required for promotion of transcription factor binding to the CD28RR.

The results above imply that the promotion of transcription factor binding may not always require DNA:HMGI(Y) interactions. In order to test this further, site-specific proline-to-alanine mutations were introduced into the second and third DNA binding domains of HMG-I (two substitutions in each binding domain) to create a form of the protein (designated HMG-I(mII,mIII)) that could not specifically bind to A:T-DNA under the assay conditions employed. We tested the ability of HMG-I(mII,mIII) to bind to the IL-2 promoter both in footprinting and in gel shift assays. At concentrations of protein where recombinant HMG-I or HMG-Y bound specifically to the CD28RR, HMG-I(mII,mIII) protein did not bind (Fig 4A). The same result was obtained in footprinting assays across the IL-2 promoter from –180 to –60 (Fig 4B). Four footprints for HMGI were observed across this region of the promoter (Fig 4B, lanes 2-5). On the other hand, HMGI(mII,mIII) did not form specific footprints across any of these regions (Fig 4B, lanes 6-9).

We also tested whether HMG-I(mII,mIII) could interact with transcription factors by generating an affinity matrix for both HMG-I and HMG-I(mII,mIII). The results of binding experiments using these affinity matrices in binding experiments demonstrated that both the wild-type HMG-I and mutant HMG-I(mII,mII) proteins could bind specifically to the AP-1 and NFAT proteins but could not interact with themselves or with each other (data not shown).

We then investigated whether HMG-I(mII,mIII) could alter transcription factor binding when added to binding assays using the CD28RR together with AP-1 and NFATp. The addition of HMG-I(mII,mIII) lead to a dose dependent increase in AP-1

binding but did not inhibit NFATp binding as did wildtype HMG-I (Fig 4C lanes 10-15). We consistently observed that HMG-I(mII,mIII) generated a more consistent dose dependence compared to the wild type HMG-I (Fig 4C cf lanes 4-9 and 10-15). c-Rel binding to the CD28RR was also increased by HMG-I(mII,mIII) (Fig 4D lanes 1 and 6-9). In addition, inhibition of transcription factor binding sometimes seen at high concentrations of HMGI was never observed with HMGI(mII,mIII). In summary, these results show that HMG-I binding to DNA is involved in the inhibition of binding but is not required for the promotion of transcription factor binding.

Alteration of HMG-I(Y) expression results in the modulation of IL-2 promoter activity in both Jurkat and primary T cells.

In order to determine if HMGI(Y) had a functional role in IL-2 gene transcription, HMGI(Y) levels were altered in the cell by antisense or overexpression studies. The pRcCMVIGMH plasmid expressing antisense HMGI was cotransfected into Jurkat T cells with pIL2luc or pHIVluc. The HIV LTR responds to PMA/ionophore (P/I) and CD28 stimulation through NF-κB sites that closely match consensus sites and contain no potential binding sites for HMG-I(Y) (unpublished results).

As previously reported (14), the expression of antisense HMG-I RNA resulted in a 72% decrease in reporter activity in P/I treated Jurkat cells and a 75% inhibition in P/I+α-CD28 treated cells at the maximum dose of antisense expression plasmid (10μg, Fig. 5A). The pHIVluc reporter transfections showed a only a small reduction in promoter activity at the maximum dose of antisense plasmid (Fig. 5A). To confirm that the antisense HMG-I RNA was reducing the level of HMG-I(Y) protein in the cells a western blot, using an anti-HMGI polyclonal antibody was performed on nuclear extracts from transfected cells either unstimulated or P/I stimulated following transfection. Densitometry scanning of the blot showed that there was a significant reduction in the levels of HMG-I(Y) protein in the antisense expressing cells (Fig. 5B).

This experiment also showed that the amount of HMGI(Y) in the nuclei of Jurkat T cells was increased almost 3 fold by P/I activation (Fig 5B).

HMG-I(Y) expression in transformed cell lines is generally high (29), hence the Jurkat T cell leukemia may display an aberrant role for HMG-I(Y) proteins in IL-2 promoter activity. In contrast, HMG-I(Y) levels are quite low or not detectable in most normal cell types. Therefore, the dependence of the IL-2 promoter on HMG-I(Y) proteins for activation of transcription was also examined in normal peripheral blood lymphoblasts (PBL). PBLs isolated from blood were cultured for four days as described to generate blasts, transfected by electroporation, rested for 24hr and restimulated with PHA. The IL-2 promoter transfected into PBLs had activity in cells that were not stimulated posttranfection most likely because of the primary stimulation with PHA prior to transfection to generate T cell blasts. These cells, however, showed a 2-3 fold increase in promoter activity when stimulated with PHA posttransfection (Fig 5C). Transfection with pRcCMVIGMH resulted in an 84% inhibition of the reporter activity in unstimulated PBLs and an 81% inhibition in PHA stimulated cells (Fig 5C). These results show that the activity of a transfected IL-2 promoter is dependent on HMG-I(Y) in either a T cell line or in primary T cells.

We also tested whether increased levels of HMGI(Y) protein affected IL-2 promoter activity. Transfection of the pcDNAHMGY expression plasmid into Jurkat T cells together with the pIL-2luc or the pHIVluc increased luciferase activity approximately 2 fold for IL-2 but had no effect on HIVLTR activity (Fig 6A). Since the mutant HMG-I(mII,mIII) protein also promoted transcription factor binding, its effect in transfection assays was also tested. Cotransfection of an expression plasmid for the HMG-I(mII,mIII) protein resulted in significant inhibition of the IL-2 promoter (60% in P/I and 73% in P/I+α-CD28 treated cells, Fig. 6B) at the highest amounts of transfected plasmid. The pHIVluc reporter plasmid showed no loss of reporter activity with expression of the HMGI(mII,mIII) protein (Fig. 6B). Thus, this non-DNA binding mutant appears to act as a dominant negative protein for IL-2 gene transcription.

Taken together these results show that modulating HMGI(Y) levels or function in either Jurkat T cells or PBLs affects IL-2 promoter activity without a general effect on transcription as measured by HIV LTR function.

IL-2 production and primary T cell proliferation are dependent on HMG-I(Y).

It is possible that the transfected IL-2 promoter may be more highly dependent on HMG-I(Y) than its endogenous counterpart because of the likely differences in chromatin configuration on chromosomal and plasmid DNA. To monitor the effect of HMGI(Y) on expression from the endogenous IL-2 gene, the amount of IL-2 protein secreted into the supernatant of cells transfected with pRcCMVIGMH and pcDNAHMG-I(mII,mIII) was assayed by ELISA. To enrich for transfected cells, 5μg of pCMVGFP was cotransfected with the HMGI plasmids above and the cells were subsequently sorted for high level GFP expression by FACS. The sorted cells were then stimulated with either PHA for PBLs or P/I+α-CD28 for Jurkat cells to induce IL-2 expression. In PBLs and Jurkats, significant inhibition of IL-2 production was detected in cells transfected with RcCMVIMGH (78% and 88% respectively) or pcDNAHMG-I(mII,mIII) (47% and 55% respectively) (Table 1). These results imply that the role of HMG-I(Y) may be extended to the endogenous IL-2 gene transcription in both Jurkat T cells and primary T cells.

If IL-2 production in PBLs is dependent on HMGI(Y), then PBL proliferation may also be affected by changes in HMGI(Y) levels in the cells. To test the effect of inhibition of HMG-I(Y) on cell growth, the IL-2 dependent PBLs and the IL-2 independent Jurkat cell line were transfected with either the parent plasmids or the expression plasmids RcCMVIGMH and pcDNAHMG-I(mII,mIII) together with the pCMVGFP plasmid to allow sorting of transfected cells by FACS. Proliferation of PBLs was inhibited by RcCMVIGMH (62%) or pcDNAHMG-I(mII,mIII) (29%) whereas the effect of these plasmids on growth of the Jurkat cell line was not significant (Fig 7A,B). In order to test whether increased HMGI(Y) levels also altered

proliferation, PBLs were transfected with the pcDNAHMGY plasmid and pCMVGFP to allow for sorting of transfected cells. Overexpression of HMG-Y in PBLs increased proliferation of these cells by approximately 2 fold (Fig 7C).

These results show that IL-2 production in both PBLs and Jurkats is dependent on the correct level of HMGI(Y). This dependence can also be seen at the level of proliferation in PBLs which are IL-2 dependent but not in Jurkats which are IL-2 independent.

DISCUSSION

The expression of the IL-2 and IL-2Rα chain genes is an essential component of the formation of the autocrine loop that drives T cell proliferation and clonal expansion following an immune stimulus. We have shown here that HMG-I(Y), a protein involved in regulating promoter architecture, is required for IL-2 promoter activity and T cell proliferation. This requirement for proliferation appears to stem from the involvement of HMG-I(Y) in controlling transcription from both the IL-2 and IL-2Rα chain genes in response to mitogenic stimuli. It has previously been shown that HMG-I(Y) participates in the inducible expression of the human IL-2Rα gene by facilitating the assembly of multiprotein, enhanceosome-like complexes on both an upstream enhancer element which has binding sites for HMG-I(Y), Elf-1 (an Ets family protein), Stat5 and a GATA family protein (51) and on the proximal promoter element which contains binding sites for HMG-I(Y), Elf-1 and NF-kB proteins (52). Likewise, the present results obtained from both *in vitro* footprinting, EMSA assays and transfection experiments strongly suggest that HMG-I(Y) facilitates the formation of a functional multiprotein complex consisting of a number of different transcription factors on the proximal promoter of the IL-2 gene during transcriptional activation *in vivo*.

Here, and elsewhere (14), we have demonstrated that HMG-I(Y) modulates the binding to the IL-2 promoter of NFAT, AP-1 and c-Rel; three transcription factor families that play important roles in IL-2 promoter activity. This has been shown both for recombinant proteins and proteins present in nuclear extracts. In each case, the transcription factor binding site constitutes a nonconsensus element that differs

markedly in sequence from the high affinity consensus sites. This is generally because of the presence of the A/T sequences required for HMG-I(Y) binding; consequently, these sites are weakly recognized by their cognate transcription factors. HMGI(Y), therefore serves to lower the threshold at which transcription factors can bind to and activate these weak sites. The presence of these nonconsensus sites in the IL-2 promoter appears to be important for the T cell restricted expression of the promoter. It has been shown that mutation of some of these sites to consensus high affinity sites weakens the induction dependence or T cell specificity of the promoter (53-55). There is no evidence that there are T cell restricted members of the transcription factor families that may have selectivity for the IL-2 promoter sites. Instead, it is likely that the weak interactions together with the need for proteins such as HMG-I(Y) and cooperative binding of many of the protein complexes is a requirement to generate these characteristics. Thus, HMG-I(Y) may play an indirect role in the T cell specificity and induction dependence of the IL-2 promoter.

The role of HMGI(Y) in the assembly of a functional enhanceosome has been best studied for the interferon-β (IFN-β) promoter (56). It has been shown that HMGI(Y) promotes the coordinate binding of members of the NF-kB, ATF and IRF families of transcription factors to the IFN-β promoter leading to the assembly of a functional complex known as an enhanceosome (56). This enhanceosome is then thought to recruit coactivator complexes such as CBP to lead to chromatin reorganization and transcription activation (57). It has recently been shown that the recruitment of transcription factors to the IFN-β promoter requires the binding of HMGI(Y) to DNA and that HMGI(Y):transcription factor interaction is not crucial for

this recruitment (58). The IFN-β promoter DNA contains an intrinsic bend that is straightened by HMGI(Y) binding and this is important to allow transcription factor binding (27). HMGI(Y):transcription factor interactions are, however, required for the completion of the enhanceosome assembly process (58). We have found here that binding of either c-Rel or c-Fos/c-Jun to the CD28RR of the IL-2 promoter does not require HMGI(Y) binding to DNA. The non-DNA binding mutant of HMGI, however, can still interact with the NFATp and AP-1 transcription factors and may promote transcription factor binding by protein:protein interactions. The intrinsic structure of the IL-2 promoter region is not known but it is possible that the promoter DNA does not require structural alteration to allow transcription factor binding. The HMG-I(mII,mIII) non-DNA binding mutant, however, acted as a dominant negative protein in transfection assays. This result implies that correct DNA binding as well as protein recruitment is important for the assembly of a functional complex. When expressed at sufficiently high levels the dominant negative mutant may compete with wildtype protein in the cells to form non-functional complexes. Thus it is likely that for both the IL-2 and IFN-β promoters, HMGI(Y) needs to both bind to DNA and interact with transcription factors in order to generate a functional complex, although the order of events may differ.

High concentrations of HMG-I appear to inhibit the binding of certain transcription factors at specific sites, e.g. AP-1 to the NFIL-2A site and NFATp to the CD28RR. This inhibition was dependent on DNA binding by HMG-I and would appear to be a direct competitive effect. It has previously been shown that HMGI(Y) can inhibit NFAT binding to the IL-4 promoter (59,60). In the case of IL-4 this inhibition of

DNA binding translates into the ability of increased HMGI(Y) to inhibit IL-4 promoter function (60). In contrast, our results strongly suggest that HMGI(Y) is an activator of the IL-2 promoter. The data which support this conclusion are 1) antisense HMG-I RNA expression strongly inhibits IL-2 promoter activity in both Jurkat T cells and PBLs, 2) increased expression of HMG-I, either in response to P/I activation or by cotransfection of an expression plasmid, correlates with increased IL-2 promoter activity and 3) production of IL-2 from the endogenous IL-2 gene is inhibited by antisense HMG-I RNA in both Jurkat T cells and PBLs. These experiments not only show that a promoter in the context of a reporter plasmid, but also the endogenous gene, in both a T cell line and normal T cells is dependent on HMGI(Y) for activity. The ability of HMGI(Y) to promote transcription factor binding may be an important aspect of the positive function of HMGI(Y) on the IL-2 promoter. Whether inhibition of transcription factor binding is an artifact of high concentrations of HMGI or plays a role in the removal of inhibitors or down regulation of the promoter following activation remains to be determined. One possible explanation for the results observed here is that at high concentrations of HMGI(Y) following T cell activation NFATp is displaced by HMGI(Y) which then promotes the binding of c-Rel. The appearance of NFATp in the nucleus () at early times following activation is likely to proceed the increased levels of HMGI(Y) and c-Rel that both require new protein synthesis (). A time-dependent change in the proteins that bind to the CD28RR may play a role in correct activation.

We have previously shown that the GM-CSF promoter (14) and the IL-3 promoter (unpublished results) are dependent on HMGI(Y) for activity. All of these cytokines are expressed following a primary T cell activation, before the development

of effector T cell function (61,62). On the other hand, IL-4 requires at least three cell divisions before expression is detected (63,64) and is the hallmark of the Th2 effector phenotype (61,62). Signaling from the IL-4 receptor has also been shown to lead to phosphorylation of HMG-I(Y) and a consequent reduction in DNA binding (65). Since IL-4 activation of T cells leads to increased IL-4 synthesis, it is intriguing to speculate that the phosphorylation of HMG-I(Y) and its removal from the DNA may be one mechanism by which this increased IL-4 production is achieved (60). The same signaling pathway may consequently decrease IL-2 production. It may be of interest to examine the role of HMG-I(Y) in expression of other Th1- or Th2-specific cytokines and to test the consequences of different levels of HMG-I(Y) on the expression of these cytokines and the development of the T cell subtypes.

Alteration of the level of HMGI(Y) in PBLs leads to an effect on proliferation of these cells. It would appear that the expression of antisense HMG-I RNA does not have a general effect on the cell cycle machinery since antisense HMG-I RNA expression had little effect on the proliferation of Jurkat T cells. This change in proliferation in PBLs is likely to be the result of changes in IL-2 expression in these cells although we have not definitively proven this link. Attempts to rescue the proliferative defect in HMG-I antisense RNA-expressing cells lead to only a small reversal of this inhibition. This may be due to the fact that the expression of the IL-2Rα chain gene is also controlled by HMGI(Y) as discussed above. The IL-2Rα chain is required for high affinity IL-2 receptor complex formation (reviewed in 66). Indeed, the PBLs expressing antisense HMG-I RNA had a reduced level of IL-2Rα chain on the cell surface as measured by FACS analysis (data not shown).

We have found here that HMGI(Y) levels increase in Jurkat T cells in response to P/I treatment. PMA treatment has previously been shown to increase HMGI(Y) levels in other cell types (67, 68) as have other signals such as exposure to growth factors such as serum (69, 70) and epidermal growth factor (71) or proinflammatory agent such as endotoxin and interleukin-1 beta (72). The increase observed in HMGI(Y) levels may directly translate into increased promoter activity mediated by HMGI(Y) interactions with the other inducible transcription factors such as c-Rel or AP-1.

The results presented here show that the architectural transcription factor HMGI(Y) is critical for the correct regulation of IL-2. The finding that HMGI(Y) not only affects the function of a transfected IL-2 promoter but also the endogenous IL-2 gene and T cell proliferation implies that the level of HMGI(Y) in cells is a critical determinant of IL-2 gene activation and that modulating such levels either by physiological or pharmacological means may provide a means of modulating the immune response.

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FOOTNOTES

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3. Abbreviations used in this manuscript.

HMGI(Y): High mobility group protein I or Y

CD28RR: CD28 response region nt -143 to – 175.

CD28RE: CD28 response element nt -156 to – 165 within the CD28RR.

P/I: PMA and calcium ionophore

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List of Figures

Figure 1.

A. DNase-I footprinting of HMG-I(Y) and AP-1 on IL-2 promoter DNA. The contents of the lanes are indicated at the top of the gels with a Maxam-Gilbert 'G' chemical cleavage of the naked DNA serving as a sequence reference marker. The right and left panels are two different loadings of the same samples on the sequencing gel that have been electrophoresed for different lengths of time. **Change....** The nucleotide numbers (Nt. #) relative to the major transcriptional start site are indicated next to the G lanes. The recognition sequences for known protein transcription factors and functional elements are indicated by vertical lines next to the free DNA lanes. The footprinted areas protected by HMG-I are indicated by a vertical thick solid line and a filled arrowhead whereas those protected by AP-1 are indicated by a vertical thick broken line and a filled circle.

B. Sequence of the IL-2 proximal promoter showing HMG-I(Y) footprints relative to known transcription factor binding sites.

Figure 2

A. HMGI(Y) promotes AP-1 and NFATp binding to the NFIL-2A region of the IL-2 promoter. Electrophoretic mobility shift assays were performed using radiolabelled oligonucleotides spanning the NFIL-2A region of IL-2 (lanes 1-10) or the GM-170 region of the GM-CSF enhancer (lanes 11-15) and recombinant truncated NFATp (0.4ng), truncated c-fos/c-jun (tAP-1) (2ng) and HMGI(Y) (1,2,4 and 8ng) proteins. The results were visualized using a Molecular Dynamics Phosphorimager. The proteins and probes used in the individual lanes are indicated above the lanes and the positions of the free DNA and the individual protein:DNA complexes are indicated.

B. HMGI(Y) promotes AP-1 but inhibits NFATp binding to the IL-2 CD28RR. EMSAs were performed as above using full length recombinant c-Fos and c-Jun (AP-1) at 12ng (lanes 1,4,7), 6ng (lanes 2,5,8) or 3ng (lanes 3,6,9) and the CD28RR radiolabelled oligonucleotide. In lanes 10-13, 0.1ng NFATp was added with 3ng AP-1. HMGI protein was added to the binding reactions at 2ng (lanes 4-6), 20ng (lanes 7-9) or 5(lane 11), 10 (lane 12) or 20ng (lane 13). EMSAs were processed and the diagram labelled as in A.

Figure 3

Binding of AP-1 from Jurkat T cell extracts requires HMGI(Y). A. EMSAs were performed with nuclear extracts prepared from Jurkat T cells either unstimulated (lanes 1,4 and 7) or activated with P/I/CD28 for 1h (lanes 2,5 and 8) or 6h (lanes 3,6 and 9) using radiolabelled CD28RR (lanes 1-4), RE mutant (lanes 5-8) or AP mutant (lanes 9-12) oligonucleotides. The inducible protein DNA complexes are indicated by an arrow. B. Competition experiments using the RE mutant as the radiolabelled probe and extracts from cells activated for 6h. The CD28RR wildtype sequence (lanes 2-4), AP mutant (lanes 5-7), RE mutant (lanes 8-10) or a TRE (AP-1 consensus binding site) (lanes 12-14) were used as competitors at 1,10 and 20ng per reaction as indicated. C. The effect of addition of HMGI(Y) antibody on complexes binding to the RE mutant (lanes 1-4), the AP mutant (lanes 5-8) and the TRE (lanes 9-11). The HMGI(Y) antibody (α-H; 1 or 2μl) or a non-specific serum (C; 2μl) was added to the binding reactions prior to the addition of probe. The AP-1 complexes are indicated. D. HMGI promotes recombinant AP-1 binding to the RE mutant. EMSAs containing 3 ng recombinant AP-1 and increasing concentrations of HMGI (0.5, 1, 2.5, 5, 10 and 20ng) were performed with either the wildtype CD28RR probe (lanes 1-8) or the RE mutant

(lanes 9-16). Lanes 1 and 9 had no protein added and lanes 2 and 10 had only AP-1. The positions of the HMGI and AP-1 bands are indicated.

Figure 4

A, B. HMGI(mII,mIII) does not bind specifically to the IL-2 promoter. A. EMSAs were performed using the IL-2CD28RR oligonucleotide as a probe and 1,10 and 20ng of HMGI (lanes 1-3), HMGY (lanes 4-6) and HMGI(mII,mIII)(lanes 7-9). The HMGI/Y bands and the free DNA are indicated. B. DNase I footprinting assay using the –180 to –60 region of the IL-2 promoter. HMGI (lanes 2-5) or HMGI(mII,mIII) (lanes 6-9) at 10, 20, 50 or 100ng were used in the footprinting reactions. Solid lines indicate the HMGI footprints and the names of the regions are shown. C, D. HMGI(mII,mIII) can promote but not inhibit transcription factor binding to the IL-2 CD28RR. Increasing amounts (1, 2.5, 5, 10 and 20ng) of HMGI (lanes 4-9) or HMGI(mII,mIII) (lanes 10-15) were added to binding reactions containing the IL-2CD28RR probe and 3ng AP-1 and 0.1ng NFATp. Reactions containing NFATp alone (lane 1), AP-1 alone (lane 2) or AP-1 and NFATp together (lane 3) were also analysed. The positions of the transcription factor:DNA complexes are indicated. D. Similar binding reactions as described in C were carried out using bacterial extract containing the RHD domain of c-Rel and increasing amounts 1, 5, 10 and 20ng) of HMGI (lanes 2-5) or HMGI(mII,mIII) (lanes 6-9). All gels were dried and exposed to X-Ray film. Digital images were produced on a Fugifilm LAS1000 Plus CCD camera and Image Gauge software.

Figure 5

Inhibition of HMGI(Y) production reduces IL-2 promoter activity in Jurkat T cells and PBLs. A. Jurkat T cells were transfected with increasing amounts of pRcCMVIGMH and the pIL-2luc or pHIVluc reporter plasmids. Transfected cells were treated with P/I (white bars) or P/I+α-CD28 (black bars) before luciferase assays were performed.

Luciferase activity is expressed as CPS readings from the Packard TopCount Luminometer. B. Western blot showing the decrease in HMGI(Y) levels in cells expressing antisense HMGI. Cells were transfected with either pRcCMV (lanes 1 and 3) or pRcCMVIGMH antisense expression plasmid (lanes 2 and 4). Protein was extracted from nuclei of either unstimulated (lanes 1 and 2) or P/I stimulated (lanes 3 and 4) cells, resolved on SDS polyacrylamide gels and HMGI(Y) detected by western blot. Recombinant HMGI protein (lane 5) was also loaded as a reference. Numbers below represent the densitometry quantitation of HMGI(Y) protein bands. C. PBLs, pretreated to generate blasts, were transfected with the pRcCMV or pRcCMVIGMH plasmids and the pIL-2luc reporter plasmid and luciferase activity measured either before or following PHA treatment. Columns represent the means of five replicate assays and the bars show the SEMs.

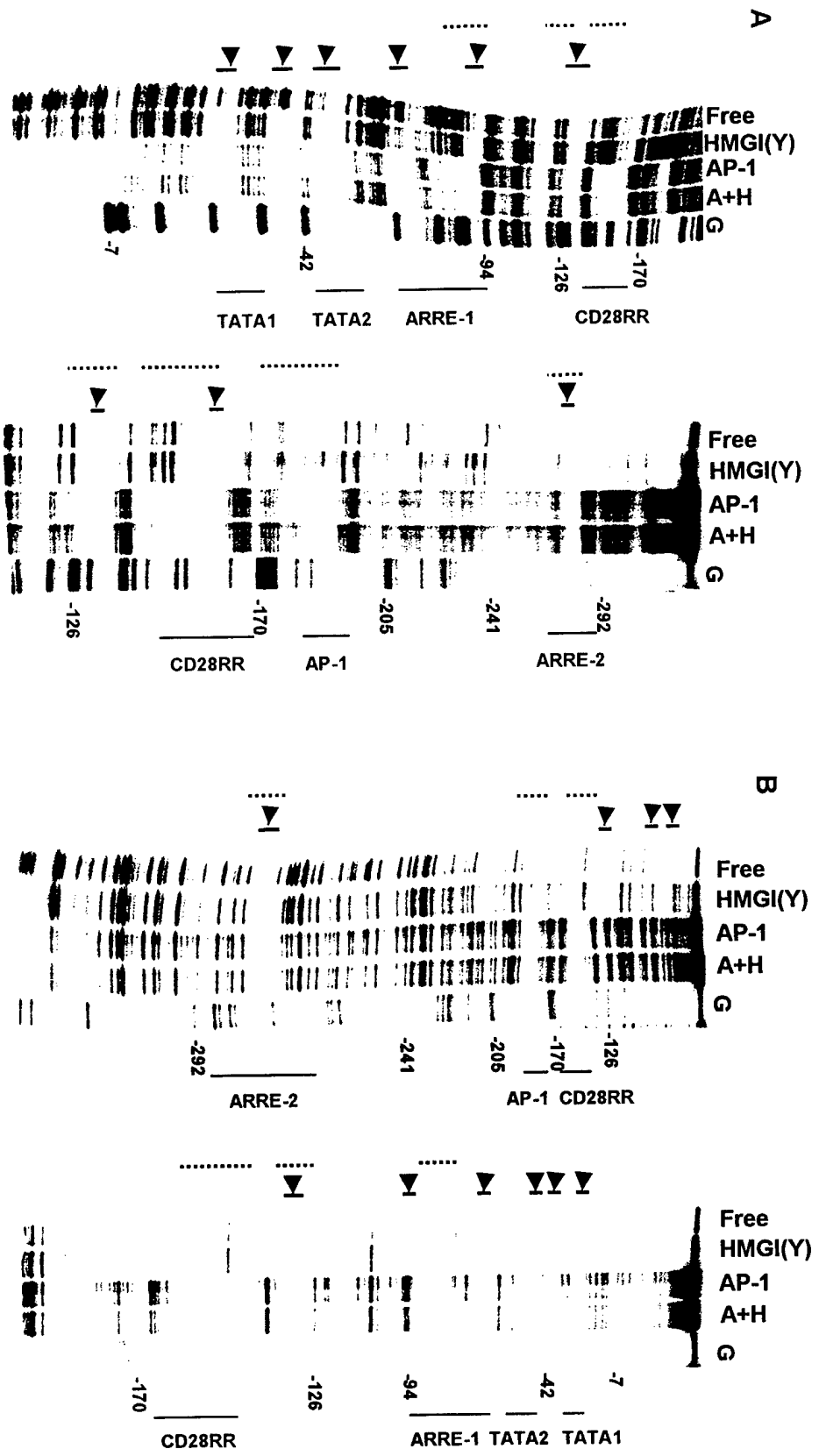
Figure 6

A. Expression of HMGY in Jurkat T cells increases IL-2 promoter activity. Jurkat cells were transfected with either the pIL-2luc or the pHIVluc reporter plasmids together with the indicated amounts of the pcDNAHMGY expression plasmid or the control pcDNA3.1 plasmid. Cells were stimulated with P/I (while bars) or P/I+α-CD28 (black bars) for 8hr before harvesting for luciferase assays. pHIVluc transfected cells were only stimulated with P/I+α-CD28. Luciferase activity is expressed as CPS as measured by a Packard Topcount scintillation counter. B. Expression of HMGI(mII,mIII) in Jurkat cells inhibits IL-2 promoter activity. The pIL-2luc or pHIVluc reporter plasmids were transfected into Jurkat T cells together with the indicated amounts of the pcDNAHMGI(mII.mIII) expression plasmid or the control pcDNA plasmid. Cells were

activated and analysed and results presented as described in A.

Figure 7

Altering HMGI(Y) levels or function affects T cell proliferation. PBLs and Jurkat cells were transfected with either 10μg of the antisense expression plasmid pRcCMVIGMH or the parent plasmid pRcCMV (A), and pcDNAHMGI(mII,mIII) or the parent plasmid, pcDNA3.1 (B) PBLs were also transfected with the pcDNAHMGY plasmid or the parent plasmid (C). All cells were cotransfected with 5μg of pCMVGFP and subsequently sorted by FACS for GFP expression. Cells were then stimulated with PHA in the presence of BrDU and assayed for proliferation using enzyme-conjugated antibody to BrDU and a chemiluminescent substrate. Values are given as counts per second measured by a 96 well plate luminometer and the columns represent the mean, and error bars the SEM, of four replicate assays.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Free
HMGI(Y)
AP-1
A+H
G
-7
-42
-94
-126
-170
TATA1
TATA2
ARRE-1
CD28RR
Free
HMGI(Y)
AP-1
A+H
G
-126
-170
-205
-241
-292
CD28RR
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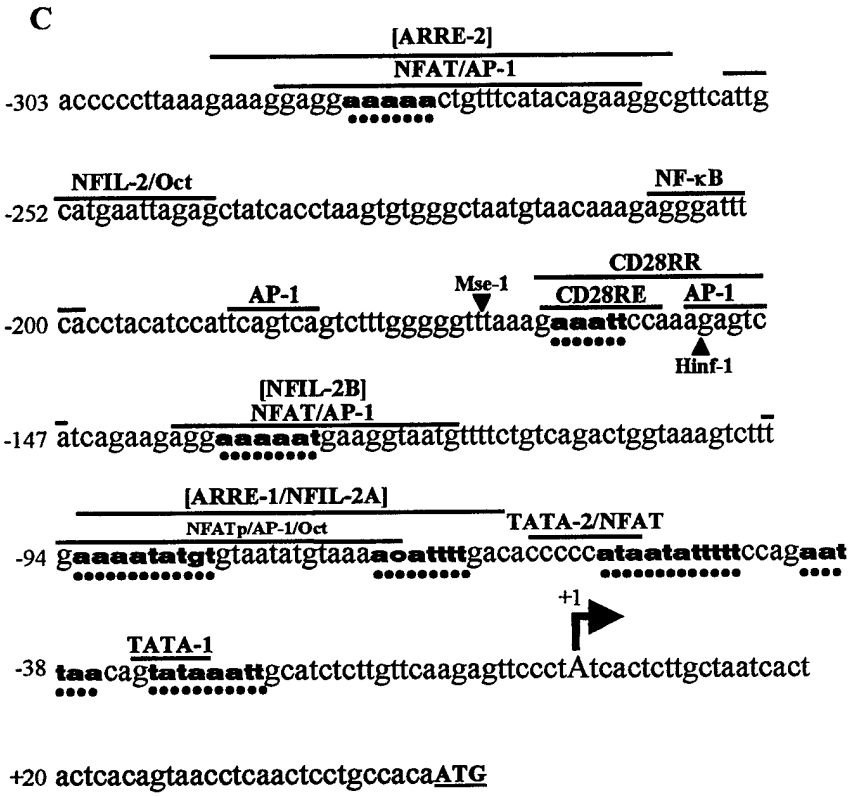
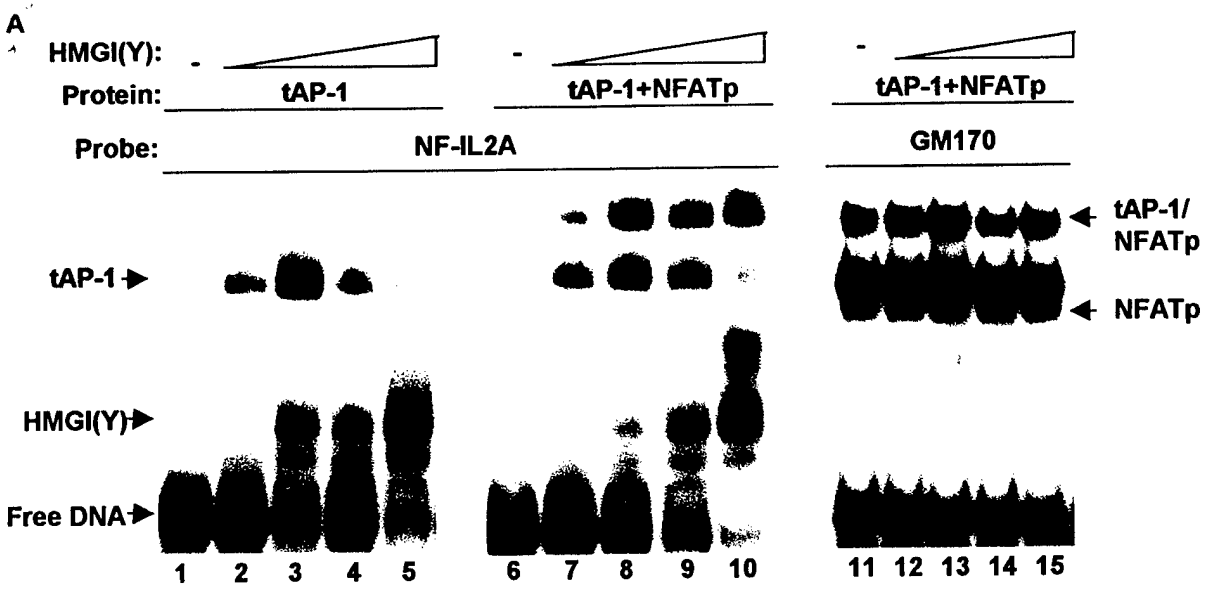
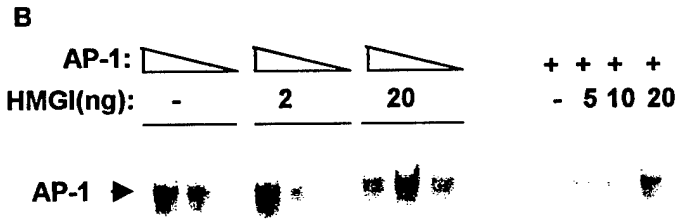
-42
-7
CD28RR
ARRE-1 TATA2 TATA1

Fig 1a,b

C


[ARRE-2]
NFAT/AP-1
-303 acccccttaaagaaaggaggaaaaactgtttcatacagaaggcgttcattg
NFIL-2/Oct
NF-κB
-252 catgaattagagctatcacctaagtgtgggctaatgtaacaaagagggattt
CD28RR
AP-1
Mse-1
CD28RE
AP-1
-200 cacctacatccattcagtcagtctttgggggtttaaagaaattccaaagagtc
Hinf-1
[NFIL-2B]
NFAT/AP-1
-147 atcagaagaggaaaaatgaaggtaatgttttctgtcagactggtaaagtcttt
[ARRE-1/NFIL-2A]
NFATp/AP-1/Oct
TATA-2/NFAT
-94 gaaaatatgtgtaatatgtaaaacoattttgacaccccataatatttttccagaat
+1
TATA-1
-38 taacagtataaattgcatctcttgttcaagagttccctAtcactcttgctaatcact
+20 actcacagtaacctcaactcctgccacaATG


A
HMGI(Y):
Protein:
tAP-1
tAP-1+NFATp
tAP-1+NFATp
Probe:
NF-IL2A
GM170
tAP-1
tAP-1/
NFATp
NFATp
HMGI(Y)
Free DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
AP-1:
HMGI(ng):
-
2
20
+ + + +
- 5 10 20
AP-1

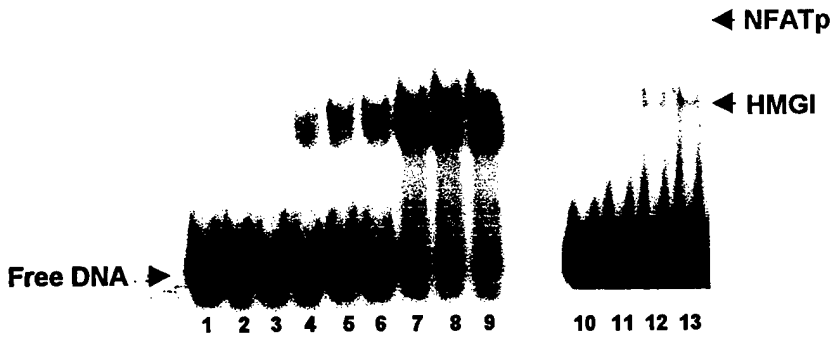
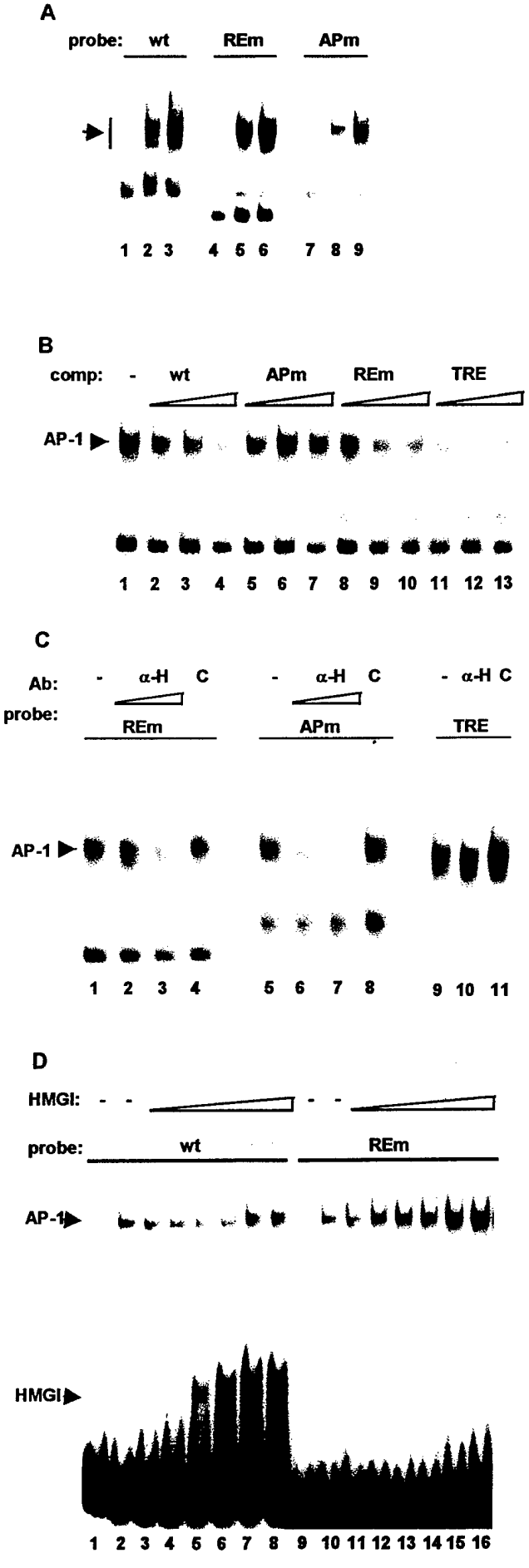

NFATp
HMGI
Free DNA
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig 3


A
probe: wt REm APm
1 2 3 4 5 6 7 8 9
B
comp: - wt APm REm TRE
AP-1
1 2 3 4 5 6 7 8 9 10 11 12 13
C
Ab: - α-H C - α-H C - α-H C
probe: REm APm TRE
AP-1
1 2 3 4 5 6 7 8 9 10 11
D
HMGI: - - - -
probe: wt REm
AP-1
HMGI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

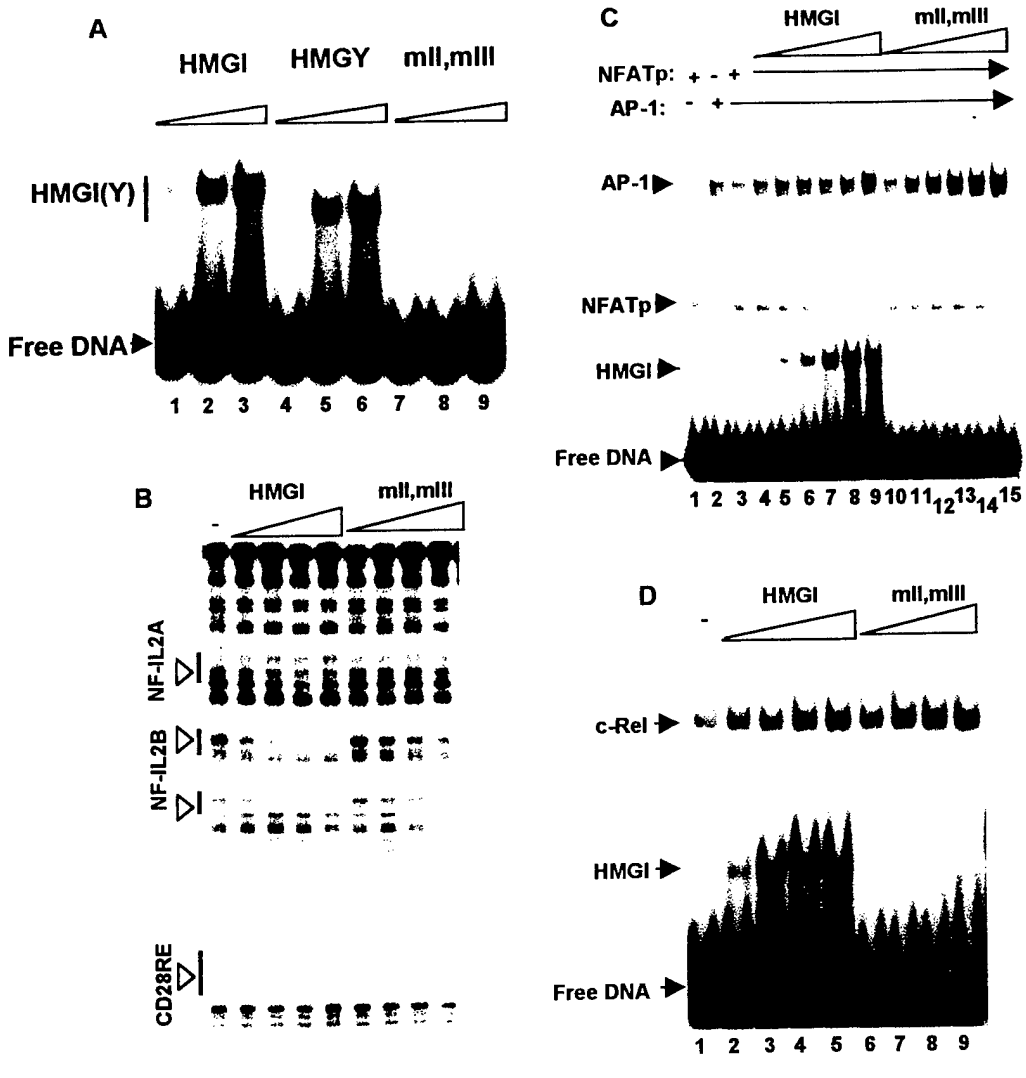
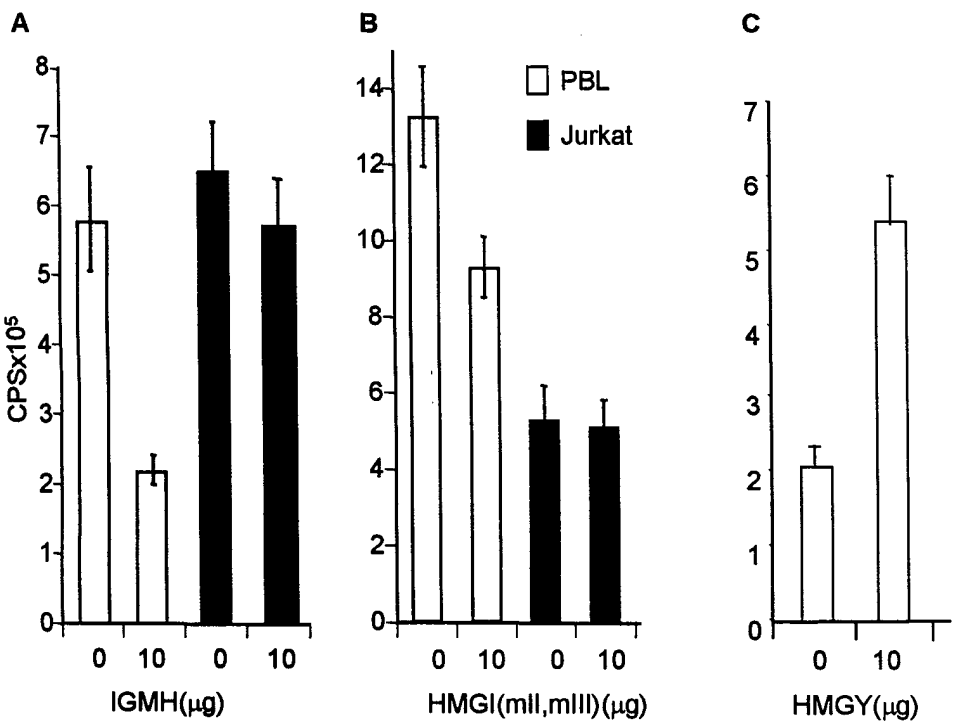

A
HMGI
HMGY
mII,mIII
HMGI(Y)
Free DNA
1 2 3 4 5 6 7 8 9
B
HMGI
mII,mIII
NF-IL2A
NF-IL2B
CD28RE
1 2 3 4 5 6 7 8 9
C
HMGI
mII,mIII
NFATp: + - +
AP-1: - +
AP-1
NFATp
HMGI
Free DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
HMGI
mII,mIII
c-Rel
HMGI
Free DNA
1 2 3 4 5 6 7 8 9

Figure 7


A
B
C
PBL
Jurkat
CPSx10⁵
0
1
2
3
4
5
6
7
8
10
12
14
0
10
0
10
IGMH(μg)
HMGI(mII,mIII)(μg)
HMGY(μg)